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DEPARTMENT OF BIO-ANALYSIS

LABORATORY OF TOXICOLOGY

DETERMINATION OF GAMMA-HYDROXYBUTYRIC ACID IN MICROVOLUMES OF BIOLOGICAL FLUIDS FOLLOWING DIRECT DERIVATIZATION AND GAS CHROMATOGRAPHY MASS SPECTROMETRY

THESIS SUBMITTED TO OBTAIN THE DEGREE OF DOCTOR IN PHARMACEUTICAL SCIENCES

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The promoter,

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LIST OF ABBREVIATIONS

1,4-BD	1,4-butanediol
6-MAM	6-monoacetylmorphine
AA	amino acids
AC	acylcarnitines
Acetyl-CoA	acetyl-coenzyme A
AHB	alpha-hydroxybutyric acid
APCI	atmospheric pressure chemical ionization
β	phase ratio
BHB	bèta-hydroxybutyric acid
Br-MMC	4-bromomethyl-7-methoxy coumarin
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C_{max}	concentration found at T_{max} following intake of a given dose
CI (GC-MS)	chemical ionization
CI	confidence interval
CZE	capillary zone electrophoresis
CZE-C^4D	capillary zone electrophoresis with contactless conductivity detection
DBS	dried blood spots
DFSA	drug facilitated sexual assault
DMS	dimethylsulphate
DUID	driving under the influence of drugs
DUS	dried urine spot
EDS	excessive daytime sleepiness
EDTA	ethylenediaminetetra-acetic acid
EI	electron impact
EMA	European Medicines Agency
ESI	electrospray ionization
FDA	Food and Drug Administration
Fluo	fluorescence detection
γ_i	activation coefficient
GAA	guanidinoacetic acid
GABA	gamma-aminobutyric acid
GBL	gamma-butyrolactone
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detection
GC-MS	gas chromatography coupled to mass spectrometry
GHB	gamma-hydroxybutyric acid
GHB-d6	deuterated gamma-hydroxybutyric acid
GHB-DH	GHB dehydrogenase
GHV	gamma-hydroxyvaleric acid
GVL	gamma-valerolactone

H_2SO_4	sulphuric acid
HCl	hydrogen chloride
HFB-OH	heptafluorobutanol
HPLC	high performance liquid chromatography
HPLC-DAD	high performance liquid chromatography-diode array detection
HPLC-fluo	high performance liquid chromatography-fluorescence detection
HPLC-UV	high performance liquid chromatography-ultraviolet detection
HS	headspace
Ht	hematocrit value
ICP-MS	inductively coupled plasma mass spectrometry
ICP-TOF-MS	inductively coupled plasma time-of-flight mass spectrometry
IMS	ion mobility spectrometry
Int	intermediate
IS	internal standard
ISR	incurred sample reanalysis
K	partition coefficient
LC	liquid chromatograph
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
LOD	limit of detection
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxy-N-methylamphetamine
MeOH	methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MTBSTFA	N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
Na_2SO_4	anhydrous sodium sulphate
NBS	newborn screening
NCI	negative chemical ionization
NMR	^1H nuclear magnetic resonance
PBDE	polybrominated diphenyl esters
PCB	polychlorinated biphenyls
PCI	positive chemical ionization
PFC	perfluorinated carbons
PTFE	polytetrafluoroethylene
QC	quality control

R^2	coefficient of determination
RE	relative error
RP	reversed phase
RSD	relative standard deviation
SA	succinyl acetone
SD	standard deviation
SIM	selected ion monitoring
S/N	signal-to-noise
SPE	solid-phase extraction
SPDE	solid-phase dynamic extraction
SPME	solid-phase micro-extraction
SSA	succinic semi-aldehyde
SSADH	succinic semi-aldehyde dehydrogenase
STA	systematic toxicological analysis
T_0	original concentration (at time point zero)
$T_{1/2}$	half-life
TBA-HSO_4	tetrabutylammonium-hydrogensulphate
TDM	therapeutic drug monitoring
THC	tetrahydrocannabinol
THC-COOH	carboxy-THC
TFAA	trifluoroacetic acid anhydride
TIAFT	The International Association of Forensic Toxicologists
TIC	total ion chromatogram
T_{max}	time when the maximum concentration is reached following intake of a given dose
TMS	trimethylsilyl
ULOQ	upper limit of quantification
U(H)PLC	ultra-performance liquid chromatography
UV	ultraviolet
Vs	sample volume
WB	whole blood

STRUCTURE AND OBJECTIVES

The aim of this work was to develop one-step procedures to identify and quantify the low molecular weight compound and drug of abuse gamma-hydroxybutyric acid (GHB) in various biological matrices using gas chromatography coupled to mass spectrometry (GC-MS). Since GHB requires derivatization prior to GC-MS, we aimed to minimize the resulting work-load by introducing direct derivatization procedures.

The first chapter of **PART I**, **Chapter I.A**, gives a brief general overview concerning GHB. Its chemical properties, metabolization, use, abuse, effects, adverse effects and current legal status are discussed. **Chapter I.B** provides a detailed overview of state-of-the-art techniques used for GHB determination in biofluids.

The second part (**PART II**) of this thesis describes the development of a GC-MS method for the determination of GHB in dried blood spots (DBS) using "on spot" derivatization. Although GHB is clinically used for treating narcolepsy associated with cataplexy (Xyrem®), it is especially notorious for its abuse as a club and date-rape drug. Given its rapid metabolism, its endogenous presence and the possibility of *ex vivo* formation, proving GHB misuse remains a challenge. The use of dried blood spots (DBS) may represent a new, minimally invasive way of sampling and storing blood from patients, abusers or victims. Amongst the advantages of DBS are the ease of sample handling and stabilization of many compounds, thereby improving long-term storage of samples. The latter is of interest for GHB since *de novo* formation of GHB in whole blood samples has been reported. First, an overview of the use of DBS in toxicology is given, with a focus on the determination of drugs of abuse (**Chapter II.A**). Derivatization procedures applied in DBS analysis are reviewed in **Chapter II.B**. In **Chapter II.C**, the determination of GHB in DBS using "on spot" derivatization and GC-MS is presented. First, the optimization of the GC-MS method is presented (**II.C.1**). Next, the development of a procedure for the determination of GHB in 50 µl-DBS is described. The complete DBS was excised for analysis, a useful approach in cases where a fixed sample volume can be easily spotted on a DBS card using capillary pipettes (**II.C.2**). Results indicated the applicability of the DBS method in routine toxicological analysis. However, as we also wished to collect capillary DBS from patients in a real-life setting, a more convenient approach was the direct collection of drops of capillary blood on a DBS card followed by punching out a fixed area (a 6-mm diameter punch). Therefore, the original method was adjusted since no longer a fixed volume, but a fixed area was analyzed (**II.C.3**). Not only the sample preparation steps were re-evaluated, but also the impact of additional parameters needed to be evaluated such as the influence of hematocrit, of the blood

spot volume and of punch localization on the measured concentration. Finally, following validation, the applicability of the DBS method was demonstrated by analyzing venous whole blood, venous DBS and capillary DBS collected simultaneously from suspected GHB-intoxicated patients and by comparing the obtained GHB concentrations. Furthermore, a proof-of-concept study was performed to evaluate the feasibility of DBS sampling in a real-life setting. Therefore, narcoleptic patients who used Xyrem® (sodium oxybate, the sodium salt of GHB) on a regular basis at night-time, were asked to collect capillary DBS during a maximum of 7 consecutive days. Results of this study are presented in **II.C.4**.

A third part of this work (**PART III**) deals with the use of headspace-trap (HS-trap) as injection technique for the GC-MS-based determination of GHB in various biofluids following "in-vial" derivatization. Similar to "on spot" derivatization, "in-vial" derivatization implies the direct addition of derivatization reagents to the biological matrix, here a small volume (100 µl) of biofluid, rather than a DBS. Combining "in-vial" derivatization with HS may lead to an extension of the application range of the HS technique to non-volatile compounds. In addition, the use of a trap enables the sampling of a larger HS fraction as compared to other available techniques, allowing small sample volumes to be analyzed, while still providing enough sensitivity. An overview of HS techniques with a focus on HS-trap and its applications found in literature is given in **Chapter III.A**. Method development, including the choice of a suitable derivatization reagent and the optimization of HS and trap parameters, is subsequently presented in **Chapter III.B**. This chapter also contains validation data and method application involving the analysis of samples from suspected GHB-intoxicated patients.

A general conclusion with future perspectives is given in the final part (**PART IV**).

PART I
GAMMA-HYDROXYBUTYRIC ACID

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*In **Chapter I.A**, a brief general introduction on gamma-hydroxybutyric acid (GHB) is given, starting with its chemical and pharmacokinetic properties, as well as a brief description of its precursors gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD). Furthermore, its general use/abuse and the effects/adverse effects of a GHB intake are described, together with a description of the onset of a GHB intoxication and the current legislation. A detailed overview of state-of-the-art techniques published since the nineties to determine GHB in various biological fluids is presented in **Chapter I.B**, where both screening and confirmation methods are summarized.*

CHAPTER I.A
GENERAL INTRODUCTION ON GHB

I.A.1 FOREWORD

Gamma-hydroxybutyric acid (GHB) or 4-hydroxybutanoic acid, a short chain fatty acid (pKa 4.6-4.8) with two polar substituent groups (hydroxyl- and carboxylgroup), was synthesized in the early sixties as a structural analogue of the neurotransmitter gamma-aminobutyric acid (GABA) that could cross the blood-brain barrier [1,2]. GHB was also found to be endogenously present in humans as a minor precursor and metabolite of GABA. Its role as endogenous compound remains unclear; it probably acts as neuromodulator or neurotransmitter through $GABA_b$ receptors and GHB specific receptors in the brain [3]. GHB can also be formed in humans from the precursors gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) (Fig. I.A.1) [4,5].

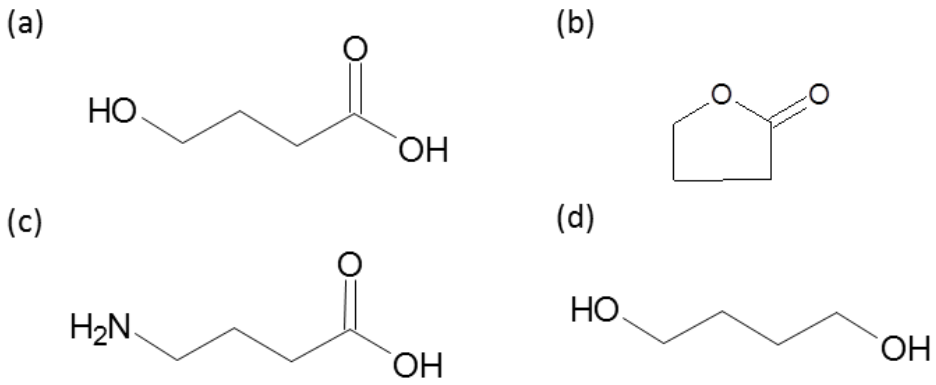

Fig. I.A.1 Structures of GHB (a), GBL (b) GABA (c) and 1,4-BD (d)

Although GHB is clinically used for e.g. treating narcolepsy associated with cataplexy (Xyrem®), it is especially notorious for its abuse as a club and date-rape drug. GHB and its precursors are often misused in combination with other drugs such as alcohol, cannabis and other club drugs such as 3,4-methylenedioxy-N-methylamphetamine (MDMA, ecstasy) and ketamine [5]. The range between oral ingestion of high recreational doses leading to desired effects and an overdose is narrow, quite commonly resulting in acute intoxications in humans [5].

I.A.2 PHARMACOKINETIC PROPERTIES AND PRECURSORS

As shown in Fig. I.A.2 -which gives an overview of the metabolization pathways of GHB- GBL, easily available as an industrial solvent, is rapidly converted to GHB *via* serum lactonases following ingestion. A half-life ($T_{1/2}$) of 1 min has been observed in rats [5,6]. Moreover, studies suggest a higher C_{max} and faster T_{max} when an equimolar dose to GHB is ingested [7]. *In vitro* conversion of GBL to GHB has also been observed, an equilibrium influenced by pH and temperature (Fig. I.A.3) [8].

Furthermore, 1,4-BD, also used as industrial solvent and during synthesis of other industrial chemicals, can also be ingested orally for recreational use because of its *in vivo* conversion to GHB [3,9]. It has been suggested that 1,4-BD is first transformed to gamma-hydroxybutyraldehyde *via* alcohol dehydrogenase, which is next converted to GHB *via* acetaldehyde dehydrogenase. An average elimination $T_{1/2}$ of 39.3 ± 11 min has been observed in a study giving volunteers 25 mg/kg 1,4-BD [3,7]. Simultaneous ingestion of ethanol competes with the biotransformation of 1,4-BD, thereby slowing down its metabolization and prolonging the effects.

GHB is both a metabolite and precursor of GABA. Via mitochondrial GABA transaminase and cytosolic succinic semialdehyde (SSA) reductase GABA can be converted to GHB. In the primary pathway of elimination, GHB is converted to SSA via GHB dehydrogenase. Next, SSA is converted to GABA or it may enter the Krebs cycle through the formation of succinate. A rare inherited metabolic disorder, succinic semi-aldehyde dehydrogenase (SSADH) deficiency, is characterized by the presence of elevated GHB-concentrations in plasma and urine [4,10]. Alternatively, GHB can be oxidized (β-oxidation) to e.g. 3,4-dihydroxybutyrate, which can be further oxidized to acetyl CoA, which also enters the Krebs cycle. Since GHB is almost completely converted to H_2O, CO_2 and energy, only 2 to 5 % of a dose is excreted unchanged in urine. No active metabolites have been identified. Recently, a new metabolite of GHB, GHB-glucuronide, has been reported to be present in urine samples [4,11,12].

Following oral ingestion, GHB has a short $T_{1/2}$ ranging from 30 min to less than 1 h, consistent with rapid gastro-intestinal absorption and elimination from the body. Depending on the dose, elimination mechanisms may be saturated, influencing the $T_{1/2}$. Less than 1 % is bound to plasma proteins [3,5,13].

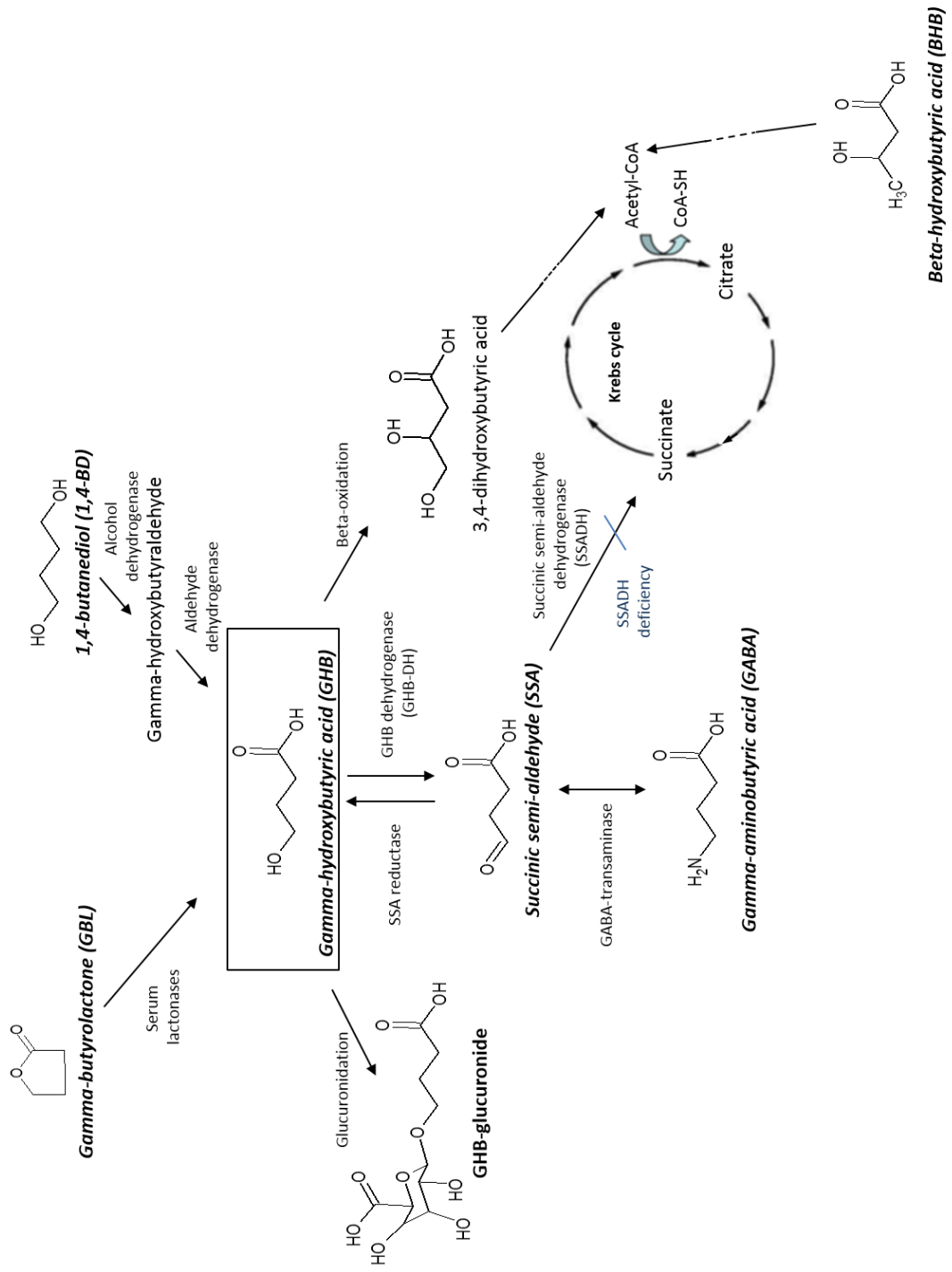

Fig.I.A.2 Overview of the metabolization pathway of GHB

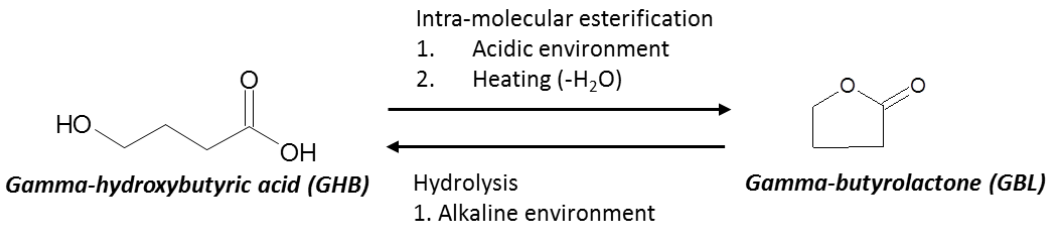

Fig. I.A.3 Interconversion of GHB and GBL *in vitro*

I.A.3 USE, ABUSE AND INTOXICATION

The sodium salt of GHB, sodium oxybate, has been used therapeutically as an anaesthetic, but this use has been abandoned in most countries because of side effects such as seizures and amnesia [14]. Furthermore, GHB/GBL containing supplements were sold to increase body muscles - since it is assumed that GHB stimulates growth-hormone release - and to improve sleep, but these supplements have been withdrawn from the market since the late nineties after reports of abuse and side effects. Nonetheless, sodium oxybate is currently being used in the treatment of narcolepsy with cataplexy and excessive daytime sleepiness (Xyrem®) and of alcohol (Alcover®, Italy) as well as opiate withdrawal [15].

In addition, illegal GHB and its precursors, GBL and 1,4-BD, have become popular amongst clubbers because of their stimulating effects. The club drug is known under street names such as liquid ecstasy, Georgia Home Boy, Grievous Bodily Harm, soap, scoop and salty water [6]. GHB and its precursors are most commonly available as solutions, in small vials or mixed in bottles with e.g. water, from which typically a capful is ingested orally per dose [4]. GHB abuse has also been reported in drug facilitated sexual assaults (DFSA), because of its strong sedative and amnesic effects. The possibility to render a victim unconscious is enabled by the chemical properties of GHB, being a colourless liquid which can be easily mixed with other liquids [16,17].

A dose typically ingested for abuse ranges from 2 to 6 g GHB, corresponding to 25 to 75 mg/ kg body weight (b.w.) [5]. Euphoria, relaxation, increased sociability, and decreased psychomotor skills are amongst the effects experienced when using GHB, showing similarity with the effects reported for moderate alcohol intoxication [5]. More in particular, effects reported following recreational use of GHB and its precursors are dual, i.e. both sedative and stimulatory, depending

on the dose. An individual dose of 1.0 to 2.0 g that is ingested orally results in effects like relaxation and euphoria, while doses of 2.5 to 3.0 g lead to side-effects such as nausea and vomiting (mild intoxication, see below). Higher doses (3.0 to 4.0 g) result in loss of consciousness, a dose of more than 4 g resulting in respiratory depression and coma (severe acute intoxication, see below) [18].

GHB has a steep dose-response curve, with a narrow margin between therapeutic or desired and toxic effects [5,19,20]. A mild intoxication is characterized by nausea, dizziness and difficulty in focussing the eyes, while vomiting, extreme dizziness, disorientation, amnesia and unconsciousness may be experienced from an severe acute intoxication [5]. Finally, side effects of a severe intoxication may evolve to convulsions, coma, and death. Fatal incidents have been reported due to the use of GHB alone or in combination with other drugs such as alcohol and MDMA [19].

First reports of abuse appeared in the early 1990s [20,21]. Since the late 1990s, early 2000s, the incidence of GHB intoxications has apparently been decreasing worldwide. For example, when evaluating GHB exposures reported to the California Poison Control System from 1999 to 2003, a decrease has been recorded, which can reflect the true incidence, but may also be due to a decrease in adverse events without a decrease in overall GHB usage or to random variability [21]. According to the annual reports of the latest drug situation and trends in the European Union and Norway, published by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), national estimates of the prevalence of GHB -where they exist- remain low. For example, in the UK, 2 % of regular clubbers reported last year use of GHB in an Internet survey [22]. Furthermore, GHB screening using automated clinical analyzers has not been routinely available until 2009. In addition, due to the rapid elimination of GHB from the human body, delayed sampling may result in GHB concentrations below the current cut-off values, thus no longer allowing proof of ingestion [23]. Therefore, true case incidence may be underestimated [5]. Moreover, more recent reports show that recreational use with limited awareness of the possibility to suddenly fall into a coma has resulted again in an increase in GHB intoxications at raves or other dance parties [19,24].

So, although the available epidemiological data has its limitations, general use may be relatively low compared to other drugs of abuse such as cannabis, amphetamines and cocaine, while a higher use has been seen in subpopulations (e.g. men having sex with men), settings and

geographical areas [25,26]. Of all commonly used illegal drugs in the Flemish nightlife across four survey years from 2003 to 2009, GHB was with only 3.0 % prevalence one of the least commonly used drugs, along with ketamine (1.9 %) (Fig. I.A.4). Nonetheless, over this four survey years of a survey set up to explore changes in illicit drug use among music festival goers and dance scene attendees in Flanders, an increase in the use of GHB has been detected [27]. It is therefore recommended to closely monitor the use of GHB, given its potential for overdose, which in addition occurs more frequently than for any other club drug, especially when combined with alcohol [27-30]. Also, European surveys between 2008 and 2010 estimated a 3.9 to 14.3 % lifetime use and last month prevalence use up to 4.3 %, while in The Netherlands a survey among partygoers in 2008 and 2009 revealed a 14 % lifetime and 5 % recent use of GHB [29,31,32]. In Australia, lifetime use of GHB is reported to be 1 % of people aged 14 years and older [30].

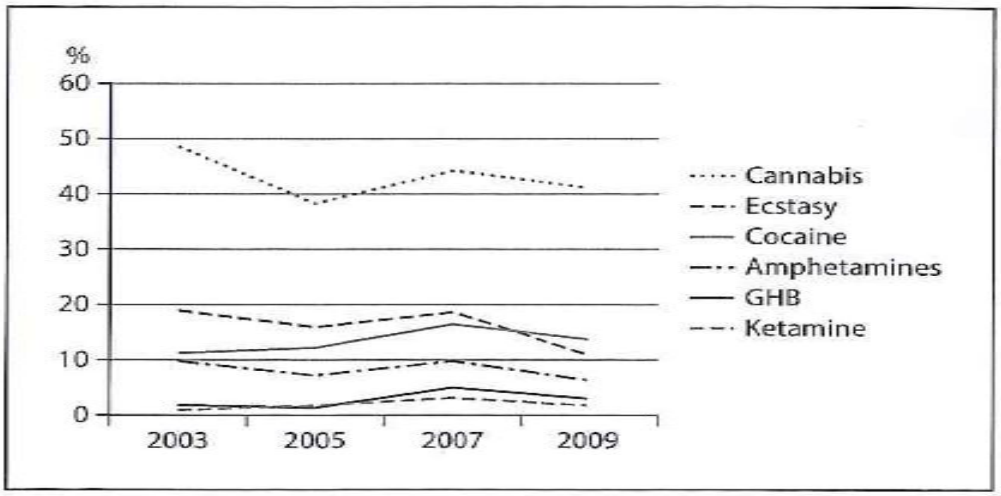

Fig.I.A.4 Last-year drug use over 4 survey years from 2003 to 2009 from a survey administered to explore changes in illicit drug use among music festival goers and dance scene attendees in Flanders. Every 2 years, a sample of party people was selected at 3 clubs, 2 dance events and 2 rock festivals, resulting in a total of 2812 respondents filling out a self-report questionnaire (Figure taken from Van havere *et al*.; 2012, [27]).

One should consider the possibility of a GHB-intoxication in the case of unexplained coma in the absence of a head injury or elevated head pressure. Treatment of GHB overdose is primarily supportive, focussing on preservation of respiratory status, with no known antidote available. Gastric lavage and induction of emesis are contra-indicated since GHB may cause a rapid loss of

consciousness [4]. Also activated charcoal is not recommended to treat GHB intoxication because of the extensive metabolism with a rapid absorption of GHB and because intubation is often required, possibly contributing to aspiration risk [1]. Benzodiazepines can be used to treat seizures, while atropine can be given to manage symptomatic bradycardia [4]. Complete recovery has mostly been observed after 6 - 8 h, with a typical abrupt awakening of the patient [33].

Furthermore, frequent ingestion (every 1 to 3 h, around-the-clock) has been shown to cause long-term problems, including dependence and severe withdrawal symptoms after abrupt discontinuation of the use of GHB or its analogues [29]. The development of chronic dependence is typically seen following GHB use during minimum 2 to 3 months, with a usage of more than 3 to 4 times per day. After discontinuation, withdrawal has been seen with clinical features similar to those seen with ethanol and/or benzodiazepine withdrawal. Most commonly observed withdrawal symptoms include agitation, anxiety, tachycardia, hypertension and delirium. These symptoms begin 1 to 6 h after the last dose, can be very severe within the first day and could last for 5 to 15 days [7,34,35]. Also a prolonged withdrawal state has been reported, lasting 3 to 6 months and characterized by e.g. memory loss and insomnia [36]. Wood *et al.* (2011) recommend to initially manage withdrawal patients with benzodiazepines, ideally administered prior to the development of the withdrawal symptoms. Second-line management options are baclofen and barbiturates [7].

I.A.4 LEGISLATION

Since the late 1990s, the Food and Drug Administration (FDA) banned all non-prescription sales of GHB and illicit GHB is since 2000 a Schedule I agent (Controlled Substances Act) in the US. The FDA has approved in 2002 the use of sodium oxybate for the treatment of narcolepsy with cataplexy (Xyrem®), so it has become available on prescription as a Schedule III agent. Also, in 2005, the European Medicines Agency (EMA) approved sodium oxybate for the treatment of narcolepsy with cataplexy. On the other hand, GBL is a List I controlled chemical, used for the manufacture of a controlled substance -GHB-, making its possession, manufacturing, or selling with the intention for ingestion illegal. The latter also applies to gamma-valerolactone (GVL) and 1,4-BD. So, the congeners of GHB are easily and moreover legally available on e.g. the internet, as long as the use is *not* intended for ingestion [4,21]. In Belgium, the possession, buying and selling of GHB is illegal since 1998, when it became listed as a psychotropic substance [37].

REFERENCES

1. Neijzen R, van Ardenne P, Sikma M, Egas A, Ververs T, van Maarseveen E. Activated charcoal for GHB intoxication: an in vitro study. *Eur J Pharm Sci*. 47(5):801-803 (2012).
2. Sakurada K, Kobayashi M, Iwase H, Yoshino M, Mukoyama H, Takatori T, Yoshida K. Production of gamma-hydroxybutyric acid in postmortem liver increases with time after death. *Toxicol Lett*. 129(3):207-217 (2002).
3. Thai D, Dyer JE, Jacob P, Haller CA. Clinical pharmacology of 1,4-butanediol and gamma-hydroxybutyrate after oral 1,4-butanediol administration to healthy volunteers. *Clin Pharmacol Ther*. 81(2):178-184 (2007).
4. Smith KM, Larive LL, Romanelli F. Club drugs: methylenedioxymethamphetamine, flunitrazepam, ketamine hydrochloride, and gamma-hydroxybutyrate. *Am J Health Syst Pharm*. 59(11):1067-1076 (2002).
5. Abanades S, Farre M, Segura M, Pichini S, Barral D, Pacifici R, Pellegrini M, Fonseca F, Langohr K, De La Torre R. Gamma-hydroxybutyrate (ghb) in humans: Pharmacodynamics and pharmacokinetics. *Ann N Y Acad Sci.* 1074:559-576 (2006).
6. LeBeau MA, Montgomery MA, Miller ML, Burmeister SG. Analysis of biofluids for gamma-hydroxybutyrate (GHB) and gamma-butyrolactone (GBL) by headspace GC-FID and GC-MS. *J Anal Tox*. 24(6): 421-428 (2000).
7. Wood DM, Brailsford AD, Dargan PI. Acute toxicity and withdrawal syndromes related to γ-hydroxybutyrate (GHB) and its analogues γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD). *Drug Test Anal*. 3(7-8):417-425 (2011).
8. Ciolini LA, Mesmer MZ, Satzger RD, Machal AC, McCauley HA, Mohrhaus AS. The chemical interconversion of GHB and GBL: forensic issues and implications. *J Forensic Sci.* 46:1315-1323 (2001).
9. Brenneisen R, Elsohly MA, Murphy TP, Passarelli J, Russmann S, Salamone SJ, Watson DE. Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. *J Anal Toxicol*. 28(8):625-630 (2004).
10. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L. Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. Mol Genet Metab. 109(3):255-259 (2013).
11. Elliott S, Lowe P, Symonds A. The possible influence of micro-organisms and putrefaction in the production of GHB in post-mortem biological fluid. Forensic Sci Int. 139(2-3):183-190 (2004).
12. Petersen IN, Tortzen C, Kristensen JL, Pedersen DS, Breindahl T. Identification of a new metabolite of GHB: gamma-hydroxybutyric acid glucuronide. *J Anal Toxicol.* 37(5):291-297 (2013).
13. Nicholson KL, Balster RL. GHB: a new and novel drug of abuse. *Drug Alcohol Depend*. 63(1):1-22 (2001).

14. Miotto K, Darakjian J, Basch J, Murray S, Zogg J, Rawson R. Gamma-hydroxybutyric acid: Patterns of use, effects and withdrawal. *Am J Addict.* 10(3):232-241 (2001).
15. Carter LP, Pardi D, Gorsline J and Griffiths RR. Illicit gamma-hydroxybutyrate (GHB) and pharmaceutical sodium oxybate (Xyrem): differences in characteristics and misuse. *Drug Alcohol Depend.* 104(1-2):1-10 (2009).
16. LeBeau MA, Montgomery MA, Morris-Kukoski C, Schaff JE, Deakin A. Further evidence of in vitro production of gamma-hydroxybutyrate (GHB) in urine samples. *Forensic Sci Int*. 169(2-3):152-156 (2007).
17. ElSohly MA, Salamone SJ. Prevalence of drugs used in cases of alleged sexual assault. *J Anal Toxicol*. 23(3):141-146 (1999).
18. Andresen H, Stimpfl T, Sprys N, Schnitgerhans T, Müller A. Liquid ecstasy - a significant drug problem. *Dtsch Arztebl Int*. 105(36):599-603 (2011).
19. Zvosec DL, Smith SW, Porrata T, Strobl AQ, Dyer JE. Case series of 226 γ-hydroxybutyrate–associated deaths: lethal toxicity and trauma. *Am J Emerg Med*. 29(3):319-332 (2011).
20. Li J, Stokes SA, Woeckener A. A tale of novel intoxication: seven cases of gamma-hydroxybutyric acid overdose. *Ann Emerg Med*. 31(6):723-728 (1998).
21. Anderson IB, Kim SY, Dyer JE, Burkhardt CB, Iknoian JC, Walsh MJ, Blanc PD. Trends in gamma-hydroxybutyrate (GHB) and related drug intoxication: 1999 to 2003. *Ann Emerg Med*. 47(2):177-183 (2006).
22. EMCDDA website: http://www.emcdda.europa.eu/publications/edr/trends-developments/2013, accessed on 30 October 2013.
23. Villain M, Cirimele V, Ludes B, Kintz P. Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and urine by gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 792(1):83-7 (2003).
24. Van Amsterdam JGC, Brunt TM, McMaster MTB, Niesink RJM. Possible long-term effects of γ-hydroxybutyric acid (GHB) due to neurotoxicity and overdose. *Neurosci Biobehav Rev*. 36(4):1217-1227 (2012).
25. Schep LJ, Knudsen K, Slaughter RJ, Vale JA, Mégarbane B. The clinical toxicology of γ-hydroxybutyrate, γ-butyrolactone and 1,4-butanediol. *Clin Toxicol*. 50(6):458-470 (2012).
26. Wood DM, Nicolaou M, Dargan PI. Epidemiology of recreational drug toxicity in a nightclub environment. *Subst Use Misuse.* 44:1495-1502 (2009).
27. Van Havere T, Lammertyn J, Vanderplasschen W, Bellis M, Rosiers J, Broekaert E. Illicit drug use in the Flemish nightlife scene between 2003 and 2009. *Eur Addict Res*. 18(4):153-60 (2012).
28. Kim SY, Anderson IB, Dyer JE, Barker JC, Blanc PD. High-risk behaviors and hospitalizations among gamma hydroxybutyrate (GHB) users. *Am J Drug Alcohol Abuse*. 33(3):429-438 (2007).

29. Brunt TM, Koeter MW, Hertoghs N, van Noorden MS, van den Brink W. Sociodemographic and substance use characteristics of gamma hydroxybutyrate (GHB) dependent inpatients and associations with dependence severity. *Drug Alcohol Depend*. 131(3):316-319 (2013).
30. Horyniak D, Degenhardt L, Smit DV, Munir V, Johnston J, Fry C, Dietze P. Pattern and characteristics of ecstasy and related drug (ERD) presentations at two hospital emergency departments, Melbourne, Australia, 2008-2010. *Emerg Med J*. [Epub ahead of print] (2013).
31. Krul J, Girbes AR. γ-Hydroxybutyrate: experience of 9 years of γ-hydroxybutyrate (GHB)-related incidents during rave parties in The Netherlands. Clin Toxicol. 49(4):311-5 (2011).
32. van Amsterdam JG, van Laar M, Brunt TM, van den Brink W. Risk assessment of gamma-hydroxybutyric acid (GHB) in the Netherlands. *Regul Toxicol Pharmacol*. 63(1):55-63 (2012).
33. Van Sassenbroeck DK, De Neve N, De Paepe P, Belpaire FM, Verstraete AG, Calle PA, Buylaert WA. Abrupt awakening phenomenon associated with gamma-hydroxybutyrate use: a case series. *Clin Toxicol*. 45(5):533-538 (2007).
34. Dyer JE, Roth B, Hyma BA. Gamma-hydroxybutyrate withdrawal syndrome. *Ann Emerg Med*. 37(2):147-153 (2001).
35. Wojtowicz JM, Yarema MC, Wax PM. Withdrawal from gamma-hydroxybutyrate, 1,4-butanediol and gamma-butyrolactone: a case report and systematic review. *CJEM*. 10(1):69-74 (2008).
36. Snead OC, Gibson KM. Y-hydroxybutyric acid. *N Engl JMed*. 352(26):2721-2732.
37. Koninklijk besluit tot houdende regeling van sommige psychotrope stoffen en betreffende risicobeperking en therapeutisch advies. 22 januari 1998, art 2. http://www.ejustice.just.fgov.be/cgi_loi/change_lg.pl?language=nl&la=N&cn=1998012251&table_name=wet, accessed on September, 11th 2013.

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CHAPTER I.B

SCREENING AND CONFIRMATION METHODS TO DETERMINE GHB IN BIOLOGICAL FLUIDS

Based on

Screening and confirmation methods to determine gamma-hydroxybutyric acid in biological fluids. Ann-Sofie ME Ingels, Willy E Lambert, Sarah Wille, Nele Samyn and Christophe P Stove. *Manuscript in preparation*

I.B.1 INTRODUCTION

Various bioanalytical methods for GHB determination have been reported since the early 1970s [1-4]. This review will focus primarily on those methods published since the 1990s, when there was an emerging need for analytical methods to measure GHB in biological fluids as part of toxicological investigations, given the first reports of GHB abuse appearing in the US [5]. Also trends, advantages and disadvantages of sample preparation and analytical techniques are discussed. First, according to the generally applied strategy in toxicology, the so-called systematic toxicological analysis (STA), screening techniques including e.g. colorimetric and enzymatic tests will be discussed. These differentiate between (presumably) positive and negative GHB samples. Positive GHB results are then confirmed using a second, independent method, mostly involving quantitation [6]. This has been achieved mostly by gas chromatography (GC), although also liquid chromatography (LC) and capillary zone electrophoresis (CZE) have been applied.

I.B.2 ANALYTES OF INTEREST

Although in a toxicological context it might be relevant to determine whether GHB positivity is the result of the intake of GHB, GBL or 1,4-BD, GHB remains the most important analyte to search for in biological fluids, owing to the fast *in vivo* biotransformation of its precursors [7,8]. Also in fatalities involving consumption of large amounts of these precursors, high GHB and only low GBL and 1,4-BD levels have been observed [9]. Other compounds that might be of interest to determine simultaneously (in the same run) are the positional isomers and isobaric compounds alpha- and beta-hydroxybutyric acid (AHB, BHB) (diabetic and post-mortem cases) [10-13], glycols (in emergency cases with coma of unknown origin when ingestion of GHB or ethyleneglycol is suspected, the latter also causing high anion gap metabolic acidosis) [13-15] and other club drugs such as MDMA or ketamine [16], as well as gamma-hydroxyvaleric acid (GHV) or its lactone, GVL (reported to be a GHB alternative) [9,17].

It needs to be kept in mind that a quantitative result may be influenced by the *in vitro* interconversion of GHB and GBL in aqueous matrices, the equilibrium depending on pH and temperature [18]. Therefore, several methods have evaluated the rate of GHB/GBL conversion during sample treatment or analysis, with different outcomes. Overall, three scenarios have been described. First, conversion was complete in one direction and was used for GHB determination [e.g. 19-21]; secondly, conversion did not occur, so absolute GHB was measured [e.g. 13,14,22];

lastly, conversion occurred but was minimal, with little or no relevance in the forensic or clinical setting [e.g. 23,24]. Therefore, the method of analysis needs to be considered when comparing existing data from e.g. post-mortem analyses. In methods involving conversion to GBL, slightly higher GHB concentrations may be observed (measured as total GBL) than in methods determining absolute GHB. This may be due to the conversion of a proportion of the (endogenous) GHB present in a post-mortem plasma or urine sample to GBL during storage, depending on sample pH [25,26]. Furthermore, if GHB is determined as GBL, samples can be analyzed in duplicate, one with and one without acidic treatment to convert GHB to GBL [e.g. 19]. Similarly, total GHB (GHB+GBL) can be determined, if actual GBL is converted completely to GHB before analysis [20,21].

I.B.3 GHB CONCENTRATIONS & BIOLOGICAL MATRICES OF INTEREST

As mentioned above, the natural presence of GHB results in measurable baseline levels in various biological matrices. Studies have been conducted in e.g. urine [e.g. 26-30], plasma [e.g. 29], serum [e.g. 30] whole blood [e.g. 10,28,29] and oral fluid [e.g. 31] samples obtained from healthy non-users. Also data from non-GHB related fatalities [e.g. 25,32], together with concentrations arising from exogenous administration have been collected. Ingestion can be intentional - for recreational use - or accidental, which both may lead to overdoses or even fatalities, illustrated by several case reports [e.g. 33-36]. Physiological concentrations of GHB, situated in the low and sub-microgram-per-milliliter range, are mostly well below concentrations found in intoxicated patients, where a narrow range exists between recreational doses and overdoses. An overlap between highly toxic and lethal concentrations has been observed, demonstrating high inter-individual variability between measured GHB concentration and effect [37,38]. According to the list of therapeutic and toxic concentrations from The International Association of Forensic Toxicologists (TIAFT), a value above 280 µg/ml of GHB in plasma may be sufficient to cause death [39]. In addition, *in-vitro* production during storage, especially in post-mortem blood samples, has been reported, further complicating the interpretation of a GHB concentration. Therefore, an appropriate storage of samples until analysis is required (recommendation: - 20 °C) [37,38]. For more detailed information concerning GHB production in post-mortem cases, we refer the interested reader to existing literature [25,32,40,41].

To differentiate between endo- and exogenous concentrations [29], cut-off levels have been established. Most authors agree on a 10 µg/ml cut-off level for GHB in ante-mortem urine [28,29,42], although suggestions of 5 [43] or 6 [30] µg/ml have been made as well. For ante-

mortem whole blood, 10 [22,29], 5 [28] or 4 [30] µg/ml has been proposed as a cut-off, while one group even proposes 1 µg/ml, if appropriate storage is guaranteed [10]. This implies that screening and confirmation methods for GHB in ante-mortem urine, whole blood and plasma preferably have a a decision limit or lower limit of quantification (LLOQ) below or equal to 4 or 5 µg/ml. Higher cut-off levels have been proposed for post-mortem matrices (20 and 30 for urine, 50 for whole blood and 12 µg/ml for vitreous humour) to exclude false positives [15,22,32]. For following up GHB concentrations in Xyrem® patients, a wide concentration range may be necessary, depending on the timing of sampling (shortly after intake *vs.* several hours later) [44]. Endogenous presence of GHB in various biological matrices not only renders true blank matrices unavailable for conducting method validation experiments, it also precludes the use of low calibrators (< 1 µg/ml) prepared in authentic matrices [11], and complicates the interpretation of a positive result.

As an alternative to the use of interpretative cut-off concentrations, continuous-flow GC-combustion-isotopic ratio MS has been used to discriminate between exogenous (i.e. synthetic) and endogenous GHB in blood samples. First findings suggest differences in the ^{13}C and ^{12}C content of the endogenous and synthetic form of GHB [45]. However, it is obvious that the cost and complexity associated with this high-end technique strongly limits its general applicability.

In addition to the endogenous presence and possible instability during storage, samples must be collected as soon as possible after ingestion, due to extensive metabolism of GHB once ingested orally (plasma $T_{1/2}$ less than 1 h) [46-48]. Otherwise, the GHB level will drop in blood and urine to endogenous concentrations within 6 to 12 h following intake, no longer allowing to prove intake of GHB, possibly leading to an underestimation of the total number of positive cases [22]. Therefore, alternative sampling strategies and alternative matrices have been evaluated. These include dried blood spots (DBS), i.e. capillary whole blood obtained by fingerprick, facilitating sample collection, as well as non-conventional matrices such as sweat and oral fluid. Only moderate results have been obtained in the latter two matrices since diffusion of the acidic drug in these has been shown to be limited. Following GHB intake (50 mg/kg sodium GHB, n=5), only 1/4 to 1/3 of the concentration found in plasma was measured in oral fluid, with an even quicker return to baseline values and high oral fluid/plasma inter-variability, while in sweat, GHB concentrations were only slightly higher than baseline values [24,48-52]. On the other hand, hair analysis has been shown useful to extend the window of detection, because of incorporation of GHB in the hair matrix. A case report has described detection even after a single use in a case of DFSA [53]. Also in hair,

endogenous GHB is present, often rendering it difficult to draw straightforward conclusions [54]. Therefore, small segments are analyzed to detect an elevation of the baseline GHB concentration owing to exogenous ingestion [54,55].

More than 95 % of an oral dose of GHB is converted to CO_2 and H_20 as it enters the Krebs cycle *via* succinate, with less than 5 % being excreted 'unchanged' in urine [46]. Until recently, no specific metabolites of GHB were known. However, Petersen *et al.* (2013) [56] demonstrated the existence of a new metabolite, GHB-glucuronide, in urine, in concentrations ranging from 0.11 to 5.0 µg/ml. Although more research such as pharmacokinetic studies following GHB administration are required, this compound is theoretically a biomarker of GHB exposure with the potential to extend the window of detection in the conventional matrix urine [56].

I.B.4 SCREENING PROCEDURES FOR THE PRESENCE OF GHB IN BIOFLUIDS

A good screening procedure allows the identification of unknown analytes in a simple, sensitive, selective and rapid way, starting from a minimal amount of sample. STA approaches typically utilize immuno- and/or enzymatic assays to screen for analytes or categories of compounds, next to GC-mass spectrometry (GC-MS) or high performance liquid chromatography- diode array detection (HPLC-DAD) for high-throughput screening for simultaneous detection of as many toxic compounds as possible. Liquid chromatography-mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) and high resolution techniques have been used to a lesser extent for such comprehensive screening but are becoming of more and more interest nowadays, sometimes even replacing the immunological and/or enzymatic tests [6,57,58]. Below, an overview of possible screening procedures for GHB is given, starting with colorimetric tests. Given the lack of commercially available immunoassays, STA using chemical analyzers did not include GHB until 2009 [12,22]. Since then, an enzymatic assay adaptable to common analyzers has become commercially available (Bühlmann laboratories, Switzerland) [59]. Furthermore, several GC methods became available and recently LC-MS/MS-based methods have been reported with the focus on high-throughput, so both techniques can therefore also be used as screening tool. A screening method preferably has a decision limit (cut-off of the applied assay) at or below the exogenous/endogenous cut-off, to allow for a reliable first differentiation between samples considered to be GHB-positive or -negative. However, since moderately to severely intoxicated GHB patients such as those brought to an emergency department in comatose state will mostly display GHB concentrations well above these cut-off levels we also consider in this review methods

with decision limits/LLOQs (well) above these cut-offs as screening methods. As with any screening test, a positive result should only be considered preliminary and needs to be confirmed using an independent, preferentially MS-based, technique such as GC- or LC-MS (/MS).

I.B.4.1 COLORIMETRIC TESTS

Badcock and Zotti [60] reported a colorimetric test that allows the identification of GHB in human urine based on the conversion of GHB to GBL. Briefly, following the addition of concentrated sulphuric acid, ammonium sulphate and nitroprusside to 250 µl of urine, an intense and instant blue/olive-green colour will appear if GHB is present in the sample [60]. Another colorimetric test, a modification of the ferric hydroxamate test for ester detection, only requires 5 min to detect GHB in 0.3 to 1 ml urine, the presence of GHB being indicated by purple colouring of the sample [61]. Although both colorimetric tests are simple and results can be obtained in less than 10 min, the prime disadvantage is the lack of sensitivity, with limits of detection of 100 or even 500 µg/ml [60,61].

I.B.4.2 ENZYMATIC ASSAYS

Enzymatic assays to determine GHB are based on the oxidation of GHB to SSA, a reaction that occurs during metabolization *in vivo via* the enzyme GHB-DH.

I.B.4.2.1 Colorimetric enzymatic assays

Bravo *et al.* (2004) [62] developed a solution-endpoint- and a dipstick-assay for the determination of GHB in human urine. The identification was possible by coupling the oxidation reaction of GHB, *via* a cloned and isolated GHB-DH, to a reduction reaction of a tetrazolium pro-dye, resulting in the formation of a colored product (absorbance at 450 nm). Although these tests are easy to perform, providing enough sensitivity remains a critical issue, only ensuring 100 % true positives when a minimum of 100 µg/ml of GHB is present in urine.

Another test strip, commercially available by Drugcheck®, can detect GHB in human urine with a cut-off level of 10 µg/ml. Results are obtained within 10 min and a colour chart on the test strip has to be used for interpretation, next to a test strip for vitamin C, this compound showing cross-reactivity with the GHB test. Although this GHB test strip is more sensitive, detecting lower GHB concentrations, only a preliminary result is provided, without indication of the degree of intoxication [63].

I.B.4.2.2 Enzymatic kit

It has become clear from the tests mentioned above that there was an urgent need for a rapid and simple screening method to detect GHB in urine and serum samples in a more sensitive and semi-quantitative way. To this end, an enzymatic kit was commercialized in 2009 [59]. This kit also utilizes a recombinant GHB-DH to oxidize GHB to SSA, while the co-factor nicotinamide adenine dinucleotide (NAD^+) is simultaneously reduced to NADH + H^+, which absorbs at 340 nm. The test is adaptable to common clinical chemistry analyzers and requires only 10 µl of sample. Quantification is performed using 2 calibrators and 2 quality controls provided by the manufacturer, with a working range from 5 to 250 µg/ml. Results are obtained in about 10 minutes and interferences as well as cross-reactivities have been evaluated. A 4 % interference of GBL has been observed, which is stated to have no relevant implication since GBL is rapidly converted to GHB once ingested. Also per 1.06 g/L ethanol, a 3.0 µg/ml linear increase of false-positive GHB concentration was observed, so GHB concentrations of 8-20 µg/ml need careful interpretation, especially since GHB is commonly ingested with alcoholic beverages. A cut-off level of 10 µg/ml for serum and 15 µg/ml for urine has been proposed [64,65].

Grenier *et al.* (2012) [66] evaluated the use of this enzymatic assay as a screening method in forensic matrices including whole blood and vitreous humour. When correlating the results of a variety of cases (sexual assaults, impaired drivers and deaths) with a GC-MS reference method, no false negatives and few false positives were observed, with post-mortem samples appearing to be more prone to testing false positive than ante-mortem samples. Although whole blood required protein precipitation with acetonitrile before analysis, analyst time savings can still be substantial compared to chromatography-based procedures. In addition, although very efficient GC-MS and LC-MS/MS procedures have been developed for GHB, integration with a battery of other tests on automated analyzers makes this assay valuable for *(clinical)* toxicology labs. However, Grenier *et al*. (2012) [66] found that a limitation of this test is that it may not be applicable to alternative matrices such as e.g. vitreous humour due to the observed high rate of false positives.
In summary, this test may be valuable for screening urine and serum samples in an emergency setting, for forensic applications and for other screening purposes [65].

I.B.4.3 OTHER SCREENING TECHNIQUES

^{1}H nuclear magnetic resonance (NMR) spectrometry has been used to detect GHB in urine and serum [67], as well as in oral fluid (600 µl) [57]. This technique is non-destructive and has little or

no sample preparation requirements, and is therefore less labour-intensive than other techniques. Similarly, ion mobility spectrometry (IMS) showed promise as a screening method for GHB and related compounds present in urine samples [68]. Via direct injection using a split/splitless injection port and thermal desorption, the sample was brought directly into the IMS configuration without chromatographic separation, reducing analysis time and resulting in an estimated detection limit of 3 µg/ml.

In addition, CZE with indirect ultraviolet (UV) detection is capable of detecting high concentrations of GHB in urine samples following a simple 1:4 dilution with water. Calibration curves ranged from 80 to 1280 µg/ml [69]. For detection, indirect UV absorption using a chromophore in an electrolyte solution was necessary because the native molecule GHB has poor UV absorption [70,71]. Small adaptations of analytical conditions (co-ion, pH, etc.) further improved method sensitivity and selectivity and enabled the analysis of not only urine but also serum samples following 1:8 dilution with 3 mM NaOH, completely converting GBL to GHB (calibration curve ranged from 25 to 500 µg/ml) [72]. Although accurate and precise results may be obtained using CZE, the LLOQ is relatively high (ranging from 25 to 80 µg/ml and 5 to 60 µg/ml, dependent on urine density), when compared with chromatographic techniques (LLOQ ranging from 0.1 to 8 µg/ml). Therefore, these CZE-based methods are considered to be more suitable as an alternative screening method for a GHB overdose, being rapid and simple, rather than as a secondary confirmatory method.

I.B.4.4 CHROMATOGRAPHIC SCREENING TECHNIQUES

When compared to colorimetric and enzymatic assays, chromatographic assays typically require more intensive and time-consuming sample preparation such as derivatization or conversion to GBL (see below). For example, Lebeau *et al.* (2000) [19] opted for a gas chromatography - flame ionization detection (GC-FID) screening method using headspace (HS) as injection technique following conversion of GHB to GBL, while confirmation of GHB (as GBL) was done by GC-MS. Also, in clinical practice, where the aim is to define a medical diagnosis and start a treatment, a non-specific detection such as GC-FID is sufficient, as stated by Blanchet *et al.* (2002) [21]. These authors determined GHB following derivatization with BF_3-butanol.

Similarly, urinary organic acid assays based on silylation and GC-MS, more readily available than GHB assays in hospital laboratories, were investigated for their use to detect GHB in urine samples. However, if these methods included acidification of the samples during sample treatment, which

favours conversion of GHB to GBL, only a small peak of GHB was visible, as can be expected [73]. In addition, silylated urea may elute closely to/co-elute with silylated GHB, having in addition similar MS properties. Therefore, it may be important to eliminate the urea interference by adding an urease treatment step to the sample preparation procedure, enabling the identification of GHB with higher confidence [74-76].

In addition, chromatographic methods used to screen for various compounds including GHB have been reported. Rasanen *et al.* (2010) [77] developed a headspace in-tube extraction GC-MS method to screen for hydroxylic methyl-derivatized organic acids, including GHB, in urine and extracted whole blood samples. In addition, a GC-MS method for the simultaneous screening in urine of 128 date-rape drugs, including GHB, 1,4-BD and GBL (using silylation), has been reported by Adamowicz and Kala (2010) [78]. Recently, an LC-MS/MS method has been reported to screen for elevated GHB concentrations in DBS obtained from newborns, to diagnose SSADH deficiency, a rare inherited metabolic disorder where GHB concentrations are increased because of a deficiency of the succinic semi-aldehyde dehydrogenase enzyme, responsible for conversion of SSA to succinate [50]. Although not intended for toxicology purposes, this methodology may also be applicable to screen DBS for exogenous GHB [79,80].

Next to these screening methods, several authors have reported simplified and rapid procedures to determine GHB with high-throughput, leading to the possibility of using actual confirmation methods also as a screening tool. Here, we mention only examples of these methods in which sample preparation is reduced or minimal. Details can be found in the next section and in Table I.B.1. For example, Van hee *et al.* (2004) [14] determined GHB (and glycols) in low volume plasma and urine samples (20 µl) using GC-MS, by adding an excess silylation reagent directly to the biological sample. This procedure was recently modified by Meyer *et al.* (2010) [13], utilizing micro-wave assisted derivatization, another approach particularly useful in hospital laboratories of emergency departments, as quantitative results for urine samples can be obtained within 30 min using one-point calibration. Other examples of procedures with minimal hands-on time are those where derivatization reagents are applied directly “on spot”(in the case of DBS) or “in-vial” (in the case of HS-sampling) [20,24,49]. More recently, a multi-analyte ultra high performance LC-MS/MS (UHPLC-MS/MS) method has been reported, which may also be useful as a screening tool because of the easy sample preparation and resulting high-throughput [81].

I.B.5 CONFIRMATION METHODS FOR CLINICAL AND FORENSIC CASES

Methods suitable for the confirmation of a presumed GHB-positive sample have preferably a LLOQ below or at the proposed cut-off level, should be selective for GHB and if they deliver quantitative results, these should be reliable and accurate. Since it may be necessary to confirm the presence of GHB in more complex biological matrices and because more sophisticated chromatographic techniques are used, sample preparation becomes more important. Sample work-up is usually more complicated than that used for colorimetric or enzymatic methods, which are primarily suited for urine and serum. Below, an overview of commonly used sample preparation procedures is given, followed by an overview of the used analytical techniques to separate and detect GHB (and analogues). Table I.B.1 provides an overview of the different published procedures (at the end of this chapter). To evaluate if a given method allows differentiation between exo- and endogenous GHB, the calibration range with the quantification limit is included. Also the choice of internal standard may influence the data quality and has therefore also been mentioned in the table [82]. As shown in the table, several compounds showing similarity with GHB have been used as internal standard. In MS-based methods, the use of a deuterated internal standard is recommended to compensate for variations during sample preparation, as well as during analysis. The deuterated form of GHB, GHB-d6, has been used widely for this purpose; a C-labelled internal standard is not commercially available (yet).

I.B.5.1 SAMPLE PREPARATION

The following techniques have been applied to treat biofluids, either alone but mostly combined: dilution, filtration, deproteinization, chemical modification, liquid-liquid extraction (LLE), solid-phase extraction (SPE), and HS extraction. These sample preparation procedures are often regarded as time-consuming and there has been a tendency to reduce manual sample handling by introducing new, fully automated techniques. It should be mentioned that the latter implies longer method development times and new skill requirements and may not always be implementable in smaller laboratories [83]. Furthermore, starting from the more traditional procedures, simplified extractionless procedures have been proposed such as dilution and direct derivatization (“on spot” and “in-vial”), together with micro-wave assisted derivatization and on-line derivatization techniques such as injection port derivatization. Some of these simplifications have been made possible due to improved separation and detection techniques such as tandem MS, resulting in procedures with minimal hands-on time. In addition, initial sample volume required for analysis may be reduced without loss of method sensitivity.

I.B.5.1.1 Dilution and filtration of the biological fluid

Using appropriate separation and detection techniques, simple dilution of urine and serum samples, with or without subsequent filtration, may be sufficient as sample preparation [12,81,84]. This has been demonstrated by several LC-MS/MS methods, capable of quantifying GHB with sufficient sensitivity in these matrices. In addition, possible extraction difficulties arising from the hydrophilic nature of GHB are avoided. For example, urine has been diluted 1:20 [12] and 1:1 [81] with water, and 1:10 with acidic 10 % MeOH [84] prior to LC-MS/MS analysis. Alternatively, urine and serum samples have been diluted 1:4 with a buffer solution prior to CZE analysis with contactless conductivity detection (CZE-C^4D) [85].

Important to note is that sufficient sensitivity has been obtained, as illustrated by Wood *et al*. (2004) [12], who compared method sensitivity (reflected in signal-to-noise (S/N)) of two sample pretreatment procedures prior to LC-MS/MS analysis. The first of these procedures consisted of a 1:20 dilution of urine samples (with deionised water containing internal standard); the second sample clean-up was based on a more time-demanding SPE extraction (OASIS® cartridges). Although an approximately 2-fold increase in sensitivity was observed when using the SPE cartridges, the authors were still able to use the simpler dilution method as it readily enabled measurement of endogenous GHB levels.

Although this dilute-and-shoot approach is simple and convenient, with minimal hands-on time, assessment of matrix effects is strongly advised, as in any LC-MS/MS-based procedure, because matrix components may strongly influence the ionization of GHB in the MS source. To compensate for any effect owing to the matrix, a stable isotopically-labelled internal standard should be included in the procedure [12]. Assuming a similar degree of alteration of the response of this internal standard, matrix effects can be compensated for.

In addition, samples have been diluted to reduce the influence of the original matrix during sample treatment and analysis. For example, the effect of the matrix during solid-phase micro-extraction (SPME) may be reduced by diluting the original sample [16,86]. Similarly, samples were diluted with water before LLE [87,88] or SPE [81,89-91]. For example, Elian *et al*. (2011) [89] assumed that a 50-µl sample and synthetic urine, consisting of inorganic salts and proteins in an aqueous medium, would act in a similar way as 50-µl deionized water, if these were all diluted in 4 ml of

water prior to SPE. As a consequence, calibrators and controls could be prepared in water instead of blank matrix, which is especially of interest for GHB, endogenously present in human samples.

I.B.5.1.2 Deproteinization of the biological fluid

For compounds with low protein binding such as GHB, protein precipitation is an adequate and easy technique to remove a variety of interferences present in blood and plasma, such as blood cells, proteins and lipids prior to analysis [10,81]. To illustrate, Shima *et al*. (2005) [10] compared protein precipitation with various SPE and LLE techniques to clean up urine samples, and found that protein precipitation led to the highest GHB recovery and cleanest chromatograms. Generally, there are 4 protein precipitation techniques: organic solvents, acids, salts and metal ions [92]. To our knowledge, for GHB analysis, only the first 3 have been applied.

As organic solvent, acetonitrile [e.g. 13,15,22,93,94-96], methanol (MeOH) [10,97] or a combination of both (ice-cold acetonitrile:MeOH 85:15 v:v [81,91]) and acetone [9,98] have been used. Also water:MeOH (3:97, v:v) has been used [99]. Placing samples in a freezer for at least 10 min before centrifugation may help to obtain complete precipitation [81].

Organic solvents and acids have also been used simultaneously to improve protein precipitation and GHB recovery. For example, the addition of sulphuric acid during protein precipitation using acetonitrile was found to increase the recovery of GHB from 50 to 90 % [11,46,48]. Similarly, acidified methanol has been used for whole blood protein precipitation [84].

Cold perchloric acid [5,7,34] has been added alone to plasma samples, thereby combining deproteinization and lactone-formation (see below). Finally, anhydrous sodium sulphate (Na_2SO_4) has been added to biological fluids prior to HS-trap analysis for protein precipitation and for salting-out purposes [20].

Similar to the above mentioned dilute-and-shoot approach, it is important to evaluate matrix effects, particularly when protein precipitation is (almost) the only sample preparation technique prior to LC -MS/MS analysis. For example, when blood or serum samples were subjected to protein precipitation, with subsequent centrifugation and 1:1 dilution of the supernatant with acidified water prior to LC-MS/MS analysis, about 40 % suppression of the GHB signal was observed. Again, the use of a deuterated internal standard such as GHB-d6 is emphasized, since it is able to

compensate for the matrix effect [84]. To our knowledge, only three sample preparation procedures merely consist of protein precipitation prior to LC-MS/MS injection, with only two evaluating matrix effects [95,96,99]. For example, one procedure that did evaluate matrix effects saw a 16 to 27 % enhancement of GHB ionization which was compensated for by the use of GHB-d6 [99].

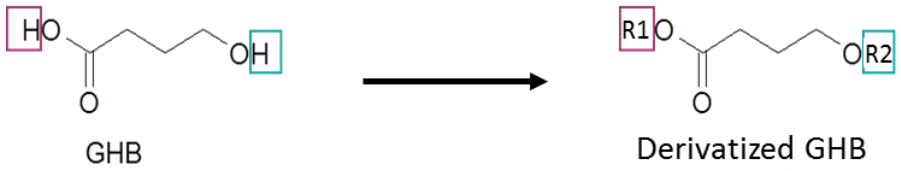
I.B.5.1.3 Chemical modification of GHB

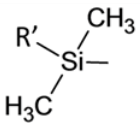
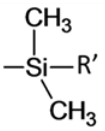
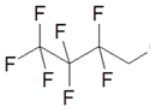
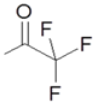
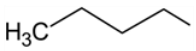
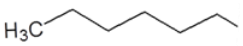
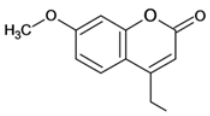
Because GHB is a polar (sometimes anionic) molecule, with lactone-formation seen at high injector-port temperatures or induced chemically (at low pH), chemical modification prior to GC is necessary for reliable quantification [57,100]. For GC analysis, two major strategies have been applied: either lactone-formation through the addition of acid, or derivatization using various derivatization reagents. Formation of a derivative prior to LC analysis and detection has proven to be useful as well; however, it has been applied to a lesser extent when compared to its use in GC-based applications. Both strategies – lactone formation and derivatization – may improve extraction properties of GHB and/or chromatographic analysis, as discussed more into detail below. Fig. I.B.1 gives a schematic overview of possible derivatization procedures, with resulting derivatives of GHB, using common derivatization reagents.

I.B.5.1.3.1 Chemical modification to improve extraction

GHB undergoes intra-molecular esterification within minutes in acidic environment [18] and the GBL formed is more easily extracted from biological matrices than GHB. Lactone-formation has been accomplished by the addition of 6 N hydrochloric acid (urine samples [5,7]; [101]), concentrated sulphuric acid [19,102], 20 % trifluoroacetic acid [103] or 1.6 or 0.8 N perchloric acid (plasma [5,7,34]) to plasma, urine or oral fluid samples. By adding perchloric acid to plasma samples, lactone-formation and deproteinization were performed in a single step. Subsequently, the acidified sample or the supernatant following centrifugation [5,7,34] were placed at room temperature [101,102] for 5 min [19], at 80 °C for 20 min [5,7] or at 75 °C for 1 h [103] to allow for complete conversion. As a consequence, differentiation between the initial GBL present in the sample and the GBL formed as a result of the acid-induced cyclization of GHB is no longer possible [87], unless two aliquots of the same sample are analyzed, one with and one without acid treatment [7,19].

In addition, derivatization reagents suitable for “in-situ” or “in-vial” derivatization can be added directly to the sample matrix, thereby allowing the samples to be analyzed by solid-phase micro extraction (SPME) or HS-based extraction (and injection) techniques by forming a more volatile derivative of GHB. Hexylchloroformate, in the presence of the catalyst pyridine, has been used to derivatize the carboxylic group of GHB [16,86], while dimethylsulphate (DMS) in alkaline medium has been utilized to modify both functional groups [20].



Type of derivatization	R1	R2
Silylation *Reagents:* *R'= -CH_3 e.g. BSTFA, MSTFA* *R'=-$C(CH_3)_3$ e.g. MTBSTFA*		
Methylation *Reagents: e.g. dimethylsulphate*		
Alkylation/Acetylation *With TFAA/HFB-OH*		
Butylation *With n-butanol*		-H
Alkylchloroformation *Reagents: e.g hexylchloroformate*		-H
To enable fluorescence detection *Reagents: e.g. Br-MMC*		-H

Br-MMC: 4-bromomethyl-7-methoxy coumarin; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; HFB-OH: heptafluorobutanol; MSTFA: N-methyl-N-(trimethylsilyl)-trifluoroacetamide; MTBSTFA: N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide; TFAA: trifluoroacetic acid anhydride

Fig. I.B.1 Overview of the applied derivatization procedures for GHB determination

To overcome the difficulties seen when extracting the hydrophilic and small analyte GHB in those methods requiring derivatization, extractionless derivatization procedures have been reported. In addition, sample preparation time, as well as organic solvent waste is reduced. Van hee *et al*. (2004) [14] were the first to report on an extractionless sample preparation, based on the direct derivatization of GHB in biofluids with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The addition of excess derivatization reagent to a 20 µl sample of biofluid (serum, plasma, urine) resulted in a simple and fast method with sufficient sensitivity for routine toxicological analysis. Similarly, starting from 1 µl oral fluid, an extractionless procedure with direct derivatization has been reported, allowing determination of exogenous GHB concentrations [51]. Furthermore, GHB has been derivatized directly ("on spot") in DBS with a mixture of trifluoroacetic acid (TFAA) and heptafluorobutanol (HFB-OH), thereby omitting the extraction step [24,49].

I.B.5.1.3.2 Chemical modification to improve chromatographic analysis and detection

Apart from improving or facilitating extraction, chemical modification may also improve chromatographic analysis and detection. GC properties of GHB are improved by conversion to its more volatile and stable lactone-form GBL, achieved by applying the same procedures as to improve extraction *via* GBL formation (see above). Secondly, various derivatization reagents have been used to increase its molecular weight, at the same time decreasing its polarity, thereby enhancing volatility, separation efficiency and/or selectivity, and consequently, method sensitivity.

As shown in Table I.B.1, silylation is widely used to derivatize GHB off-line in GC-based applications. Mainly BSTFA [e.g. 9,10,11,13,14,22,23,29,30,40,42,43,46,48,51,73,93,94,104-107,109] has been applied, next to N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) [87,97,107] and N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) [15,88]. Using these reagents, the hydroxyl and carboxyl functional groups of GHB are derivatized simultaneously, thereby avoiding lactone-formation since no acidic conditions are used [10]. Moreover, to avoid GBL formation and GHB losses during evaporation, Kimura *et al.* (2003) [73] alkalinized urine samples prior to derivatization, producing the non-volatile salt form of GHB. Furthermore, the resulting di-trimethylsilylderivative of GHB (Fig. I.B.1) can be injected directly into the GC-MS, without removal of excess reagent. Of course, the latter requires increased maintenance of the injection port and MS source to prevent contamination between runs. Another issue is the possibility of co-eluting di-TMS urea, requiring baseline separation of GHB and the urea di-TMS derivative under the GC

conditions used. As silylating reactions mostly require heating for 5 to 30 min, injection-port [88] and micro-wave assisted silylation [13] may offer valuable alternatives to reduce technical time. Also the aforementioned derivatization reactions improve chromatographic analysis and detection.

Although derivatization is primarily known for its use in GC applications, it may also be applied in LC-based separations. For example, to allow fluorescence detection, the carboxylic group has been derivatized by adding 4-bromomethyl-7-methoxy coumarin (Br-MMC) to an aqueous-free sample residue in the presence of dibenzo-18-crown-6-ether acting as a catalyst to improve the reaction yield [71]. Furthermore, butylation of the carboxylic function of GHB using HCl n-butanol improved detection with ion-trap mass spectrometry [110].

I.B.5.1.4 Liquid-liquid extraction (LLE) of GHB or GBL

I.B.5.1.4.1 Liquid-liquid extraction of GHB

Solvents commonly used to extract GHB from biological fluids include ethylacetate [29,30,40,42,43,88,104-110], t-butylmethylether [87,110] and hexane [23]. Because the physical properties of GHB make it a poor candidate for LLE, various approaches to enhance the transfer of GHB to the organic solvent have been reported. GHB has to be in its uncharged or neutral form to obtain an optimum extraction yield and selectivity, influenced by the choice of solvent, pH and additives [83]. Therefore, the charge of the carboxylic group (pKa 4.6-4.8) has been influenced by the addition of 0.1 M HCl or cold 0.1 N H_2SO_4 to urine, serum and blood samples, enhancing its transfer to ethylacetate [42,104,110]. Also, for whole blood samples, Pan *et al.* (2001) [15] reported the use of water scavenging material such as dimethoxypropane and N,N-dimethylformamide (80:20) to facilitate GHB extraction. Furthermore, salting-out approaches have been reported, whereby the ionic strength of the aqueous phase increases, improving the partitioning of relatively water-soluble analytes between two immiscible phases [87]. For this purpose, saturated salt solutions such as saturated ammoniumchloride buffer [31,107,109] have been added to the test tubes or NaCl (solid salt) has been pre-loaded [87] prior to extraction.

I.B.5.1.4.2 Liquid-liquid extraction of GBL

Following lactone-formation (see above), GBL has been extracted from biologic fluids with methylenechloride [19], chloroform [101,103] or benzene [5], solvents that are preferentially avoided in the modern laboratory. Since GBL may be protonated under the acidic conditions

required for complete conversion, recoveries can be improved by adding sodium chloride to the solution for salting-out purposes, but also by neutralizing (pH 6-7) the initial acidic pH (pH 1) using e.g. phosphate buffer and sodium hydroxide [5,34,103]. Following LLE, the mixture is generally centrifuged and the supernatant subsequently concentrated, but not completely evaporated since GBL may be lost during evaporation to dryness, being more volatile than the free acid [74]. As an example, it was found essential to evaporate with low nitrogen flow and at low temperatures (max 35 °C) to avoid unacceptable losses of GBL [5].

I.B.5.1.5 Solid phase extraction (SPE) of GHB

A first type of SPE sorbent used to extract GHB from biofluids is (strong) anion exchange. When using this type of cartridges, the classical SPE procedure of conditioning, loading, washing, drying and eluting has been followed. The interaction is based on ion exchange chemistry, whereby the organic moiety or quaternary ammonium material bonded to the solid matrix maintains its positive charge over the whole pH range, allowing pH-dependent interaction with GHB. At neutral pH, the carboxylic group will be negatively charged (pKa 4.6-4.8), and will interact with the positively charged sorbent. To elute GHB, it is necessary to neutralize its negative charge using an acidic elution solvent [89,90,111].

In addition, SPE cartridges can also be used to retain interfering substances, allowing the analyte of interest to pass through the sorbent and collecting the resulting eluate for further analysis. For this purpose, Clean Screen® SPE cartridges have been applied to clean-up vitreous humour, blood and urine. The collected eluate contained GHB without substances that could interfere during subsequent analysis [9,23,108]. In addition, a (strong) cation exchange sorbent can be used for sample clean-up of whole blood samples following protein precipitation. Introducing this additional clean-up resulted in improved peak shape of GHB and in reduced baseline noise [81,91].

An advantage of SPE is that it can be automated more easily than current precipitation or derivatization techniques which typically require off-line manual operations [89]. To illustrate this, an automated SPE (Oasis® HLB 30) using a 96-well plate has recently been applied for the extraction of GHB from whole blood samples, following protein precipitation [81]. Combining this automated SPE with LC-MS/MS resulted in a high-throughput method suitable for screening more than 6000 samples a year [81].

Also SPME, as a modification of the more classical SPE, has been introduced. In contrast to conventional extraction methods, which use multi-step techniques and excess organic solvents, SPME only consists of one solvent-free step to concentrate the analytes of interest. This technique has been applied to determine GHB (derivatized with hexylchloroformate) in urine samples, using a fused silica fibre coated with a stationary phase absorbing the analytes of interest. The SPME fiber can be brought directly in the sample, or alternatively, in the headspace (see further, headspace extraction of GHB) [37,86].

I.B.5.1.6 Headspace extraction of GBL or derivatized GHB

Only a minority of the presented GC methods uses HS as extraction and injection technique. The reason for this may lie not only in the more complex optimization of these procedures but also in the fact that typically a larger sample volume is required to obtain similar sensitivity as compared to more traditional sample preparation procedures such as LLE or SPE [7,86]. Also, the requirement for a more specific configuration, which also may imply the use of a more specific analytical column (see Table I.B.1) limits its general use. Nonetheless, these techniques have the advantage that GHB, in a derivatized form or as GBL, can be extracted directly from the aqueous sample, requiring less manual operations, being fully automatable, consuming less solvent (being solvent-free) and saving technical time [16,86]. Sample preparation is mostly limited to adding the following to a HS vial: an aliquot of the sample, anhydrous salt to enhance the transfer of the analyte of interest to the headspace, derivatization reagents or acid for lactone-formation. Next, after proper sealing of the vial, it can be placed in the HS oven for analysis.

I.B.5.1.6.1 Headspace extraction of derivatized GHB

Combining “in-vial” derivatization with headspace injection techniques may extend the application range normally reserved for volatile compounds to semi- or non-volatile analytes such as GHB. Following derivatization with hexylchloroformate or dimethylsulphate, derivatized GHB has been extracted using SPME or HS-trap, respectively [16,20]. Both methods have minimal sample preparation time. The method using SPME is one of the most sensitive methods reported, having an LLOQ of 0.1 µg/ml, starting from 0.5 ml urine. The HS-trap method is suited for the determination of GHB in various biological fluids, requiring only 100 µl of sample.

I.B.5.1.6.2 Headspace extraction of GBL

A static HS method has been described for the determination of GHB, based on LLE of 1 ml urine or whole blood samples, followed by conversion to GBL [19]. Headspace SPME and solid-phase dynamic extraction (SPDE) have also been applied to determine GHB as total GBL in plasma and urine samples, resulting in methods with sufficient sensitivity (LLOQ from 1 - 5 µg/ml) but requiring relatively large sample volumes (ranging from 0.5 to 1.0 ml) compared to other sample preparation techniques (0.02 – 0.5 ml) [7,102].

I.B.5.2 CHROMATOGRAPHIC ANALYSIS AND DETECTION

I.B.5.2.1 Gas chromatography

Although the nature of GHB does not favour the use of GC (see above), it remains the most popular separation technique of the last two decades, enabled by the use of appropriate sample preparation techniques. Toxicological analyses are commonly performed using an analytical column with a stationary phase consisting of silica with 95 % methyl - 5 % phenyl groups, which is also well suited for the determination of derivatized GHB and/or GBL (and analogues), reflected in its wide use. The majority of GC-based methods focus on the detection of GHB, either in derivatized form or in the form of GBL, while a few methods also include simultaneous analysis of glycols, BHB, GHV, 1,4-BD and/or SSA [9,13,14,17,105,106].

I.B.5.2.1.1. Gas chromatography – flame ionization detection

Although various authors use this universal detector to initially screen for GHB, followed by confirmation using GC-MS [19,36], Jones *et al*. (2007) [98] used GC- flame ionization detection (FID) to quantify GHB as GBL in blood samples within a wide calibration range, starting at 8 µg/ml.

I.B.5.2.1.2 Gas chromatography – mass spectrometry

To unequivocally demonstrate and determine GHB in biological fluids, GC is preferably used in conjunction with mass spectrometry. It has been used in electron impact ionization (EI) and positive or negative chemical ionization mode (PCI or NCI). For quantification, the MS operates in SIM (selected ion monitoring) mode, following those *m/z* ions typical for GBL or derivatized GHB. Derivatization using silylating or other derivatizing reagents is generally advantageous for MS detection, by increasing the molecular weight and the fragments' masses. Hence, more selective ions are formed than those formed from GBL (*m/z* 42, 56 and 86 in EI mode). In addition,

fragmentation of the di-TMS-derivative via CI instead of EI results in mass spectra with more abundant and higher molecular weight ions [42]. PCI has been used by Kerrigan (2002) [42] and Chen *et al.* (2003) [93] to quantify GHB in various biofluids following silylation and by Lenz *et al.* (2009) [102] and Frison *et al.* (2000) [7] following GHB conversion to GBL. Although one method had a relatively lower LLOQ of 0.4 µg/ml, no relevant gain in sensitivity has been observed when compared to GC methods where the MS performs in EI mode. On the other hand, using the MS in NCI mode to quantify GHB as GBL in plasma samples has been shown suitable for the determination of endogenous concentrations, with a calibration range situated in the low µg/ml range [101].

Although not routinely performed using GC-based methods, simultaneous analysis of GHB and 1,4-BD or other compounds such as BHB and SSA is possible, as was done by Lora-Tamayo *et al*. and Sakurada *et al.*, [105,106] respectively, who only slightly modified the method of Couper and Logan (2000) [104]. GHV and GHB can be analyzed simultaneously [9] and recently, Andresen-Streichert *et al*. (2013) [17] reported a GC-MS method for the simultaneous analysis of GHB and GHV in urine samples, with an extraction and derivatization procedure based on the method published by Kerrigan (2002) [42].

I.B.5.2.1.3 Gas chromatography - tandem mass spectrometry

Coupling tandem MS to GC enables the monitoring of a selected transition from a parent ion to (a) specific daughter ion(s), which may reduce the requirement for time-consuming sample clean-up techniques. However, although very high sensitivity as a result of increased selectivity may be valuable for hair analysis, the advantage of being able to detect low GHB levels by MS/MS techniques is not crucial for blood and urine samples, since GHB is readily endogenously present at relatively high concentrations (sub- and low- microgram-per-milliliter range). Nonetheless, MS/MS still may offer improvements in peak shape required for reliable integration [11]. Although tandem MS may have the advantage over existing methodologies of providing spectra free from background contaminants and thus being more selective, it remains or becomes even more important to evaluate if the di-TMS derivative of GHB is free from interferences from compounds with the same precursor ion (*m/z* 233), such as its positional isomers, AHB and BHB [11].

I.B.5.2.2 Liquid chromatography

The minority of confirmatory methods uses LC to determine GHB in biofluids. This may be due to historical reasons, since GC has been longer and more widely available for routine analyses in toxicological laboratories, but also because of practical reasons, since poor retention of the native molecule on classical reversed phase (RP) columns is expected. Nonetheless, LC techniques may offer advantages over existing GC methods. For one, although similar sensitivity has been obtained, workload and use of toxic solvents may be reduced, since the introduction of tandem MS has resulted in simpler sample preparations, such as dilute-and-shoot, without the requirement for derivatization or conversion prior to analysis. The fact that no conversion is required makes that several LC methods can detect GHB and its precursor GBL simultaneously, while most of the reported GC methods require additional analyses [81]. Finally, introduction of ultra-high performance LC (UHPLC), having higher efficiency than traditional high performance LC (HPLC), and automated sample preparation techniques have further led to the development of highly useful high-throughput LC-MS/MS methods [12,50,81,84].

I.B.5.2.2.1 Liquid chromatography – ultraviolet detection/ fluorescence detection

Since GHB has no chromophoric group, UV-detection is only possible at a low wavelength (220 nm), as reported by De Vriendt *et al.* (2001) [90]. Starting from 60-µl plasma samples, quantification was possible in a range from 10 to 750 µg/ml, the LLOQ being 5 to 10-fold higher than the majority of confirmatory methods reported here. Introducing an UV-active or fluorescent group through derivatization should allow for enhanced sensitivity and improved certainty of identification, as illustrated by Zacharis *et al.* (2004) [71]. These authors derivatized GHB, producing a highly fluorescent derivative starting from 500-µl oral fluid samples, with the lowest calibrator corresponding to 0.25 µg/ml.

I.B.5.2.2.2 Liquid chromatography - tandem mass spectrometry

UHPLC-MS/MS has the potential for shorter run times and improved sensitivity and precision compared to more traditional separation methods such as HPLC-UV or HPLC-Fluo, also facilitated by the possibility to use a stable isotopically labelled internal standard. To illustrate, Fung *et al*. (2004) [111] modified the LC-UV method described above [90] to a method suitable for LC-MS/MS, and although a slightly higher initial sample volume was required -100 instead of 60 µl - the run time was reduced to 5 min and sensitivity was increased 100-fold (LLOQ equal to 0.1 µg/ml).

Also, LC-MS/MS may allow for the simultaneous analysis of GHB and its precursors, GBL and 1,4-BD [12], using isocratic elution (with 10 % MeOH or acetonitrile) or a slightly rising gradient. Adequate baseline separation of not only GBL and 1,4-BD but also of AHB, BHB and GVL from GHB has been shown [12,50,84]. This baseline separation of GHB and its positional isomers is particularly important for adequate identification of GHB using one parent and one product ion. Moreover, since under some conditions in ESI(+), the molecule might lose water within the instrument source with the formation of GBL, it is of interest that the method can distinguish between in-source generated GBL or GHB-H_2O^+ and actual GBL in a sample [12,84,91]. Interesting to note is that one method [84] used this in-source conversion of GHB to GBL to obtain sufficient sensitivity for GHB determination in whole blood samples, while others [12,91] only observed a relatively low conversion (factor 6 %) unsuitable for GHB quantification.

Alternatively, to counter the detection of small *m/z* ions typical for GHB (*m/z* parent ion= 103), recently, an LC-MS/MS method for GHB in human serum has been reported, where quantification was based on the fragmentation of adducts formed with components of the mobile phase, more specifically on the fragmentation of the GHB sodium acetate adduct in ESI(-) (*m/z* 185) [99] .

Tandem MS has been used in both atmospheric pressure chemical ionization (APCI) and ESI mode, with ESI(+) producing only one product ion with significant abundance and ESI(-) revealing three abundant transition products. The latter is more beneficial for method sensitivity and selectivity [89,91]. On the other hand, reversed phase C_{18} columns frequently used for GHB separation require acidified mobile phases to better control the retention of GHB (being a weak acid with a pKa 4.6 it is only uncharged in acidic mobile phases) [81]. This may lead to a restriction to work in the ESI (+) mode, since the acidic conditions used may reduce the response of GHB in ESI(-) mode [81,91]. However, Forni *et al*. (2013) reported lower background noise under their chromatographic conditions with the MS/MS operating in ESI(-) as compared to ESI(+) [50].

Sørensen *et al.* (2012) [87] and Lott *et al*. (2012) [96] suggested the use of a hydrophilic interaction liquid chromatography (HILIC) as an alternative to overcome this problem and to improve retention and chromatographic separation of small and polar molecules. HILIC allows for chromatography to be performed under neutral conditions, optimal for separation of GHB and analogues, which in addition also prevents inter-conversion between GHB and GBL [91]. To compare, using a typical C_{18} reversed phase column, GHB elutes first, followed by 1,4-BD and GBL,

while using HILIC the elution order is reversed, which simplifies optimization of the retention time of GHB by adjusting the composition of the mobile phase [91]. Despite these advantages, to reduce analysis costs of high-troughput methods, one may opt not to use HILIC methods with acetonitrile, given its higher toxicity and price than e.g. MeOH. Furthermore, also practical reasons, such as instrumental back-up, may influence the choice to use RP-columns and -chromatographic separation rather than HILIC-based chromatography [81].

I.B.5.3 NON-CHROMATOGRAPHIC TECHNIQUES

Gong *et al*. (2008) [85] reported a CZE-C^4D method to determine GHB in urine and serum samples. Although not commonly used for toxicological purposes, this technique is well-suited for the determination of small and ionic molecules such as GHB (anionic form) [100]. Separation and detection of AHB, BHB and GHB was achieved without preceding extraction or derivatization but only by a simple 1:4 dilution with an optimized separation buffer with a pH > 4.7 to deprotonate the analytes and to obtain them in anionic form. In addition, the more alkaline pH also inhibits conversion of GHB to GBL. The method showed sufficient sensitivity to discriminate between endo- and exogenous GHB levels in urine samples (cut-off 10 µg/ml). Also, instrumentation is less expensive than other instruments used in clinical and forensic laboratories and a portable instrument may allow for on-site analysis of urine samples from suspected GHB-intoxications [85].

I.B.6 CONCLUSION

To conclude, various screening and confirmation methods are available to determine GHB (and analogues if required) in biological fluids. GHB screening/analysis in a toxicological laboratory is mostly performed based on a suspected ingestion of this club drug, supported by information of the police or physician, rather than on a routine basis, as is the case for more widely abused drugs such as cannabinoids, amphetamines and opioids [34,98]. However, since a few years, routine screening has become possible, not only by the commercialization of an enzymatic kit for GHB adaptable on common chemical analyzers, but also by the availability of more simplified GC-MS methods and more sophisticated techniques such as UHPLC-MS/MS, which - when combined with automated sample preparation procedures - allow high-throughput. To confirm the presence of GHB in biological fluids, GC has remained the most widely used separation technique during the last twenty years, despite the small and polar nature of GHB, requiring conversion to GBL or derivatization to a more volatile and stable form. However, LC-based applications coupled to tandem MS are increasingly gaining interest as they may offer the advantage of more simple

sample preparation techniques (e.g. no derivatization) or dilute-and-shoot. Of course, when sample preparation is minimal, matrix effects require special consideration. Furthermore, despite the advantages of reduced workload and shortened analysis time that tandem MS-techniques may offer, baseline separation of GHB from GBL and from its isomers AHB and BHB, achieved by adequate chromatography, remains important to avoid interference (respectively by in-source formation of GBL during MS/MS analysis and by similar transitions) [11]. As to method sensitivity, both GC- and LC-based applications offer similar LOQ's, but as Kankaanpää *et al*. (2007) has nicely pointed out "the challenge is not to reach as low GHB concentration levels as possible, but to interpret the results correctly being able to make a distinction between use of GHB and endogenous levels" [87]. Indeed, once a result has been obtained using the above mentioned screening and confirmation methods, the interpretation is a second challenge for the toxicologist, whereby the analysis of different matrices may be useful for correct interpretation.

Table I.B.1 Overview of confirmation methods to determine GHB in biological fluids, sorted by analytical technique

GC *Unless specified: GC-MS: 1 µl injected in splitless injection, MS EI ionization mode and helium as carrier gas; *If method includes conversion of GHB to GBL: without acidification of the sample: determination of original GBL concentration possible*

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Abanades et al., 2006 [46]; Abanades et al., 2007 [48]	GHB	Urine Plasma Oral fluid (100 µl) (Sweat)	**PP**: 150 µl acetonitrile +50 µl 0.1 M sulphuric acid **Derivatization**: 50 µl (BSTFA + 1% TMCS) 70 °C 30 min	5 % phenyl – 95 % methylpolysiloxane 12 m x 0.2 mm, 0.33 µm (14 min)	GHB-d6	0.2-300 µg/ml	
Andresen et al., 2010 [30]	GHB	Urine Serum (100 µl)	**LLE**: 200 µl 0.1 M HCl + 1 ml ethylacetate **Derivatization**: 100 µl ethylacetate + 50 µl (BSTFA + 1% TMCS)	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (22.7 min)	GHB-d6	2-12 µg/ml	
Andresen-Streichert et al., 2013 [17]	GHB GHV	Urine (100 µl)	**LLE**: 200 µl 0.1 M HCl + 1 ml ethylacetate **Derivatization**: 50 µl acetonitrile + 25 µl (BSTFA + 1% TMCS)	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (16.22 min)	GHB-d6	(NR for GHB)	
Blair et al., 2000 [86]	GHB	Urine (0.5 ml)	**Derivatization**: 40 µl pyridine + 24 µl hexylchloroformate 40 °C 5 min **Solution SPME**: + 2ml deionized water + 1 ml pH 7 buffer; PDMS SPME fiber 12 min 40 °C	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (23.33 min)	GHB-d6	5-500 µg/ml	SPME GC-Q-trap
Bosman and Lusthof, 2004 [34]	GHB*	Urine Blood (1 ml)	**PP + Conversion of GHB to GBL**: 1 ml 1 M perchloric acid 1 ml supernatant: 80 °C 20 min **LLE of GBL**: 300 mg NaCl, pH 6.5 (1 ml 1.5 N phosphate buffer + 350 µl 2.5 M NaOH), 5 ml chloroform	100 % polydimethylsiloxane 12 m x 0.2 mm, 0.33 µm (15 min)	GHB-d6	5-40 µg/ml	Case reports Split injection (10:1)

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Brenneisen et al., 2004 [103]	GHB*	Urine (2 ml) Plasma (0,5 ml) Oral fluid (1 ml)	**Conversion of GHB to GBL**: 0.5 ml 20 % trifluoroactetic acid, 75 °C 1 h **LLE of GBL**: 0.55 mL 2 M NaOH (pH adjusting to 6.5) + 3 ml chloroform	5 % phenyl – 95 % methylpolysiloxane 25 m x 0.2 mm, 0.33 µm (10.1 min)	GVL	10-50 µg/ml	4 µl injected
Brown et al., 2007 [16]	GHB MAMP MDMA KET	Urine (1 ml)	**Derivatization**: 10 µl hexylchloroformate + 40 µl pyridine **Headspace SPME of derivatized GHB**: 0.5 ml derivatized sample + 1 ml water; 100 µm PDMS fiber 90 °C 20 min; 1 min desorption at 225 °C	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.10 µm (12 min)	GHB-d6	0.1-20 µg/ml	SPME
Chen et al., 2003 [93]	GHB	Plasma (100 µl)	**PP**: 2ml acetonitrile 50 µl supernatant evaporated **Derivatization**: 100 µl BSTFA 75 °C 15 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 1 µm (9 min)	GHB-d6	2.5-250 µg/ml	GC-PCI-MS (ammonia as reagent gas)
Couper and Logan, 2000 [104]	GHB	Urine Plasma (1 ml)	**LLE**: 250 µl cold 0.1N sulphuric acid + 6 ml ethylacetate (2 x) **Derivatization**: 30 µl (BSTFA + 1% TMCS) + 60 µl acetonitrile 70°C 15 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.33 µm (14 min)	diethyleneglycol	1-200 µg/ml urine 1-100 µg/ml plasma	2 µl injected
Crookes et al., 2004 [43]	GHB	Urine (2 ml)	**LLE**: + 0.36 M sulphuric acid to obtain pH 2.75; + 3 ml ethylacetate **Derivatization**: 100 µl (BSTFA + 1% TMCS) 60°C 30 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (26.2 min)	GHB-d6	0.2-5 µg/ml	2 µl injected
De Paoli and Bell, 2008 [51]	GHB	Oral fluid (1 µl)	**Derivatization**: 97 µl (BSTFA + 1 % TMCS) 50 °C 30 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.5 µm (16 min)	GHB-d6	0.5-50 µg/ml	Split injection (10:1)
Duer et al., 2001 [97]	GHB	Urine Blood Vitreous humour (25 µl)	**PP**: 1.225 ml GHV methanolic solution **Derivatization**: 100 µl MSTFA + 100 µl ethylactetate 60 °C	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (24.7 min)	GHV GHB-d6	Standard addition (10-500 µg/ml)	*GBL determination:* conversion to GHB in alkaline conditions (using 1 ml 10mM

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Duer et al., 2001 [97] ***continued***			30 min				NaOH in methanol, 1 h 60 °C) following GHB analysis; *1,4-BD determinatio*n: di(dinitrobenzoyl) derivative using HPLC-UV
Elian, 2000 [107]	GHB	Urine (50 µl)	**LLE**: 50 µl IS + 0.5 ml saturated ammonium chloride buffer + 3 ml ethylacetate **Derivatization**: 50 µl (BSTFA + 1% TMCS) + 50 µl ethylacetate 30 min RT	100 % polydimethylsiloxane 12 m x 0.2 mm, 0.33 µm (9.7 min)	GHB-d6	2-50 µg/ml	2 µl injected
Elian, 2001 [109]	GHB	Blood (50 µl)	**LLE**: 0.5 ml saturated ammonium chloride buffer + 3 ml ethylacetate **Derivatization**: 20 µl (BSTFA + 1% TMCS) + 80 µl ethylacetate 70 °C 20 min	100 % polydimethylsiloxane 12m x 0.2mm, 0.33µm (11.0 min)	GHB-d6	1-200 µg/ml	2 µl injected
Elie et al., 2012 [88]	GHB	Urine (300 µl)	**LLE** : 300 µl urine + 900 µl ethylacetate **Derivatization** : 50 µl acetonitrile + 50 µl (MTBSTFA+1%TBCS)	100 % polydimethylsiloxane 30 m x 0.25 mm, 0.25 µm (15 min)	*Trans*-4-hydroxycrotonic acid	0.17-1.67 µg/ml	Injection port silylation: split/splitless injector with programmable pneumatic control
Elliott, 2004 [36]	GHB	Urine Plasma (1 ml)	**LLE**: 250 µl 0.05 M sulphuric acid + 6.0 ml ethylacetate **Derivatization**: 75 µl (BSTFA + 1% TMCS) 90 °C 5 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (9 min)	GHB-d6	5-200 µg/ml	1) GC-FID screening method for GHB identification *via* GBL conversion 2) GC-MS determination *via* derivatization (presented in detail)

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Elliott, 2004 [25]; Elliott et al., 2004 [40]	GHB	Urine Blood Vitreous humour (100 µl)	**LLE**: 50 µl cold 0.05 M sulphuric acid + 0.5 ml ethylacetate **Derivatization**: 75 µl (BSTFA + 1% TMCS) 90 °C 5 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (9 min)	diethyleneglycol GHB-d6	6.25-100 µg/ml (urine) 1-100 µg/ml (plasma)	Comparison with GC-FID method [25]
Ferrara et al., 1993 [5]	GHB*	Urine Plasma (2 ml)	**Conversion GHB to GBL**: 2 ml cold 0.8 N perchloric acid, supernatant (plasma **PP**) 0.2 ml 6 N HCl (urine) 80 ° C 20 min **LLE of GBL**: 300 mg NaCl, 1 ml pH 6.5 (1 ml 1.5 N phosphate buffer + 5 N NaOH), 8 ml (plasma) or 6 ml (urine) benzene	100 % polydimethylsiloxane 12 m x 0.2 mm, 0.33 µm (15.6 min)	δ-valerolactone	2-150 µg/ml urine 2-200 µg/ml plasma	
Frison et al., 2000 [7]	GHB*	Urine Plasma (0.5 ml)	**Conversion GHB to GBL**: 100 µl cold 1.6 N perchloric acid, supernatant (plasma **PP**) 25 µl 6 N HCl (urine) 80 ° C 20 min **Headspace SPME of GBL**: 500 mg solid phosphate buffer 50-µm CW/TPR SPME fiber 10 min 70 °C	acid-modified polyethylene glycol phase 25 m x 0.2 mm, 0.3 µm (9.8 min)	GBL-d6	5-150 µg/ml urine 1-100 µg/ml plasma	SPME GC-PCI-MS (methane as reagent gas)
Fukui et al., 2003 [101]	GHB*	Plasma (200 µl)	**Conversion GHB to GBL**: 0.5 ml 6 M HCl **LLE of GBL**: 2 ml dichloromethane (2 x) evaporation to 100 µl	polyethyleneglycol 30 m x 0.32 mm, 0.25 µm (11.5 min)	GBL-d6	0.01-1 µg/ml	GC-NCI-MS
Ingels et al., 2010 [24]; Ingels et al., 2011 [49]	GHB	DBS (50 µl [24] /6-mm punch [49])	**Derivatization**: 100 [24] or 50 µl [48] TFAA/HFB-OH (2:1)	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (12.3 min)	GHB-d6	2-100 µg/ml	
Ingels et al., 2013 [20]	GHB	Urine Plasma/serum Blood/lyzed blood	**PP**/salting-out: 100 mg Na_2SO_4 **Derivatization**: 30 µl DMS +	94 % dimethyl – 6 % cyanopropylphenyl polysiloxane	GHB-d6	5-150 urine 2-150 plasma/serum	

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Ingels et al., 2013 [20] ***continued***		(100 µl)	30 µl NaOH (0.5 M) **Headspace-trap extraction of derivatized GHB**: 30 min 90 °C	30 m x 0.25 mm, 1.4 µm (15 min)		3.5-200 blood/lyzed blood	
Jones et al., 2007 [98]	GHB*	Blood (0.5 g)	**PP**: acetone **Conversion GHB to GBL**: sulphuric acid **LLE of GBL**: dichloromethane evaporation to 50-100 µl	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (NR)	GVL	8-1000 µg/ml	2 µl injected GC-FID
Kankaanpää et al., 2007 [87]	GHB GBL 1,4-BD	Urine Blood (200 µl)	**LLE**: 400 µl water + 5 ml t-butylmethylether + 0.5g NaCl **Derivatization**: 100 µl acetonitrile + 30 µl MSTFA	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.32 mm, 1.0 µm (12 min)	benzylalcohol	3-75 µg/ml	*GBL determination:* similar sample prep: without salting –out and derivatization + different GC-MS method 3 µl injected
Kerrigan, (2002) [42]; Mazzar-Proo and Kerrigan, (2005) [108]	GHB	Urine Blood Vitreous humour (0.5 ml)	**LLE**:250 µl 0.1 M HCl + 2 ml ethylacetate (2 x) **Derivatization**: 100 µl (BSTFA + 1% TMCS) [42]; 50 µl MSTFA [104]	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (13 min)	GHB-d6	0.4-250 µg/ml	GC-PCI-MS (methane as reagent gas)
Kimura et al., 2003 [73]	GHB	Urine (0.01mg creatinine)	Urease 37 °C 30 min Alkalinization: 10 µl 0.1 N NaOH **Derivatization**: 60 µl (BSTFA + 1 % TMCS) 80 °C 30 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (20.9 min)	dimethylsuccinic acid	0.006-0.0127 µg/ml	
Lebeau et al., 2000 [19]	GHB*	Urine Blood (1ml)	**Conversion GHB to GBL**: 0.15 ml concentrated sulphuric acid **LLE of GBL**: 5 ml dichloromethane **Static headspace injection**: 20 min 90 °C	94 % dimethyl – 6 % cyanopropylphenyl polysiloxane 30 m x 0.25 mm, 1.4 µm (15 min)	α-methylene- γ-butyrolactone (screening) GHB-d6 (single-point quantification)	5-1000 µg/ml	1) headspace GC-FID screening 2) headspace GC-MS confirmation nitrogen as carrier gas
Lenz et al., 2009 [102]	GHB*	Urine Serum (0.5 ml)	**Conversion GHB to GBL**: 100 µl sulphuric acid **SPDE of GBL**: + 1 g sodium sulphate; SPDE PDMS/AC	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 1.0 µm (10.5 min)	GHB-d6	2-200 µg/ml	SPDE GC-PCI-MS (methane as reagent gas)

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Lenz et al., 2009 [102] *continued*			coating; 150 extraction strokes at 50 °C; sample agitation 500 rpm				
Lora-Tamayo et al., 2003 [105]	GHB 1,4-BD	Urine Blood (1 ml)	**LLE**: 250 µl cold 0.1N sulphuric acid + 6 ml ethylacetate **Derivatization**: 30 µl (BSTFA + 1% TMCS) + 60 µl acetonitrile 70°C 15 min; + 200 µl ethylacetate	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.33 µm (14 min)	GHB-d6	2.5-85 µg/ml urine	
Louagie et al., 1997 [94]	GHB	Serum (20 µl)	**PP**: 40 µl acetonitrile **Derivatization**: 20 µl MSTFA 90°C 10 min	100 % polydimethylsiloxane 12 m x 0.2 mm, 0.33 µm (9.6)	valproic acid	2-200 µg/ml	
Marinetti et al., 2005 [9]	GHB GHV	Urine Blood Vitreous humour (200 µl)	**PP**: 1 ml acetone **SPE** *(CLEAN SREEN GHB)*: conditioning: 3 ml methanol, 3 ml deionized water, 3 ml 100 mM sodium phosphate buffer (pH 6); sample loading (reconstituted in 250 µl 100 mM sodium phosphate buffer (pH 6)); elution: 1 ml 99:1 methanol/ammonium hydroxide **Derivatization**: 100 µl (BSTFA + 1 % TMCS) 55 °C 30 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (13.0 min)	GHB-d6	5-150 µg/ml	Post-mortem cases
McCusker et al., 1999 [23]	GHB	Urine (200 µl)	**SPE** *(CLEAN SREEN GHB)*: conditioning: 3 ml methanol, 3 ml deionized water, 0.5 ml sodium phosphate buffer (pH 6); sample loading ; elution: 1 ml 99:1 methanol/ammonium hydroxide **LLE**: DMF + hexane **Derivatization**: 100 µl ethylacetate + 100 µl (BSTFA+ 1% TMCS) 60 °C 5 min	100 % polydimethylsiloxane 30 m x 0.25 mm, 0.25 µm (10.97 min)	GHB-d6	5-500 µg/ml	

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Meyer et al., 2010 [13]	GHB, DL-lactic acid, glycolic acid, ethylene glycol, and other glycols	Urine Plasma (50 µl)	**PP**: 50 µl acetonitrile **Derivatization**: 300 µl BSTFA + 20 µl DMF	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (12 min)	GHB-d6	10-200 µg/ml	Micro-wave assisted silylation
Pan et al., 2001 [15]	GHB Ethyleneglycol	Urine Blood (100 µl)	**PP**: 80 µl water + 200 µl acetonitrile **LLE**: supernatant 150 µl + 500 µl DMP/DMF (80:20) **Derivatization**: 50 µl MTBSTFA + 50 µl ethylacetate 70°C 60 min	5 % phenyl – 95 % methylpolysiloxane 25 m x 0.25 mm, 0.25 µm (16.3 min)	2-hydroxy-3-methylbutyric acid	0-500 µg/ml	6 case histories
Paul et al., 2006 [11]	GHB	Urine Blood (50 µl)	**PP**: 150 µl acetonitrile +50 µl 0.1 M sulphuric acid **Derivatization**: 100 µl BSTFA 60 °C 20 min	5 % phenyl – 95 % methylpolysiloxane 15 m x 0.25 mm, 0.25 µm (9.40 min)	GHB-d6	2.5-100 µg/ml	GC-EI-MS/MS
Sakurada et al., 2002 [106]	GHB 1,4-BD SSA BHB	Urine Blood (1 ml)	**LLE**: 400 µl cold 0.1N sulphuric acid + 8 ml ethylacetate **Derivatization**: 50 µl (BSTFA + 1% TMCS) + 60 µl acetonitrile 70°C 15 min;+ 40 µl ethylacetate	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (14 min)	diethyleneglycol	0-200 µg/ml	2 µl injected
Shima et al., 2005 [10]	GHB BHB AHB	Urine (100 µl)	**PP**: 500 µl methanol **Derivatization**: 50 µl (BSTFA + 1% TMCS) + 50 µl acetonitrile 60 °C 20 min	50 % phenyl - methylpolysiloxane 30 m x 0.32 mm, 0.25 µm (17 min)	2-hydroxycaproic acid	0.01-30 µg/ml	
Van hee et al., 2004 [14]	GHB, DL-lactic acid, glycolic acid, ethylene glycol, and other glycols	Urine Plasma Serum (20 µl)	**Derivatization**: 750 µl BSTFA + 20 µl DMF 70°C 15 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (9.25 min)	GHB-d6	3.2-200 µg/ml	Split injection (60:1)
Villain et al., 2003 [22]	GHB	Urine Blood (20 µl)	**PP**: 45 µl acetonitrile **Derivatization**: 35 µl BSTFA + 1% TMCS 70 °C 25 min	5 % phenyl – 95 % methylpolysiloxane 30 m x 0.25 mm, 0.25 µm (11.5 min)	GHB-d6	1-200 µg/ml	

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Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Dahl et al., 2012 [81]	GHB GBL 1,4-BD Pregabaline BHB	Urine Blood (100 µl)	Urine: **Dilution/Filtration**: 1) 1:1 dilution: + 100 µl water 2) 1.75 ml 0.2 % formic acid 3) 500 µl: mini-UniPrep filter 0.2 µm filter membrane Blood: **Dilution**: + 100 µl water **PP**: 400 µl ice-cold acetonitrile: methanol (85:15, v/v), 10 min freezer **SPE**: 96-well plate (30 mg OASIS HLB): conditioning: methanol and water; sample loading: supernatant + 250 µl 0.4 % formic acid solution	HSS T3 column (100 mm x 2.1 mm, 1.7 µm) gradient elution MP A: 0.2 % formic acid MP B: methanol (4.5 min)	GHB-d6	2.6-312.3 µg/ml blood	UHPLC-MS/MS ESI(+) Injection volume: 3 µl blood/2 µl urine
de Vriendt et al., 2001 [90]	GHB	Rat plasma (60 µl)	**SPE** (SAX Bond elut cartridges): conditioning: 1 ml methanol, 6 ml 0.5 M formic acid, 1 ml water; sample loading: 60 µl; washing: 0.5 ml water, 0.5 ml water-methanol (1:1), 0.3 ml methanol; elution: 600 µl acetonitrile with 6 % acetic acid	C_{18} Aqua column (150 mm x 4.6mm, 5µm) isocratic elution MP: 100% potassium dihydrogenphosphate solution 20mM (10 min)	(tested different IS)	10-750 µg/ml	HPLC-UV (220 nm) injection volume: 100 µl
Dresen et al., 2007 [95]	GHB	Serum (100 µl)	**PP**: 200 µl cold acetonitrile	Polar-enca**pp**ed phenylpropyl RP (Synergy Polar-RP) (50 mm x 2 mm, 4 µm) gradient elution MP A: 0.1 % formic acid with 1 mmol/L ammonium formate MP B: acetonitrile with 0.1 %	GHB-d6	1-200 µg/ml	HPLC-MS/MS ESI(-) injection volume: 20 µl No evaluation of matrix effect

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Dresen et al., 2007 [95] *continued*				formic acid 95:5 (v/v) with 1 mmol/L ammonium formate (15 min)			
Dziadosz et al., 2013 [99]	GHB	Serum (100 µl)	**PP**: 1 ml water/methanol (3/97 v/v) 15 min shaking	Luna 5 µm C18 (2) 100 A (150 mm x 2 mm) gradient elution MP A: water/methanol (95/5) + 10 mM ammonium acetate + 0.1 % acetic acid (pH 3.2) MP B: water/methanol (3/97) + 10 mM ammonium acetate + 0.1 % acetic acid (pH 3.2) (3 min)	GHB-d6	5-100 µg/ml	UHPLC-MS/MS ESI(-) injection volume: 10 µl Adduct supported detection
Elian et al., 2011 [89]	GHB	Urine (50 µl)	**Dilution**: 1ml water, mix, + 3 ml water **SPE** (SAX, CUQAX 6 ml 500 mg): conditioning: 3 ml methanol, 3 ml water; sample loading; washing: 3 ml deionized water, 3 ml methanol; elution: 3 ml 6 % acetic acid in methanol	Allure biphenyl (150 mm x 4.6 mm, 5 µm) gradient elution MP A: 0.1 formic acid in water MP B: 0.1 % formic acid acetonitrile (4.1 min)	GHB-d6	0.5-10 µg/ml	HPLC-MS/MS APCI(-) injection volume: 10 µl
Forni et al., 2013 [50]	GHB	DBS (3 x 4.6 mm)	**Extraction**: 200 µl methanol 30 min	HSS T3 column (100 mm x 2.1 mm, 1.8 µm) gradient elution MP A: 0.1 % formic acid in water MP B: 0.1 % formic acid in acetonitrile (4 min)	GHB-d6	1-128 µg/ml	UHPLC-MS/MS ESI(-) injection volume: 15 µl
Fung et al., 2004 [111]	GHB	Rat plasma (100 µl)	**SPE** (SAX Bond elut cartridges, 100 mg 1 ml): conditioning: 1 ml methanol, 6 ml 10 % acetic acid, 1 ml water; sample	C_{18} Aqua column (150 mm x 4.6mm, 5µm) gradient elution MP A: 90 % 5mM formic acid	GHB-d6	0.1-10 µg/ml	HPLC-MS/MS APCI(-) injection volume: 10 µl

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Fung et al., 2004 [111] *continued*			loading: 100 µl; washing: 0.5 ml water, 0.5 ml water-methanol (1:1), 0.3 ml methanol; elution: 3 ml acetonitrile with 10 % acetic acid	MP B: 10 % acetonitrile (5 min)			
Johansen and Windberg, 2011 [84]	GHB GBL 1,4-BD GVL	Urine (20 µl) Blood (0.2 g)	Blood: **PP**: 260 µl acidic methanol **Dilution**: 50 µl supernatant diluted 1:1 acidic water Urine: **Dilution**: 10-fold dilution with acidic 10 % methanol	Zorbax SB C_{18} (150 mm x 2.1 mm, 3.5 µm) gradient elution MP A:acidic water MP B: acidic methanol (19 min)	GHB-d6	1-100 mg/kg blood	HPLC-MS/MS ESI(+) injection volume : 5 µl
Kaufmann and Alt, 2007 [110]	GHB	Urine Serum (250 µl)	**LLE**: 1) 125 µl 0.1 M HCl + 1 ml ethylacetate 2) 750 µl t-butylmethylether **Derivatization**: 50 µl 3 M HCl n-butanol 50 °C 5 min	C_{18} zorbax SB-18 Agilent (30 mm x 2.1 mm, 3.5 µm) gradient elution MP A: 5 mM ammonium formate in water MP B: 5 mM ammonium formate in acetonitrile (NR)	GHB-d6	2-100 µg/ml	HPLC-MS/MS Ion trap injection volume: 10 µl
Lott et al., 2012 [96]	GHB	Serum (100 µl)	**PP**: 200 µl acetonitrile	Nucleodur HILIC column (NS, 3 µm) isocratic elution 80 % acetonitrile 20 % water with 5 mM ammonium acetate (15 min)	GHB-d6	2-50 µg/ml	HILIC, HPLC-MS/MS APCI(-) injection volume: 10 µl
Sørensen and Hasselstrøm, 2012 [91]	GHB GBL 1,4-BD	Blood (200 µl)	**PP**: + 100 µl methanol + 600 µl acetonitrile **SPE** (3- ml strata-X-C 60 mg SCX): conditioning: 1 ml methanol, 1 ml water, 1 ml 1 M sodium dihydrogen phosphate solution, 1 ml water; sample loading: 600 µl supernatant + 250 µl water	SeQuant ZIC HILIC (100 mm x 2.1 mm, 5 µm) gradient elution MP A: 1mM ammonium acetate MP B: acetonitrile (10 min)	GHB-d6	5-200 µg/ml	HILIC, UHPLC-MS/MS ESI(+) and ESI(-) injection volume: 10 µl

Ref.	Analyte	Matrix (sample volume)	Sample preparation	Stationary phase (total run time)	IS GHB	Calibration Range GHB	Remarks
Wood et al., 2004 [12]	GHB GBL 1,4-BD	Urine (250 µl)	**Dilution**: 1:20 with deionized water	Atlantis C_{18} column (100 mm x 3 mm, 5 µm) isocratic elution MP: 0.1 % aqueous formic acid: methanol (90:10) (11 min)	GHB-d6	1-80 µg/ml	HPLC-MS/MS ESI(+) injection volume: 20 µl
Zacharis et al., 2008 [71]	GHB	Oral fluid (500 µl)	Oral fluid sample evaporated; residu reconstituted 200 µl DMF (water removal using preheated molecular sieves) **Derivatization**: 1) 100 µl Br-MMC 70 °C 70 min 2) 100 µl anthracene + 1ml acetonitrile	C_{18} Kromasil (250 mm x 4.6 mm, 4µm) gradient elution methanol/phosphate buffer (40 mM, pH 3) (25 min)	anthracene	0.25-7.5 µg/ml	HPLC-Fluorescence detection (330 (λ_{ex}) -390 (λ_{em}) nm) injection volume: 25 µl

Other confirmation method

Ref.	Analyte	Matrix (sample volume)	Extraction	Separation buffer	Calibration Range GHB	Remarks
Gong et al., 2008 [85]	GHB BHB AHB	Urine Serum	**Dilution**: 1:4 with separation buffer	20 mM arginine; 10 mM maleic acid; 30 µM cetyltrimethylammoniumbromide 5 mM vancomycin	2-400 µg/ml urine 5-150 µg/ml serum	Capillary electrophoresis with contactless conductivity detection

1,4-BD: 1,4-butanediol; AHB: alpha-hydroxybutyric acid; APCI: atmospheric pressure chemical ionization; BHB: bèta-hydroxybutyric acid; Br-MMC: 4-bromomethyl-7-methoxy coumarin; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; CW/TPR: carbowax/templated resin; DMF: dimethylformamide; DMS: dimethylsulphate; EI: electron impact; ESI: electrospray ionization; FID: flame ionization detection; GBL: gamma-butyrolactone; GC: gas chromatography; GHB: gamma-hydroxybutyric acid; GHV: gamma-hydroxyvaleric acid; GVL: gamma-valerolactone; HCl: hydrogen chloride; HFB-OH: heptafluorobutanol; HILIC: hydrophilic interaction liquid chromatography; HPLC: high performance liquid chromatography; IS: internal standard; KET: ketamine; LLE: liquid-liquid extraction; MAMP: methamphetamine; MDMA: 3,4-methylenedioxymethamphetamine; MP: mobile phase; MS: mass spectrometry; MS/MS: tandem mass spectrometry; MSTFA: N-methyl-N-(trimethylsilyl)-trifluoroacetamide; MTBSTFA: N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide; NaCl: sodium chloride; NaOH: sodium hydroxide; Na_2SO_4: anhydrous sodium sulphate; NCI: negative chemical ionization; NR: not reported; PCI: positive chemical ionization; PDMS: polydimethylsiloxane; PDMS/AC: PDMS/cyanopropyl; PP: protein precipitation; RP: reversed phase; SPDE: solid-phase dynamic extraction; SPE: solid-phase extraction; SPME: solid-phase micro-extraction; SSA: succinic semi-aldehyde; TBCS: butyldimethylchlorosilane; TFAA: trifluoroacetic acid anhydride; TMCS: trimethylchlorosilane; UHPLC: ultra high performance liquid chromatography; UV: ultraviolet

REFERENCES

1. Doherty JD, Hattox SE, Snead OC, Roth RH. Identification of endogenous gamma-hydroxybutyrate in human and bovine brain and its regional distribution in human, guinea pig and rhesus monkey brain. *J Pharmacol Exp Ther*. 207(1):130-139 (1978).
2. Lettieri J, Fung HL. Improved pharmacological activity via pro-drug modification: comparative pharmacokinetics of sodium gamma-hydroxybutyrate and gamma-butyrolactone. *Res Commun Chem Pathol Pharmacol*. 22(1):107-118 (1978).
3. Vree TB, Van der Kleijn E, Knop HJ. Rapid determination of 4-hydroxybutyric acid and 2-propylpentanoate in human plasma by means of gas-liquid chromatography. *J Chrom*. 121:150–2 (1976).
4. Hoes MJ, Vree TB, Guelen PJ. Circadian rhythm in plasma concentrations of gamma-hydroxybutyric acid in alcoholics. *Int J Addict*. 16(6):1071-5 (1981).
5. Ferrara SD, Tedeschi L, Frison G, Castagna F, Gallimberti L, Giorgetti R, Gessa GL, Palatini P. Therapeutic gamma-hydroxybutyric acid monitoring in plasma and urine by gas chromatography-mass spectrometry. *J Pharm Biomed Anal*. 11(6):483-487 (1993).
6. Maurer HH. Position of chromatographic techniques in screening for detection of drugs or poisons in clinical and forensic toxicology and/or doping control. *Clin Chem Lab Med*. 42(11):1310-1324 (2004).
7. Frison G, Tedeschi L, Maietti S, Ferrara SD. Determination of γ-hydroxybutyric acid (GHB) in plasma and urine by headspace solid-phase microextraction and gas chromatography/positive ion chemical ionization mass spectrometry. *Rapid Com Mass Spectr*. 14(24): 2401-2407 (2000).
8. Lenz D, Rothschild MA, Kröner L. Intoxications due to ingestion of gamma-butyrolactone: organ distribution of gamma-hydroxybutyric acid and gamma-butyrolactone. *Ther Drug Monit*. 30(6):755-761 (2008).
9. Marinetti LJ, Isenschmid DS, Hepler BR, Kanluen S. Analysis of GHB and 4-methyl-GHB in postmortem matrices after long-term storage. *J Anal Toxicol*. 29(1):41-47 (2005).
10. Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H. Endogenous level and in vitro production of GHB in blood from healthy humans, and the interpretation of GHB levels detected in antemortem blood samples. *J Health Sci*. 51 (2):147-154 (2005).
11. Paul R, Tsanaclis L, Kingston R, Berry A, Guwy A. GC-MS-MS determination of gamma-hydroxybutyrate in blood and urine. *J Anal Toxicol*. 30(6):375-379 (2006).
12. Wood M, Laloup M, Samyn N, Morris MR, de Bruijn EA, Maes RA, Young MS, Maes V, De Boeck G. Simultaneous analysis of gamma-hydroxybutyric acid and its precursors in urine using liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 1056(1-2):83-90 (2004).

13. Meyer MR, Weber AA, Maurer HH. A validated GC-MS procedure for fast, simple, and cost-effective quantification of glycols and GHB in human plasma and their identification in urine and plasma developed for emergency toxicology. *Anal Bioanal Chem*. 400(2):411-414 (2011).
14. Van hee P, Neels H, De Doncker M, Vrydags N, Schatteman K, Uyttenbroeck W, Hamers N, Himpe D, Lambert WE. Analysis of gamma-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. *Clin Chem Lab Med*. 42(11):1341-1345 (2004).
15. Pan YM, Gill GN, Tilson CS, Wall WH, McCurdy HH. Improved procedure for the analysis of gamma-hydroxybutyrate and ethylene glycol in whole blood. *J Anal Toxicol*. 25(5):328-332 (2001).
16. Brown SD, Rhodes DJ, Pritchard BJ. A validated SPME-GC–MS method for simultaneous quantification of club drugs in human urine. *Forensic Sci Int.* 171(2-3):142-150 (2007).
17. Andresen-Streichert H, Jungen H, Gehl A, Müller A, Iwersen-Bergmann S. Uptake of gamma-valerolactone--detection of gamma-hydroxyvaleric acid in human urine samples. *J Anal Toxicol*. 37(4):250-254 (2013).
18. Ciolini LA, Mesmer MZ, Satzger RD, Machal AC, McCauley HA, Mohrhaus AS. The chemical interconversion of GHB and GBL: forensic issues and implications. *J Forensic Sci*. 46:1315-1323 (2001).
19. LeBeau MA, Montgomery MA, Miller ML, Burmeister SG. Analysis of biofluids for gamma-hydroxybutyrate (GHB) and gamma-butyrolactone (GBL) by headspace GC-FID and GC-MS. *J Anal Tox.* 24(6): 421-428 (2000).
20. Ingels ASME, Neels H, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in biofluids using a one-step procedure with “in-vial” derivatization and headspace-trap gas chromatography-mass spectrometry. *J Chrom A*. 1296:84-92 (2013).
21. Blanchet B, Morand K, Hulin A, Astier A. Capillary gas chromatographic determination of 1,4-butanediol and gamma-hydroxybutyrate in human plasma and urine. *J Chromatogr B Analyt Technol Biomed Life Sci*. 769(2):221-226 (2002).
22. Villain M, Cirimele V, Ludes B, Kintz P. Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and urine by gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 792(1):83-87 (2003).
23. McCusker RR, Paget-Wilkes H, Chronister CW, Goldberger BA. Analysis of gamma-hydroxybutyrate (GHB) in urine by gas chromatography-mass spectrometry. *J Anal Toxicol*. 23(5):301-305 (1999).
24. Ingels AS, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct “on spot” derivatization. *Anal Bioanal Chem*. 398(5):2173-2182 (2010).

25. Elliott SP. Further evidence for the presence of GHB in postmortem biological fluid: implications for the interpretation of findings. *J Anal Toxicol*. 28(1):20-26 (2004).
26. LeBeau MA, Miller ML, Levine B. Effect of storage temperature on endogenous GHB levels in urine. *Forensic Sci Int*. 119(2):161-167 (2001).
27. LeBeau MA, Christenson RH, Levine B, Darwin WD, Huestis MA. Intra- and interindividual variations in urinary concentrations of endogenous gamma-hydroxybutyrate. *J Anal Toxicol*. 26(6):340-346 (2002).
28. Elian AA. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Sci Int*. 128(3):120-122 (2002).
29. Elliott SP. Gamma hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. *Forensic Sci Int*. 133(1-2):9-16 (2003).
30. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S. Gamma-hydroxybutyrate in urine and serum: Additional data supporting current cut-off recommendations. *Forensic Sci Int*. 200(1-3):93-99 (2010).
31. De Paoli G, Walker KM, Pounder DJ. Endogenous γ-hydroxybutyric acid concentrations in saliva determined by gas chromatography-mass spectrometry. *J Anal Toxicol*. 35(3):148-152 (2011).
32. Kintz P, Villain M, Cirimele V, Ludes B. GHB in postmortem toxicology. Discrimination between endogenous production from exposure using multiple specimens. *Forensic Sci Int*. 143(2-3):177-181 (2004).
33. Ingels M, Rangan C, Bellezzo J, Clark RF. Coma and respiratory depression following the ingestion of ghb and its precursors: Three cases. *J Emerg Med*. 19(1):47-50 (2000).
34. Bosman IJ, Lusthof KJ. Forensic cases involving the use of GHB in the Netherlands. *Forensic Sci Int*. 133(1-2):17-21 (2003).
35. Kintz P, Villain M, Pelissier AL, Cirimele V, Leonetti G. Unusually high concentrations in a fatal ghb case. *J Anal Toxicol*. 29(6):582-585 (2005).
36. Elliott SP. Nonfatal instances of intoxication with gamma-hydroxybutyrate in the United Kingdom. *Ther Drug Monit*. 26(4):432-440 (2004).
37. Morris-Kukoski CL. γ-Hydroxybutyrate. Bridging the clinical-analytical gap. *Toxicol Rev.* 23(1):33-43 (2004).
38. Andresen H, Stimpfl T, Sprys N, Schnitgerhans T and Muller A. Liquid ecstasy - a significant drug problem. *Dtsch. Arztebl. Int*. 105(36), 599-603 (2008).
39. TIAFT list of therapeutic and toxic concentrations; http://www.tiaft.org/, accessed Spetember 9th 2013.
40. Elliott S, Lowe P, Symonds A. The possible influence of micro-organisms and putrefaction in the production of GHB in post-mortem biological fluid. *Forensic Sci Int*. 139(2-3):183-190 (2004).

41. Kugelberg FC, Holmgren A, Eklund A, Jones AW. Forensic toxicology findings in deaths involving gamma-hydroxybutyrate. *Int J Legal Med*. 124(1):1-6 (2010).
42. Kerrigan S. In vitro production of gamma-hydroxybutyrate in antemortem urine samples. *J Anal Toxicol*. 26(8):571-574 (2002).
43. Crookes CE, Faulds MC, Forrest AR, Galloway JH. A reference range for endogenous gamma-hydroxybutyrate in urine by gas chromatography-mass spectrometry. *J Anal Toxicol*. 28(8):644-649 (2004).
44. Ingels AS, Hertegonne KB, Lambert WE, Stove CP. Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home: an exploratory study. *CNS Drugs*. 27(3):233-237 (2013).
45. Saudan C, Augsburger M, Kintz P, Saugy M, Mangin P. Detection of exogenous GHB in blood by gas chromatography-combustion-isotope ratio mass spectrometry: implications in postmortem toxicology. *J Anal Toxicol*. 29(8):777-781 (2005).
46. Abanades S, Farre M, Segura M, Pichini S, Barral D, Pacifici R, Pellegrini M, Fonseca F, Langohr K, De La Torre R. Gamma-hydroxybutyrate (GHB) in humans: Pharmacodynamics and pharmacokinetics. *Ann N Y Acad Sci*. 1074:559-576 (2006).
47. Palatini P, Tedeschi L, Frison G, Padrini R, Zordan R, Orlando R, Gallimberti L, Gessa GL, Ferrara SD. Dose-dependent absorption and elimination of gamma-hydroxybutyric acid in healthy volunteers. *Eur J Clin Pharmacol*. 45(4):353-356 (1993).
48. Abanades S, Farré M, Segura M, Pichini S, Pastor A, Pacifici R, Pellegrini M, de la Torre R. Disposition of gamma-hydroxybutyric acid in conventional and nonconventional biologic fluids after single drug administration: issues in methodology and drug monitoring. *Ther Drug Monit*. 29(1):64-70 (2007).
49. Ingels AS, De Paepe P, Anseeuw K, Van Sassenbroeck DK, Neels H, Lambert WE, Stove CP. Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis.* 3:2271-2281 (2011).
50. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L. Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. *Mol Genet Metab*. 109(3):255-259 (2013).
51. De Paoli G, Bell S. A rapid GC-MS determination of gamma-hydroxybutyrate in saliva. *J Anal Toxicol*. 32(4):298-302 (2008).
52. Kintz P, Goullé JP, Cirimele V, Ludes B. Window of Detection of γ-Hydroxybutyrate in Blood and Saliva. *Clin Chem*. 47(11):2033-2034 (2001).
53. Kintz P, Cirimele V, Jamey C, Ludes B. Testing for GHB in hair by GC/MS/MS after a single exposure. Application to document sexual assault. *J Forensic Sci*. 48(1):195-200 (2003).

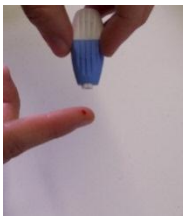
54. Goullé JP, Chèze M, Pépin G. Determination of endogenous levels of GHB in human hair. Are there possibilities for the identification of GHB administration through hair analysis in cases of drug-facilitated sexual assault? *J Anal Toxicol*. 27(8):574-80 (2003).
55. Cirimele V, Baumgartner M, Vallet E, Duez M. Interpretation of GHB concentrations in hair. *Ann Toxicol Analyt.* 22(4):161-164 (2010).
56. Petersen IN, Tortzen C, Kristensen JL, Pedersen DS, Breindahl T. Identification of a new metabolite of GHB: gamma-hydroxybutyric acid glucuronide. *J Anal Toxicol*. 37(5):291-297 (2013).
57. Grootveld M, Algeo D, Silwood CJ, Blackburn JC, Clark AD. Determination of the illicit drug gamma-hydroxybutyrate (GHB) in human saliva and beverages by 1H NMR analysis. *Biofactors*. 27(1-4):121-136 (2006).
58. Marquet P. LC-MS vs. GC-MS, online extraction systems, advantages of technology for drug screening assays. *Methods Mol Biol*. 902:15-27 (2012).
59. Bühlmann laboratories, http://www.buhlmannlabs.ch/core/clinical-chemistry/ghb-kinetic/ accessed on 26/08/2013.
60. Badcock NR, Zotti R. Rapid screening test for gamma-hydroxybutyric acid (GHB, Fantasy) in urine. *Ther Drug Monit*. 21(3):376 (1999).
61. Alston WC 2nd, Ng K. Rapid colorimetric screening test for gamma-hydroxybutyric acid (liquid X) in human urine. *Forensic Sci Int*. 126(2):114-117 (2002).
62. Bravo DT, Harris DO, Parsons SM. Reliable, sensitive, rapid and quantitative enzyme-based assay for gamma-hydroxybutyric acid (GHB). *J Forensic Sci*. 49(2):379-387 (2004).
63. Drugcheck, http://www.drugcheck.com/dc_ghb.html accessed on 26/08/2013.
64. Sciotti MA, Hasan L, Scholer A, Jermann TM, Weber JM, Gygax D. Development and characterization of an enzymatic method for the rapid determination of gamma hydroxybutyric acid. *Chimia*. 64(11):793-798 (2010)
65. Hasan L, Jermann TM, Weber JM, Abrahamsson L, Sciotti MA, Böttcher M, Jöchle W, Gygax D, Scholer A. An enzymatic method to determine γ-hydroxybutyric acid in serum and urine. *Ther Drug Monit*. 33(6):757-765 (2011).
66. Grenier V, Huppé G, Lamarche M, Mireault P. Enzymatic assay for GHB determination in forensic matrices. *J Anal Toxicol*. 36(7):523-528 (2012).
67. Del Signore AG, McGregor M, Cho BP. 1H NMR analysis of GHB and GBL: further findings on the interconversion and a preliminary report on the analysis of GHB in serum and urine. *J Forensic Sci*. 50(1):81-86 (2005).
68. Mercer J, Shakleya D, Bell S. Applications of ion mobility spectrometry (IMS) to the analysis of gamma-hydroxybutyrate and gamma-hydroxyvalerate in toxicological matrices. *J Anal Toxicol*. 30(8):539-544 (2006).

69. Gottardo R, Bortolotti F, Trettene M, De Paoli G, Tagliaro F. Rapid and direct analysis of gamma-hydroxybutyric acid in urine by capillary electrophoresis-electrospray ionization ion-trap mass spectrometry. *J Chromatogr A*. 1051(1-2):207-211 (2004).
70. Baldacci A, Theurillat R, Caslavska J, Pardubská H, Brenneisen R, Thormann W. Determination of gamma-hydroxybutyric acid in human urine by capillary electrophoresis with indirect UV detection and confirmation with electrospray ionization ion-trap mass spectrometry. *J Chromatogr A*. 990(1-2):99-110 (2003).
71. Zacharis CK, Raikos N, Giouvalakis N, Tsoukali-Papadopoulou H, Theodoridis GA. A new method for the HPLC determination of gamma-hydroxybutyric acid (GHB) following derivatization with a coumarin analogue and fluorescence detection: application in the analysis of biological fluids. *Talanta*. 75(2):356-361 (2008).
72. Bortolotti F, De Paoli G, Gottardo R, Trattene M, Tagliaro F. Determination of gamma-hydroxybutyric acid in biological fluids by using capillary electrophoresis with indirect detection. J Chromatogr B Analyt Technol *Biomed Life Sci*. 800(1-2):239-244 (2004).
73. Kimura M, Hasegawa Y, Nakagawa K, Kajita M, Watanabe K, Yamaguchi S. A sensitive method for 4-hydroxybutyric acid in urine using gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 792(1):141-144 (2003).
74. Shinka T, Inoue Y, Ohse M, Ito A, Ohfu M, Hirose S, Kuhara T. Rapid and sensitive detection of urinary 4-hydroxybutyric acid and its related compounds by gas chromatography-mass spectrometry in a patient with succinic semialdehyde dehydrogenase deficiency. *J Chromatogr B Analyt Technol Biomed Life Sci*. 776(1):57-63 (2002).
75. Quang LS, Levy HL, Law T, Desai MC, Maher TJ, Boyer EW, Shannon MW, Woolf AD. Laboratory diagnosis of 1,4-BD and GHB overdose by routine urine organic acid analysis. *Clin Toxicol*. 43(4):321-323 (2005).
76. Garg U, Scott D, Kiscoan M, Knoblauch J, Frazee CC 3rd, Wasserman G. Use of urine organic acids method for the analysis of gamma-hydroxybutric acid (GHB): importance of urease in eliminating urea interference. *Clin Toxicol*. 45(2):194-6 (2007).
77. Rasanen I, Viinamäki J, Vuori E, Ojanperä I. Headspace in-tube extraction gas chromatography-mass spectrometry for the analysis of hydroxylic methyl-derivatized and volatile organic compounds in blood and urine. *J Anal Toxicol*. 34(3):113-121 (2010).
78. Adamowicz P, Kała M. Simultaneous screening for and determination of 128 date-rape drugs in urine by gas chromatography-electron ionization-mass spectrometry. *Forensic Sci Int*. 198(1-3):39-45 (2010).
79. Stove CP, Ingels AS, Lambert WE. Quantitation of gamma-hydroxybutyric acid in dried blood spots in newborns screening. *Mol Genet Metab*. 110(1-2):195 (2013).

80. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L. Response to Stove and colleagues concerning newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency in dried blood spots. *Mol Genet Metab*. 110(1-2):196 (2013).
81. Dahl SR, Olsen KM, Strand DH. Determination of γ-hydroxybutyrate (GHB), β-hydroxybutyrate (BHB), pregabalin, 1,4-butane-diol (1,4BD) and γ-butyrolactone (GBL) in whole blood and urine samples by UPLC-MSMS. *J Chromatogr B Analyt Technol Biomed Life Sci*. 885-886:37-42 (2012).
82. Kraemer T, Paul LD. Bioanalytical procedures for determination of drugs of abuse in blood. *Anal Bioanal Chem*. 388(7):1415-35 (2007).
83. Smith RM. Before the injection--modern methods of sample preparation for separation techniques. *J Chromatogr A*. 1000(1-2):3-27 (2003).
84. Johansen SS, Windberg CN. Simultaneous determination of γ-Hydroxybutyrate (GHB) and its analogues (GBL, 1.4-BD, GVL) in whole blood and urine by liquid chromatography coupled to tandem mass spectrometry. *J Anal Toxicol*. 35(1):8-14 (2011).
85. Gong XY, Kubán P, Scholer A, Hauser PC. Determination of gamma-hydroxybutyric acid in clinical samples using capillary electrophoresis with contactless conductivity detection. *J Chromatogr A*. 1213(1):100-104 (2008).
86. Blair S, Song M, Hall B, Brodbelt J. Determination of gamma-hydroxybutyrate in water and human urine by solid phase microextraction-gas chromatography/quadrupole ion trap spectrometry. *J Forensic Sci*. 46(3):688-693 (2001).
87. Kankaanpää A, Liukkonen R, Ariniemi K. Determination of gamma-hydroxybutyrate (GHB) and its precursors in blood and urine samples: a salting-out approach. *Forensic Sci Int*. 170(2-3):133-138 (2007).
88. Elie MP, Baron MG, Birkett JW. Injection port silylation of γ-hydroxybutyrate and trans-hydroxycrotonic acid: conditions optimisation and characterisation of the di-tert-butyldimethylsilyl derivatives by GC-MS. *Analyst*. 137(1):255-262 (2012).
89. Elian AA, Hackett J. Anion exchange SPE and liquid chromatography-tandem mass spectrometry in GHB analysis. *J Chromatogr B Analyt Technol Biomed Life Sci*. 879(31):3752-3758 (2011).
90. de Vriendt CA, van Sassenbroeck DK, Rosseel MT, van de Velde EJ, Verstraete AG, Vander Heyden Y, Belpaire FM. Development and validation of a high-performance liquid chromatographic method for the determination of gamma-hydroxybutyric acid in rat plasma. *J Chromatogr B Biomed Sci Appl*. 752(1):85-90 (2001).
91. Sørensen LK, Hasselstrøm JB. A hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry method for the simultaneous determination of γ-hydroxybutyrate and its precursors in forensic whole blood. *Forensic Sci Int*. 222(1-3):352-359 (2012).

92. Polson C, Sarkar P, Incledon B, Raguvaran V, Grant R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 785(2):263-275 (2003).
93. Chen M, Andrenyak DM, Moody DE, Foltz RL. Stability of plasma gamma-hydroxybutyrate determined by gas chromatography-positive ion chemical ionization-mass spectrometry. *J Anal Toxicol*. 27(7):445-458 (2003).
94. Louagie HK, Verstraete AG, De Soete CJ, Baetens DG, Calle PA. A sudden awakening from a near coma after combined intake of gamma-hydroxybutyric acid (GHB) and ethanol. *J Toxicol Clin Toxicol*. 35(6):591-594 (1997).
95. Dresen S, Kempf J, Weinmann W. Prevalence of gamma-hydroxybutyrate (GHB) in serum samples of amphetamine, metamphetamine and ecstasy impaired drivers. *Forensic Sci Int*. 173(2-3):112-116 (2007).
96. Lott S, Musshoff F, Madea B. Estimation of gamma-hydroxybutyric acid (GHB) co-consumption in serum samples of drivers positive for amphetamine or ecstasy. *Forensic Sci Int*. 221: 98-101 (2012).
97. Duer WC, Byers KL, Martin JV. Application of a convenient extraction procedure to analyze gamma-hydroxybutyric acid in fatalities involving gamma-hydroxybutyric acid, gamma-butyrolactone, and 1,4-butanediol. *J Anal Toxicol*. 25(7):576-582 (2001).
98. Jones AW, Holmgren A, Kugelberg FC. Gamma-hydroxybutyrate concentrations in the blood of impaired drivers, users of illicit drugs, and medical examiner cases. *J Anal Toxicol*. 31(9):566-572 (2007).
99. Dziadosz M, Weller JP, Klintschar M, Teske J. Adduct supported analysis of γ-hydroxybutyrate in human serum with LC-MS/MS. *Anal Bioanal Chem*. 405(20):6595-6597 (2013).
100. Van Bocxlaer JF. Recent trends in analytical procedures in forensic toxicology. *Ther Drug Monit*. 27(6):752-755 (2005).
101. Fukui Y, Matsusima E, Muramoto K, Nagai N, Ohama K, Yamashita K. Validation of a simple gas chromatographic-mass spectrometric method for the determination of gamma-butyrolactone in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 785(1):73-80 (2003).
102. Lenz D, Kröner L, Rothschild MA. Determination of gamma-hydroxybutyric acid in serum and urine by headspace solid-phase dynamic extraction combined with gas chromatography-positive chemical ionization mass spectrometry. *J Chromatogr A*. 1216(18):4090-4096 (2009).
103. Brenneisen R, Elsohly MA, Murphy TP, Passarelli J, Russmann S, Salamone SJ, Watson DE. Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. *J Anal Toxicol*. 28(8):625-630 (2004).

104. Couper FJ, Logan BK. Determination of gamma-hydroxybutyrate (GHB) in biological specimens by gas chromatography--mass spectrometry. *J Anal Toxicol*. 24(1):1-7 (2000).
105. Lora-Tamayo C, Tena T, Rodríguez A, Sancho JR, Molina E. Intoxication due to 1,4-butanediol. *Forensic Sci Int*. 133(3):256-259 (2003).
106. Sakurada K, Kobayashi M, Iwase H, Yoshino M, Mukoyama H, Takatori T, Yoshida K. Production of gamma-hydroxybutyric acid in postmortem liver increases with time after death. *Toxicol Lett*. 129(3):207-217 (2002).
107. Elian AA. A novel method for GHB detection in urine and its application in drug-facilitated sexual assaults. *Forensic Sci Int.* 109(3):183-187 (2000).
108. Mazarr-Proo S, Kerrigan S. Distribution of GHB in tissues and fluids following a fatal overdose. *J Anal Toxicol.* 29(5):398-400 (2005).
109. Elian AA. GC-MS determination of gamma-hydroxybutyric acid (GHB) in blood. *Forensic Sci Int.* 122(1):43-47 (2001).
110. Kaufmann E, Alt A. Determination of GHB in urine and serum by LC/MS using a simple one-step derivative. *Forensic Sci Int*. 168(2-3):133-137 (2007).
111. Fung HL, Haas E, Raybon J, Xu J, Fung SM. Liquid chromatographic-mass spectrometric determination of endogenous gamma-hydroxybutyrate concentrations in rat brain regions and plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 807(2):287-291 (2004).



PART II
DETERMINATION OF GHB IN DRIED BLOOD SPOTS USING "ON SPOT" DERIVATIZATION AND GC-MS

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*In this part, we aimed to determine GHB in dried blood spots (DBS) using a simple GC-MS method. First, a brief description of DBS and their use in toxicology is given, with the focus on the determination of drugs of abuse (**Chapter II.A**). To adequately quantify the club drug in DBS, chemical modification of this small and polar molecule was required. Since we preferred to derivatize GHB, derivatization techniques used in combination with DBS analysis are discussed more into detail in **Chapter II.B**.*

*We chose a direct derivatization approach - addition of the reagent(s) directly on the DBS, thereby omitting the extraction step - which resulted in the set-up of a quick and efficient protocol. We coined this approach "on spot" derivatization. Thus, following the development and optimization of the GC-MS method used to determine derivatized GHB (**Chapter II.C.1**), the development and validation of the GC-MS method to quantify GHB in complete DBS with a fixed volume of 50 µl is presented in **Chapter II.C.2**.*

*To address the need for a more practical approach, which is the collection of drops of blood directly on the filter paper, the first method was adjusted, as discussed in **Chapter II.C.3**. Validation was performed, with the evaluation of additional parameters*

typical for analysis of DBS punches, where no longer a fixed volume is used but a fixed diameter. These parameters include the influence of punch localization, hematocrit and blood spot volume. Finally, applicability of this method was demonstrated by analyzing venous blood and capillary DBS, collected simultaneously from GHB-intoxicated patients.

Chapter II.C.4 *concludes with a study, conducted to evaluate the feasibility of the DBS sampling technique at home. Therefore, DBS were collected by patients who use the sodium salt of GHB (sodium oxybate, Xyrem®). Samples were sent by mail to our laboratory and analyzed using the method described in II.C.3.*

CHAPTER II.A
DBS IN TOXICOLOGY: FOCUS ON DRUGS OF ABUSE

Based on

Dried Blood Spots in Toxicology: from the Cradle to the Grave? *Crit. Rev. Toxicol*, 42(3):230-243 (2012). Christophe P Stove, Ann-Sofie ME Ingels, Pieter MM De Kesel and Willy E Lambert

II.A.1 INTRODUCTION

A dried blood spot (DBS) is capillary whole blood obtained by a finger or heel prick and collected on a filter paper. This sampling technique ensures an easy and rapid collection of a representative sample without specific handling and storage requirements. These advantages as to a venepuncture make it a cost-effective choice for the collection, transport and storage of blood samples (Fig. II.A.1) [1,2]. Already about a century ago, the potential of using DBS for biomonitoring was recognized by Ivar Bang, who demonstrated its usefulness for glucose monitoring [3]. However, it took another 50 years before the use of DBS became more widespread, ignited by the seminal paper by Guthrie and Susi, who demonstrated the applicability of screening newborn DBS for phenylketonuria [4]. Since then, an ever increasing amount of biomarkers has been included in DBS newborn screening programs worldwide [5-9]. Apart from its use for newborn screening, DBS sampling has also been applied in animals, children, adults and even post mortem, its applications rising rapidly the last few years, covering the analysis of DNA (e.g. HIV, serotyping of bacteria, genotyping), proteins (e.g. enzyme activity or antibody-based analysis), small molecules (endogenous or exogenous, e.g. amino acids or therapeutic drugs), as well as trace elements (e.g. lead).

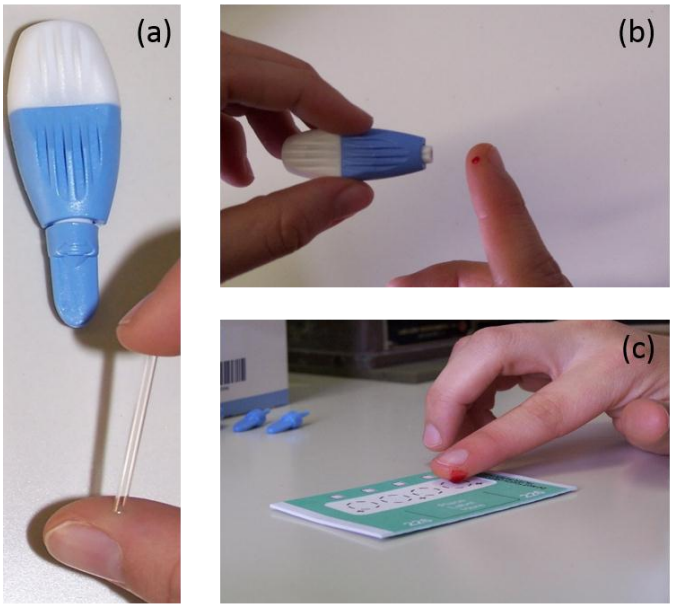


Fig. II.A.1

(a) An automatic lancet (top) and a 50-µl precision capillary (bottom)

(b) The fingertip is pricked with the help of an automatic lancet (single use)

(c) Drop of blood can be collected with the help of a precision capillary (a, bottom) or directly onto the filter paper

The different subdisciplines of toxicology for which DBS sampling has been reported include the application of DBS for the analysis of therapeutic drugs (toxicokinetics), drugs of abuse, environmental contaminants, toxins and (trace) elements. The main focus of this overview is the application of DBS for the analysis of drugs of abuse. Topics not covered are the use of DBS for therapeutic drug monitoring (TDM) and for metabolic screening, two fields which, although having some overlap with toxicology because toxic effects may be encountered, are considered as distinct disciplines.

The interest in using DBS for the purpose of TDM (including clinical toxicology), with sampling either in the clinic or at the patient's home, has recently shown a strong increase. Also here, apart from follow-up and reassuring that therapeutic concentrations are reached in patients, toxicology may come into play when considering the purpose to monitor (and avoid) supratherapeutic (potentially toxic) concentrations. However, it is beyond the scope of this review to provide a full overview of all therapeutic drugs for which DBS sampling has been applied. The reader is referred to other, comprehensive reviews on this topic (e.g. [2,10,11]).

Metabolic screening programs based on DBS sampling aim at identifying disturbed balances in endogenous biomolecules, which may lead to toxic effects. A forensic application worth mentioning in this context is the "metabolic autopsy", which can be performed on DBS to screen for inherited metabolic disorders in cases of sudden infant death syndrome or sudden unexplained death syndrome [12]. These DBS can either be obtained post mortem or can be those that were obtained at birth in cases where only later a metabolic disorder is suspected in deaths of previously unknown cause.

II.A.2 Dried Blood Spots – Sources

In developed countries, typically five DBS, each corresponding to about 80 µl of blood, are obtained by heel stick from the vast majority (>95%) of newborns within the first 1-3 days of life. With the exception of the 'positive' cases, only a limited amount of this material is used for newborn screening programs, which primarily focus on inborn errors of metabolism. Thus, a substantial amount of valuable material is left behind. These remainders have proven to be a useful matrix for assessing certain exposures at birth. The prime aim of these assessments is to monitor prenatal exposure to toxic compounds capable of crossing the foetoplacental barrier.

However, as in most cases no information is available with respect to breastfeeding, it needs to be remarked that postpartum exposure of the newborn via mother milk cannot be excluded. Moreover, when interpreting the results in the context of epidemiological studies, several potential sources of bias need to be kept in mind [13]. First, although newborn screening is almost universal in developed countries, non-participation is unlikely to be random (e.g. infants may have died before DBS sampling or parents may have refused DBS sampling). Second, less (or no) material may be left from those newborns that tested positive in newborn screening programs.

As analyses are typically performed on material that has been archived up to several years, contamination has to be excluded and analyses are limited to analytes with long-term stability in DBS. In addition, parameters potentially influencing the analytical result, such as hematocrit, blood volume spotted and site of punching (peripheral versus central) [14,15] have not been examined in many cases. Nevertheless, keeping these limitations in mind, newborn DBS can be valuable material for screening and may provide important retrospective information on the extent of exposure to a wide array of chemicals or elements. Given the fact that these early life exposures may be relevant to disease later in life, this information may not only result in a close follow-up of 'positively scored' newborns, but may also form the basis for intervention studies, targeting women at specific locations and/or belonging to specific (social) groups.

Apart from DBS sampling for newborn screening, more recently, this sampling technique has increasingly gained interest for its use in both animal and human studies. In humans (both adults and children), DBS are mostly obtained by finger prick. The resulting DBS, which may be directly applied from the finger onto the filter paper or via a precision capillary (Fig. II.A.1), are in general smaller than those obtained by heel stick. Advantages associated with the sampling itself primarily include its ease and its minimal invasiveness, facilitating sampling in remote areas and in paediatric studies by non-specialized individuals. Sampling from animals at specified time intervals after administration of a given drug is generally performed using microsampling devices (e.g. precision capillaries). The blood collected with these devices can be used to generate DBS, or it can be frozen, diluted in another solution or centrifuged to prepare plasma [16,17]. Applications include pharmaco- and toxicokinetics, TDM and clinical, forensic and environmental toxicology.

II.A.3 Analysis of Therapeutic Drugs - Toxicokinetics

Currently, many pharmaceutical companies undertake major efforts to implement DBS rather than classical plasma samples as starting material for bioanalytical measurements. These efforts are situated in the preclinical phase of the drug discovery process (e.g. toxicokinetics) as well as in later phases (pharmacokinetics and TDM). Here, we will primarily focus on the use of DBS for toxicokinetics, determining the relationship between systemic exposure of an animal to a compound and the harmful effects (toxicity) of this compound. A preliminary safety assessment can be derived from parameters such as bioavailability and dose proportionality, serving as a basis to decide which doses can be used in future studies.

From the point of view of animal welfare, DBS sampling conforms very well to the '3R principle (Replacement, Reduction, Refinement)' in toxico- and pharmacokinetic studies. The fact that finer needles can be used to obtain DBS and that there is no need to warm the animals prior to sampling, causes less distress to the animals. Besides this refinement, resulting in less animal burden, the implementation of DBS sampling also leads to a strong reduction in the number of animals needed in early drug discovery and preclinical studies. More specifically, DBS sampling requires less blood to be taken at each time point than is the case when analyses are performed using the 'classical' matrices plasma or serum. For the latter, the number of samples that can be collected per animal is limited by both ethical and physiological constraints. These constraints are greatly relieved by 'microsampling', resulting in the generation of DBS and allowing serial sampling from a strongly reduced amount of laboratory animals, including small rodents such as mice. This allows the replacement of composite pharmaco- or toxicokinetic profiles (obtained from different animals) by serial profiles (obtained within individual animals), which leads to higher data quality [18-20]. Apart from a large improvement in animal welfare, DBS sampling is also beneficial for the (pharmaceutical) companies involved. From a financial point of view, there is a serious reduction in costs associated with animal studies (including amount of test compound that should be available) and with sample handling. The latter includes both transportation costs to a bioanalytical facility and storage costs. Both transportation and storage are often facilitated, as experience has learned that stability, though requiring analyte-specific evaluation, is generally good. Thus, in many cases prolonged storage, even at ambient temperatures, is possible. Despite these many advantages, however, pharmaceutical companies initially somewhat restrained from utilizing DBS as an alternative to plasma or serum. Importantly, the use of DBS instead of plasma or serum

necessitated a rethinking of bioanalytical procedures, particularly in the pre-analytical phase. This not only includes the selection of the filter paper card, but ideally also encompasses evaluation of the influence of spotting temperature, anticoagulant, the spotting device used, the volume spotted, the site of punching and the hematocrit, in addition to evaluation of the "on spot stability" and the effect of drying and storage conditions. Additionally, one should also dispose of means for detecting contamination, such as evaluation of blanks and/or incurred sample reanalysis [21-26]. However, setting up new bioanalytical procedures is (was) not the main problem for the 'switch' from plasma to DBS. More important are regulatory constraints and the fact that plasma and serum have been used for decades as the gold standard, with all currently available toxico- and pharmacokinetic data having been obtained in these matrices. The latter implies that care should be taken, not only in evaluating how plasma concentrations correlate with blood or DBS concentrations, but also if and how capillary concentrations correlate with venous concentrations [27]. This may be particularly relevant when evaluating early time points in kinetic experiments [28]. Another factor to consider is the anticipated concentration: when in the low- or sub-ng/ml range, the limited amount of available material may impose analytical challenges that have to be dealt with. Recent improvements in analytical equipment, with primarily LC-MS/MS becoming more widespread available, have catalyzed progress in this field. Currently, major efforts are also being undertaken to render DBS analysis high-throughput-capable. Examples include the automated analysis of DBS, the on-line extraction and analysis of DBS ('direct elution') and direct desorption of DBS (e.g. [20,29]). These new developments have recently been reviewed elsewhere [30,31].

II.A.4 Analysis of Drugs of Abuse

Several publications and meeting abstracts demonstrate (or suggest) the potential of DBS for detecting exposure to drugs of abuse (Table II.A.1 and Appendix 1). Analytes measured include both legal drugs (scheduled drugs available on prescription) and illegal drugs. It needs to be mentioned, though, that some of these reports focus on the potential of determining these drugs for TDM (e.g. narcotic painkillers) or for newborn screening (e.g. monitoring exposure to cocaine), rather than for forensic purposes. In addition, several reports have demonstrated the possibility to identify drugs of abuse, as well as ethyl glucuronide, a marker for alcohol abuse, in blood spotted on different surfaces, rather than on filter paper. As in these cases the resulting blood spots are bloodstains rather than DBS, we do not consider these as true 'DBS applications' [32-34]. Overall,

two sources of DBS can be distinguished for monitoring drugs of abuse: firstly DBS obtained from adults, where the application can be classified under 'forensic toxicology', and secondly DBS from newborns, where the aim is to assess exposure prior to birth. Owing to the low concentrations to be detected in minute amounts of material, LC-MS/MS has been the method of choice in the vast majority of applications, although also GC-MS has been applied in some cases (Table II.A.1).

II.A.4.1 FORENSIC TOXICOLOGY

There is a substantial number of reports describing DBS applications for drugs of abuse (for an overview, see Table II.A.1). Analytes of particular forensic interest that have been measured in DBS include benzodiazepines (alprazolam, clonazepam, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam, phenazepam, temazepam), zolpidem, zopiclone, zaleplon, MDMA, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDEA), amphetamine, methamphetamine, cocaine, tetrahydrocannabinol (THC), opiates (6-monoacetylmorphine, morphine, codeine, hydromorphone, hydrocodone, oxycodone, noroxycodone), tramadol, methadone, buprenorphine, fentanyl, ketamine and their respective metabolites, gamma-hydroxybutyric acid (GHB) and novel psychoactive substances [35-68]. Also interesting from a forensic point of view is the potential to monitor alcohol abuse *via* the determination of ethylglucuronide and ethylsulfate or phosphatidylethanol in DBS [69-71].

Table II.A.1 Overview of the analytes discussed in this review, with referral to the utilized analytical techniques. (*Update of (the markers of) drugs of abuse until July 2013)*.p72-75.

Analyte	Technique	Selected References
(MARKERS OF) DRUGS OF ABUSE		
Amphetamine	LC-MS/MS	Jantos and Skopp, 2011 [51]; Lauer et al., 2011 [55]; Saussereau et al., 2012 [56]; Ambach et al., 2013 [57]
	GC-MS	Langel et al., 2011 [54]
MDMA, MDA	LC-MS/MS	Skopp, 2007 [58]; Jantos and Skopp, 2011 [51]; Jantos et al., 2011b [53]; Lauer et al., 2011 [55]; Thomas et al., 2012 [59]; Saussereau et al., 2012 [56], Déglon et al., 2012a [30], Ambach et al., 2013 [57]
	GC-MS	Langel et al., 2011 [54]
MDEA	LC-MS/MS	Lauer et al., 2011 [55]; Saussereau et al., 2012 [56]; Déglon et al., 2012a [30]; Ambach et al., 2013 [57]
Methamphetamine	LC-MS/MS	Lauer et al., 2011 [55]; Saussereau et al., 2012 [56]; Déglon et al., 2012a [30]; Ambach et al., 2013 [57]
	GC-MS	Langel et al., 2011 [54]
Novel psychoactive substances	LC-MS/MS	Ambach et al., 2013 [57]

Cocaine, benzoylecgonine and other metabolites	RIA, GC-MS	Henderson et al., 1993 [35]
	RIA, LC-MS	Henderson et al., 1997 [37]
	LC-FLUO	Mercolini et al., 2010 [45]
	LC-MS/MS	Sosnoff et al., 1996 [36]; Alfazil and Anderson, 2008 [38]; Lauer et al., 2011 [55]; Thomas et al., 2012 [59]; Saussereau et al., 2012 [56]; Déglon et al., 2012a [30]
	GC-MS	Langel et al., 2011 [54]
Benzodiazepines	LC-MS/MS	Alfazil and Anderson, 2008 [38]; Havard et al., 2010 [42]; Thomas et al, 2010 [46]; Jantos and Skopp, 2011 [51]; Lauer et al., 2011 [55]; Déglon et al., 2012a,b [30,60]
	GC-MS	Langel et al., 2011 [54]
Zolpidem	LC-MS/MS	Hudson et al., 2011 [49]; Lauer et al., 2011 [55]; Déglon et al., 2012a,b [30,60]
Zopiclone	LC-MS/MS	Jantos and Skopp, 2011 [51]; Lauer et al., 2011 [55]; Jantos et al., 2012 [61]; Déglon et al., 2012a,b [30,60]
	GC-MS	Langel et al., 2011 [54]
Zaleplon	LC-MS/MS	Déglon et al., 2012b [60]
Ketamine and norketamine	LC-MS/MS	Moll et al., 2009 [40]; Ambach et al., 2013[57]
Gamma-hydroxybutyric acid	LC-MS/MS	Forni et al., 2013 [68]
	GC-MS	Ingels et al., 2010 [43]; Ingels et al.,2011 [50]
Opiates and metabolites	LC-MS/MS	Skopp et al., 2007 [58]; Garcia Boy et al., 2008 [39]; Thomas et al., 2010 [46]; Marin et al., 2010 [44]; Clavijo et al., 2011a [47]; Jantos et al., 2011a [52]; Lauer et al., 2011 [55]; Saussereau et al., 2012 [56]; Déglon et al., 2012a [30]; Mommers et al., 2013 [63]
	GC-MS	Langel et al., 2011 [54]
Buprenorphine and metabolites	LC-MS/MS	Thomas et al., 2010 [46]; Marin et al., 2010 [44], Lauer et al., 2011 [55]
	GC-MS	Langel et al., 2011 [54]
Methadone and metabolites	LC-MS/MS	Clavijo et al., 2010 [41], Lauer et al., 2011 [55]; Déglon et al., 2012a [30]
	LC-coulometry	Addolorate Saracino et al., 2012 [62]
	GC-MS	Langel et al., 2011 [54]
Fentanyl and metabolites	LC-MS/MS	Clavijo et al., 2011b [48]; Jantos et al., 2011a [52], Lauer et al., 2011 [55]; Déglon et al., 2012a [30]
Tramadol	LC-MS/MS	Déglon et al., 2012a [30]
	GC-MS	Langel et al., 2011 [54]
Tetrahydrocannabinol and metabolites	LC-MS/MS	Thomas et al., 2010 [46], Thomas et al., 2012 [59]; Déglon et al., 2012a [30]; Mercolini et al., 2013 [64]
	GC-MS	Langel et al., 2011[54]
Cotinine	LC-MS/MS	Murphy et al., 2013 [65]
	GC-MS	Spector et al., 2007 [84]
Ethylglucuronide – ethylsulfate	LC-MS/MS	Hernández Redondo et al., 2011 [58], Hernández Redondo et al., 2013 [66]
Phosphatidylethanol	LC-MS/MS	Faller et al., 2011 [56]; Jones et al., 2011 [57]; Faller et al., 2013 [67]
ENVIRONMENTAL CONTAMINANTS		
Benzene oxide	GC-MS	Funk et al., 2008 [86]
Organochlorine pesticides	GC-ECD	Dua et al., 1996 [87]; Burse et al., 1997 [88]
	GC-HRMS	Shlosberg et al., 2012 [89]
Perfluoroalkyl compounds	LC-MS/MS	Spliethoff et al., 2008 [80]; Kato et al., 2009 [91], Shlosberg et al., 2012 [89]

Polychlorinated biphenyls	GC-HRMS	Shlosberg et al., 2012 [89]; Lu et al., 2012 [93]
Polybrominated diphenyl esters	GC-HRMS	Shlosberg et al., 2012 [89]; Lu et al., 2012 [93]
Perchlorate	IC-MS/MS	Otero-Santos et al., 2009 [95]
Bisphenol A	LC-MS/MS	Leonard et al., 2011 [96]
Cholinesterase Inhibitors	Cholinesterase activity measurement	Heilbronn, 1953 [98]; Augustinsson and Heimburger, 1953 [97]; Augustinsson and Holmstedt, 1965 [99]; Holmstedt and Oudart, 1966 [100]; Collombel and Perrot, 1970 [101]; Oudart and Holmstedt, 1970 [102]; Augustinsson et al., 1978 [103]; Eriksson and Faijersson, 1980 [104]; Rhyanen et al., 1984 [105]; Hilborn and Padilla, 2004 [106]; Trudeau et al., 2007 [72]; Quandt et al., 2010 [107]
BIOTOXINS		
Domoic acid	C-ELISA	Maucher and Ramsdell, 2005 [116]
Brevetoxins	R-binding assay	Fairey et al., 2001 [112]; Woofter et al., 2003 [113]
	RIA	Woofter et al., 2003 [113]; Woofter et al., 2005 [114]
	C-ELISA	Maucher et al., 2007 [117]
Ciguatoxin	Cytotox. assay	Bottein Dechraoui et al., 2005 [119]
(TRACE) ELEMENTS		
As	ICP-MS	Shlosberg et al., 2012 [89]
Ba	LA-ICP-MS	Hsieh et al., 2011 [124]
Be	LA-ICP-MS	Hsieh et al., 2011 [124]
Bi	LA-ICP-MS	Hsieh et al., 2011 [124]
Ca	LA-ICP-TOF-MS	Cizdziel, 2007 [122]
	SF-ICP-MS	Langer et al., 2010 [109]
Cd	ICP-MS	Chaudhuri et al., 2009 [123]; Shlosberg et al., 2012 [89]
	SF-ICP-MS	Langer et al., 2010 [109]
	LA-ICP-MS	Hsieh et al., 2011 [124]
Co	LA-ICP-MS	Hsieh et al., 2011 [124]
Cr	SF-ICP-MS	Langer et al., 2010 [109]
Cs	SF-ICP-MS	Langer et al., 2010 [109]
Cu	LA-ICP-TOF-MS	Cizdziel, 2007 [122]
	SF-ICP-MS	Langer et al., 2010 [109]
	LA-ICP-MS	Hsieh et al., 2011 [124]
Fe	LA-ICP-TOF-MS	Cizdziel, 2007 [122]
	SF-ICP-MS	Langer et al., 2010 [109]
Hg	ICP-MS	Chaudhuri et al., 2009 [123]; Shlosberg et al., 2012 [89]
K	SF-ICP-MS	Langer et al., 2010 [109]
Li	SF-ICP-MS	Langer et al., 2010 [109]
Mg	SF-ICP-MS	Langer et al., 2010 [109]
	LA-ICP-MS	Hsieh et al., 2011 [124]
Mn	LA-ICP-MS	Hsieh et al., 2011 [124]
Mo	SF-ICP-MS	Langer et al., 2010 [109]
Na	SF-ICP-MS	Langer et al., 2010 [109]
Ni	SF-ICP-MS	Langer et al., 2010 [109]
	LA-ICP-MS	Hsieh et al., 2011 [124]
P	SF-ICP-MS	Langer et al., 2010 [109]
Pb	AAS	Cernik et al., 1971 [129]; Verebey et al., 1991 [132]
	GFAAS	Shen et al., 2003 [125]
	SS-GFAAS	Resano et al., 2007 [121]
	ICP-MS	El-Hajjar et al., 2007 [135]; Chaudhuri et al., 2009 [123]; Shlosberg et al2012 [89]
	LA-ICP-TOF-MS	Cizdziel, 2007 [122]

	SF-ICP-MS	Langer et al., 2010 [109]
	LA-ICP-MS	Hsieh et al., 2011 [124]
Rb	SF-ICP-MS	Langer et al., 2010 [109]
S	SF-ICP-MS	Langer et al., 2010 [109]
Sb	LA-ICP-MS	Hsieh et al., 2011 [124]
Se	AAS	Lombeck et al., 1989 [140]
	ICP-MS	Shlosberg et al., 2012 [89]
Tl	LA-ICP-MS	Hsieh et al., 2011 [124]
V	LA-ICP-TOF-MS	Cizdziel, 2007 [122]
Zn	LA-ICP-TOF-MS	Cizdziel, 2007 [122]
	SF-ICP-MS	Langer et al., 2010 [109]
	LA-ICP-MS	Hsieh et al., 2011 [124]

AAS: atomic absorption spectrometry; C-ELISA: competitive enzyme-linked immunosorbent assay; GC-ECD: gas chromatography with electron capture detection; GC-HRMS: gas chromatography with high resolution mass spectrometric detection; GC-MS: gas chromatography with mass spectrometric detection; IC-MS/MS: ion chromatography with tandem mass spectrometric detection; ICP-MS: inductively coupled plasma mass spectrometry; LA-ICP-MS: laser ablation inductively coupled plasma mass spectrometry; LA-ICP-TOF-MS: laser-ablation inductively coupled plasma time-of flight mass spectrometry; LC-MS: liquid chromatography with mass spectrometric detection; LC-MS/MS: liquid chromatography with tandem mass spectrometric detection; R-binding assay: receptor-binding assay; RIA: radio-immuno assay; SF-ICP-MS: sector-field inductively coupled plasma mass spectrometry; SS-GFAAS: solid sampling graphite furnace atomic absorption spectrometry

Although most reports have included patient samples, two important remarks need to be made. First, various reports utilize DBS prepared by pipetting venous blood onto a paper card (e.g. [39,41,43,53,69,70]), with only a limited amount of reports describing the analysis of true capillary DBS (e.g. [36,45,50]). Although the latter can be obtained by using a precision capillary [45], our experience learned that this significantly complicates the procedure and ideally requires some training. Instead, direct application of the blood drop from the pricked fingertip onto the paper is generally found to be easy, also for a non-trained individual. However, as DBS obtained in this way do not represent a fixed volume, quantitative evaluation requires the analysis of DBS punches rather than of complete DBS. This brings us to the second remark. Analysis of (very) large spots, obtained from spotting up to 100 µl, has been performed in a substantial number of publications (e.g. [38,39,51,69]). These volumes are not easily obtained by finger prick; in our experience a spot applied directly from a pricked fingertip onto paper corresponds typically to less than 40 µl of blood. Moreover, the non-volumetric application also has the consequence that disks (typically 3, 6 or 6.35 mm diameter) need to be punched from the paper, further reducing the amount of material available for analysis. Important to mention in this context is that the validation of methods starting from disk punches rather than from complete blood spots requires the

evaluation of additional parameters such as punch location, hematocrit and volume spotted [10,50]. Thus, although promising results have been obtained, suggesting more widespread applicability in forensic toxicology, true 'on-field' studies are needed for a substantial amount of compounds, in which DBS are obtained by a finger prick.

Below we discuss more into detail two specific advantages associated with DBS sampling and the opportunities these offer for implementation in forensic toxicology: the ease of sampling (facilitating rapid sampling) and the stabilizing effect of DBS.

II.A.4.1.1 Ease of Sampling

Although legislation in most countries does not (yet) allow non-medical staff to obtain DBS from someone else, the ease with which DBS can be taken renders it in principle possible to acquire a blood sample with a minimal loss of time. As this would imply that, at least in some instances, the sampling is done outside a medical facility or a laboratory, e.g. at the road-side, also care has to be taken to let the filter paper dry properly, as an analyte's stability may be impacted by the drying time and drying conditions. In such situations, the paper can be dried by e.g. putting it in a box or bag with desiccant, taking care that the blood spots do not come into contact with other surfaces. This approach of pro-active drying has already been applied in field studies where blood was sampled from wild birds [72]. Furthermore, sufficient experience and frequent sampling most likely contribute to collection of high quality DBS.

Rapid sampling is particularly relevant in cases in which the half-life of a drug is short. Examples include cocaine, heroin (and its metabolite 6-monoacetylmorphine, 6-MAM) and GHB [35-39,43,45,50,68]. Whereas cocaine intake can be demonstrated by virtue of its metabolite benzoylecgonine, heroin abuse cannot be simply deduced from the presence of its hydrolysis end-product, morphine (see also below). An even more difficult case is presented by GHB, which is also endogenously present and is rapidly cleared from the circulation. GHB or one of its precursors is sometimes used in cases of drug-facilitated sexual assault (DFSA). In these cases, there is most often readily a delay before the victim presents at the police station, thus sampling should be done as fast as possible (without needing to wait for a doctor to arrive). DBS sampling may be a good option in these cases.

The ease and speed of sampling also allows to investigate the epidemiology of drug abuse in a nightclub environment, where DBS could be obtained in “first aid” rooms (e.g. [73]). As in these cases informed consent needs to be obtained from individuals who are under the influence of drugs, this may pose bio-ethical issues, which can be dealt with *via* informed consent from a relative or *via* delayed informed consent.

DBS sampling in the context of DUID (driving under the influence of drugs) has only been evaluated to a limited extent [74]. Although for DUID testing, oral fluid has become the matrix of choice for both screening and confirmation in countries such as Belgium, Australia and Spain, some controversy exists whether the obtained concentrations always closely mirror blood concentrations (supposedly best correlating with intoxication) and whether falsification by e.g. mouth washing may be possible [75,76]. DBS sampling does not suffer from these drawbacks and combines the advantages (relevance and reliability) of obtaining the ultimate specimen for determination of drug concentrations - i.e. blood - with an easy and rapid collection procedure by non-specialized staff. A key issue related to DBS sampling in this context is the exclusion of contamination.

For the follow-up of drug and alcohol addicts, DBS sampling may be useful to control abstinence from drugs and/or intake of substitution medication. Here, the use of DBS, though offering a more restricted window of detection, may offer an alternative for urine testing, which is now routinely used. Importantly, DBS sampling is gender neutral and is not hampered by privacy issues, which often lead to unsupervised sampling (and possibly adulteration) of urine. As there is also no need for medical staff, one may envisage a system in which unwittingly (former) addicts get a phone call at irregular time intervals and have to present themselves at a given centre to provide DBS under supervision. Moreover, given the higher prevalence of viral infections (e.g. hepatitis and HIV) in people with a history of intravenous drug abuse, the low biohazard risk posed by the resulting DBS is an additional important advantage. Once a DBS has been collected (using safety measurements similar to venous sampling), no further manipulation of the blood is required (in contrast to e.g. the preparation of plasma, requiring centrifugation) and DBS can be transported *via* regular mail with no risk of breaking or leaking, thus minimizing the risk of transmitting blood-borne viruses and overcoming the need for taking special safety precautions [77]. In fact, viruses such as HIV-1

lose their infectivity as their envelope is disrupted upon drying, which has led to the use of DBS for routine HIV monitoring in screening and follow-up programs in developing countries [78].

II.A.4.1.2 Stabilizing Effect

Multiple publications have pointed out that DBS may have a stabilizing effect (e.g. [79,80]). In a forensic context, this can be exemplified by several examples. First, the stabilizing effect on drugs having e.g. an ester function was demonstrated by the increased stability (reduced hydrolysis) of cocaine and 6-MAM (a metabolite of heroin) in DBS, as compared to whole blood [35,38,39]. This is of particular importance as identification of 6-MAM unequivocally demonstrates heroin use (whereas the presence of morphine alone does not). Secondly, DBS may also overcome the problem of *ex vivo* formation of a given compound. Whereas *ex vivo* formation of the club drug GHB in whole blood has been reported [81,82], prolonged storage of DBS at room temperature (up to 6 months) demonstrated no significant changes [50]. Similarly, whereas the presence of ethanol in blood may result in the *ex vivo* generation of phosphatidylethanol (1-palmitoyl-2-oleyl-sn-glycero-4-phosphoethanol) upon storage [83,70], this *ex vivo* formation does not take place in DBS [70]. Thus, DBS sampling is able to counter a serious drawback associated with classical venous sampling, thereby increasing the trustworthiness of the result.

In the forensic toxicology laboratory, the use of DBS may also offer the possibility to preserve small amounts of sample in an economical way in 'closed cases', where all other evidence is to be discarded. When, for one reason or another, a case is to be reopened, there is at least some material left, potentially allowing targeted analysis. A similar approach has also been suggested for other biological matrices, such as urine [84]. Obviously, a limitation is that only analytes can be detected that remain stable for an extended period of time [84].

II.A.4.2 Newborn Screening

Benzoylecgonine and cotinine, which are metabolites of cocaine and nicotine, respectively, have been determined in newborn DBS to assess the prevalence of the use of cocaine and tobacco products among childbearing women [35-37,85]. An inherent limitation here is that positive results will only indicate the mother's use of cocaine or tobacco near the time of delivery, thus only offering a limited view on the use during pregnancy. On the other hand, a factor likely extending the interval for detecting positive cases, is the immature liver function in newborns.

Although immunological assays have been found to be a useful tool for initial screening of benzoylecgonine in DBS, confirmation is required using other techniques, such as mass spectrometry (GC-MS or LC-MS/MS) [35-37]. With respect to decision-making, any positive signal (above the limit of detection, LOD, or lower limit of quantification, LLOQ) may raise an alert. This implies that the lower the LOD or LLOQ of a given method, the higher the expected detection rate. Implementing a cut-off value in DBS testing of newborns for drugs of abuse may facilitate the inter-laboratory comparison of prevalences. While defining this decision limit, the potential error caused by the possible effect of e.g. varying hematocrit and volume spotted should be taken into account. However, as the cut-off would necessarily be above the LOD or LLOQ, the % of false negatives will undoubtedly increase.

II.A.5 Analysis of Environmental Contaminants

Screening for environmental contaminants has been performed using DBS from both humans (primarily newborns) and animals. Examples of analytes that have been monitored include environmental pollutants such as benzene oxide (a metabolite of benzene, monitored via its adducts with hemoglobin) [86], organochlorine pesticides [87-89], perfluoroalkyl compounds (PFCs) [89-92], polychlorinated biphenyls (PCBs) [89,93], polybrominated diphenyl esters (PBDEs) used as flame-retarding chemicals [89,93,94], perchlorate [95], heavy metals, as well as certain toxins. Although no published reports are available, yet, DBS have also been suggested to be useful for monitoring bisphenol A [92,96].

An alternative, indirect way for assessing the exposure to a contaminant, is the monitoring of a biological activity directly influenced by this contaminant (via a so-called 'biomarker of effect'). Insecticides like organophosphates and carbamates are good candidates for this approach, as exposure can be assessed by virtue of their inhibition of cholinesterase activity. The first reports on the determination of cholinesterase activity in blood samples absorbed on filter paper readily date back to 1953 [97,98], a decade before Guthrie and Susi published on the detection of phenylalanine in newborn DBS [4]. It needs to be remarked, though, that sensitivity is rather limited and ideally one should know an individual's enzyme activity prior to exposure, with only considerable intoxications resulting in a significant decrease in enzyme activity. Yet, multiple other publications have shown the potential to use (dried) blood and plasma spots for monitoring cholinesterase activity, primarily for occupational surveillance of exposed workers [99-107].

Several of the DBS applications for monitoring environmental contaminants are discussed more into depth below.

II.A.5.1 Newborn Screening

The organochlorine dichlorodiphenyldichloroethylene (a metabolite of dichlorodiphenyltrichloroethane, DDT) and the PFCs perfluorooctane sulphonate and perfluorooctanate, as well as benzene oxide and perchlorate, have been detected in all evaluated newborns' DBS [86,88,90,91,95], mirroring their general spread in ecosystems and their presence in virtually 100% of the adult population, including pregnant women [108]. Interestingly, a sharp decline in perfluoroalkyl content in DBS from newborns after the year 2000, coinciding with the phasing-out of perfluorooctane sulphonate in the US, nicely demonstrates the utility of this approach for assessing temporal trends in exposure to environmental chemicals [90]. Newborn DBS have also been demonstrated to have the potential for monitoring exposure to supraphysiological levels of trace elements (e.g. lead), allowing the extraction of (semi)quantitative information [109]. However, most of these studies have not been performed within the context of newborn screening and are therefore discussed in a separate paragraph.

II.A.5.2 Biomonitoring of Animals

Intoxication of animals with cholinesterase inhibitors (e.g. organophosphate and carbamate insecticides) may occur via ingestion (e.g. of exposed prey) or via dermal contact. Assessment of cholinesterase activity in DBS of avian species has been found sensitive enough to serve as a diagnostic tool for identifying exposure to cholinesterase-inhibiting pesticides. DBS sampling of animals allows the collection of samples at remote areas and in non-specialized centres, where no special equipment like a centrifuge is available and where proper storage of a blood sample is difficult [72].

A recent initiative in the context of monitoring exposure of animals to toxic substances, somewhat paralleling the efforts done for evaluating a newborn's exposure to environmental contaminants via DBS, is DABSE ("Database for avian blood spot examination") [110]. This biomonitoring project aims at setting up reference values for exposure of wild birds to five groups of environmental contaminants: trace elements, organochlorine pesticides, PCBs, PFCs and PBDEs. Referral to the values within this database should help to pinpoint a possible cause in cases in which an individual

bird or a bird population presents with a problem. A first application of this biomonitoring project was performed on griffon vultures, demonstrating detectable levels of several contaminants in DBS obtained from these birds [89].

Environmental health can also be assessed by monitoring the exposure of top predators, acting as sentinels. In coastal waters in the Gulf of Mexico, this approach has been used for monitoring the exposure of bottlenose dolphins to the marine algal biotoxins domoic acid and brevetoxins, respectively produced by members of the diatom genus Pseudo-nitzschia and by the dinoflagellate Karenia brevis [111]. Both biotoxins have been measured in DBS, obtained by spotting filter paper cards with 100 µl of blood, obtained from either exposed laboratory test animals (mice, rats or the fish species striped mullet) or from free-living dolphins [111-115]. Toxin detection in DBS extracts has been performed using receptor-binding assays [112] and radio immuno-assay (RIA, for brevetoxins) [113] and, more recently, by competitive enzyme-linked immunosorbent assay (ELISA), either detecting brevetoxins and their metabolites, or domoic acid [116,117]. Ciguatoxins are another class of highly potent neurotoxins, sharing with brevetoxins the binding site 5 on the α-subunit of voltage-gated sodium channels as effector site [118]. Using a neuroblastoma cytotoxicity assay, ciguatoxins have been determined in DBS extracts from exposed mice [119].

II.A.6 Elemental Analysis

Biomonitoring of toxic trace elements (metals and metalloids) in human blood has been applied for decades. Examples include lead, which exerts neurological toxicity, and arsenic, cadmium, mercury, chromium, copper, nickel and vanadium, all of which have distinct toxicity profiles. When aiming at (primarily) single-element analysis, analysis is usually performed by atomic absorption spectrometry, or more recently by (solid sampling) graphite furnace atomic absorption spectrometry [120,121]. Inductively coupled plasma mass spectrometry (ICP-MS) has been used for both single-element and for multi-element analysis, with more recent developments being laser ablation-ICP-(TOF-)MS and sector-field-ICP-MS [109,122-124].

Considering the analysis of trace elements in DBS, most attention has been given to the determination of Pb in DBS obtained from children. Although the determination of venous Pb concentrations is considered the gold standard, venepuncture of infants and toddlers is impractical, may be traumatic for the children and in many countries is not widely accepted by the

parents as a screening test for asymptomatic children [125]. As children are particularly sensitive to Pb and in most countries Pb concentrations peak at approximately 2 years of age [126,127], a minimally invasive technique such as DBS sampling offers many advantages for obtaining a representative blood sample. Micro-sampling of blood for Pb determination in DBS was first reported in the early seventies [128,129]. Although since then, many reports have been published on the determination of Pb in DBS, this approach has also been the subject of controversy, given the risk of contamination that may take place, as opposed to blood collection by venepuncture [120,130-134]. Indeed, given the ubiquitous presence of Pb in the environment, special care has to be taken to avoid contamination at every step, from paper handling, sampling, and drying, over transport to analysis. More specifically, falsely elevated Pb concentrations may result from contamination by Pb present on the skin (thus necessitating suitable cleansing before sampling) and/or by improper paper handling [131,133,135].

In contrast to a controlled clinical environment, in which the issue of contamination can be dealt with from sampling to analysis, one has to be aware that DBS sampling 'on-field', with less control on pre-analytical variables, potentially suffers from an increased risk of contamination. Yet, especially in developing countries, where studies have shown that the threshold limit of Pb poisoning is exceeded in a large percentage of children [136-138], the lack of resources renders DBS sampling one of the most feasible ways for screening large populations [125]. Sampling can be done on-site by a relatively untrained collector and samples can be sent by mail to an analytical laboratory. To correct for possibly inhomogeneous Pb distribution on the filter paper, analysis of 5 replicates (3.2-mm punches obtained from a single 50-µl blood spot) has been recommended by Resano and colleagues [121]. However, in practice, blood spots often correspond to smaller blood volumes, which may limit the number of punches and/or may pose a problem when larger punches (e.g. 6 or 6.35 mm diameter) are to be analyzed [139]. The Pb concentrations determined in DBS have been shown to be independent from the volume spotted and from the site of punching (excluding the area near the perimeter, where concentrations are higher owing to a higher amount of red blood cells). Moreover, a good correlation was found between Pb concentrations in DBS and those in venous blood [121,135]. DBS obtained from subjects with strongly deviating hematocrit values, however, may give rise to discordant results [135].

Apart from Pb, also other toxic metals, as well as elements of clinical or forensic interest, have been determined (or have been shown to be detectable) in DBS, including As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Rb, S, Sb, Se, Tl, V and Zn [89,109,123,124,140]. Quantification of several of these elements may lead to the generation of an individual's "metallic profile", from which exposure to a certain contamination source may be deduced [141].

An important obstacle for fully quantitative analysis of a substantial amount of elements, however, is the variable contribution by the filter paper (both within and between lots) and possible contamination, requiring adequate control of blank filter paper. This implies the control of different lots of unexposed blank paper, directly from the manufacturer, as well as the control of paper ("internal blanks") at some distance from the DBS. Yet, still, one cannot fully exclude the scenario in which contamination within, but not near the DBS took place [109,123]. The background values obtained from the controls can either be used for subtracting (possibly causing a negative bias) or can merely be used for evaluating the overall extent of contamination. Either way, replicate analysis of the same DBS (punch) and/or analysis of another DBS (punch) from positive cases is recommended to reduce the reporting of false positives [122,123]. In this respect, the technique of laser ablation ICP-TOF-MS, providing a "line scan" with several data points per blank and per DBS, allows easy discrimination of potential random contamination [122]. Moreover, as reported by Cizdziel, the use of isotope ratio's determined by this technique may also allow to discriminate contamination extraneous to the blood sample [122].

To overcome the major problem of contamination encountered in elemental analysis of DBS and, at the same time, to account for possible variations in hematocrit and/or volume spotted, normalization may be another possible future improvement. This can be done using one or multiple elements, having a narrow physiological distribution and/or being (almost) absent in blank filter paper. As suggested by Langer *et al*., one such candidate could be potassium [109]. Finally, it is important to mention that decision-making in the case of environmental pollution (including the analysis of trace elements) is somewhat distinct from that in the case of drugs of abuse. Whereas for the latter any positive signal (above the LOD, LLOQ or a certain cut-off) can raise an alert, positivity for the former can in many cases be considered as 'normal', with only levels exceeding a certain threshold warranting further follow-up.

II.A.7 Conclusion and Future Perspectives

DBS sampling is being applied in a wide range of applications in toxicology, covering fields as toxicokinetics, epidemiology and environmental and forensic toxicology. The analytes measured in DBS include therapeutic drugs, drugs of abuse, environmental contaminants and (trace) elements. Among the advantages associated with DBS sampling, the ease of collecting a representative sample with minimal discomfort is of particular importance for its application in toxicology. This holds true for sampling of animals, newborns, children, but also for adults, considering the potential of DBS sampling at home or in the context of DFSA, DUID or the follow-up of drug addicts. The stabilizing effect of DBS, largely preventing both *ex vivo* degradation and *de novo* formation of analytes, is another significant advantage associated with this sampling technique, facilitating sample handling and transport and often allowing long-term storage of samples. Despite these -as well as other- important advantages, also some remarks should be made with respect to the use of DBS for toxicological purposes. A first remark is the issue of contamination, which primarily (but not only) is a problem in the field of elemental analysis. Although this issue can be largely dealt with in a tightly controlled environment, contamination can never be excluded, especially in the case of 'on-field' sampling. Given the bioanalyst's awareness of this problem, various avenues are being explored to increase the confidence one may have in a positive result. As mentioned above, these include e.g. the analysis of blank controls, the acquisition of multiple data points from a single spot or from replicate spots and/or attempts to normalize for e.g. hematocrit using one or multiple elements. A second remark is that for many analytes the influence of parameters such as hematocrit, volume spotted and site of punching has not been examined. Lack of knowledge about the influence of these (as well as other) parameters adds an additional, often neglected, factor of uncertainty to the reported analytical result. Thirdly, although promising results have been obtained in e.g. forensic toxicology, the approaches followed are often not fully compatible with the collection of true 'on-field' capillary blood samples, requiring more extensive validation. Apart from these points of attention, requiring more work to be done, it is our feeling that the largest contribution of DBS sampling in toxicology may lie in the field of drug development. There, its implementation of 'refinement' and 'reduction', allowing "small sampling of small animals" closely follows the 3R principle and is even accompanied by improved data quality. Also in (pre)clinical studies, the implementation of DBS sampling may be an incentive, e.g. by facilitating patient recruitment. As this evolution will

evidently lead to a large amount of samples to be analyzed, current efforts are now being focused on automation and rapid, direct analyses of DBS.

REFERENCES

1. McDade TW, Williams S, Snodgrass JJ. What a drop can do: Dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography*. (4):899-925 (2007).
2. Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: Methods, assays, and pitfalls. *Ther Drug Monit.* 31 (3):327-336 (2009).
3. Bang I. Der Blutzucker. Wiesbaden, Germany (1913).
4. Guthrie R, Susi A. A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants. *Pediatrics*. 32:338-343 (1963).
5. Seashore MR, Seashore CJ. Newborn screening and the pediatric practitioner. *Semin Perinatol.* 29:182-188 (2005).
6. Watson MS, Mann MY, Lloyd-Puryear MA, Rinaldo P, Rodney Howell R. Newborn screening: Toward a uniform screening panel and system - Executive summary. *Pediatrics.* 117:S296-S307 (2006).
7. Garg U, Dasouki M. Expanded newborn screening of inherited metabolic disorders by tandem mass spectrometry: clinical and laboratory aspects. *Clin Biochem*. 39:315-332 (2006).
8. Bodamer OA, Hoffmann GF, Lindner M. Expanded newborn screening in Europe 2007. *J Inherit Metab Dis*. 30:439-444 (2007).
9. Chace DH. Mass spectrometry in newborn and metabolic screening: historical perspective and future directions. *J Mass Spectrom*. 44:163-170 (2009).
10. Li W, Tse FL. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed Chromatogr*. 24:49-65 (2010).
11. Stolk LM, Edelbroek PM. Characteristics of new TDM assays, described in literature, based on dried blood spot methods. [Online] Available at: http://www.iatdmct.org/images/File/Update%20review%20TDM14072010.pdf (2010).
12. Chace DH, DiPerna JC, Mitchell BL, Sgroi B, Hofman LF, Naylor EW. Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death. *Clin Chem*. 47:1166-1182 (2001).
13. Searles-Nielsen S, Mueller BA, De Roos AJ, Checkoway H. Newborn screening archives as a specimen source for epidemiologic studies: Feasibility and potential for bias. *Ann Epidemiol*. 18:58-64 (2008).
14. Holub M, Tuschl K, Ratschmann R, Strnadova KA, Muhl A, Heinze G, Sperl W, Bodamer OA. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. *Clin Chim Acta*. 373:27-31 (2006).
15. O'Mara M, Hudson-Curtis B, Olson K, Yueh Y, Dunn J, Spooner N. The effect of hematocrit and punch location on assay bias during quantitative bioanalysis of dried blood spot samples. *Bioanalysis*. 3:2335-2347 (2011).

16. Smith C, Skyes A, Robinson S, Thomas E. Evaluation of blood microsampling techniques and sampling sites for the analysis of drugs by HPLC-MS. *Bioanalysis*. 3:145-156 (2011).
17. Stokes AH, Moose TA, Parry SP, Barfield M, Lovatt CA, Dopson WJ, Melich D, Overvold CR, Gade SD, Spooner N. Determination of drug concentrations using dried blood spots: investigation of blood sampling and collection techniques in Crl:CD(SD) rats. *Lab Anim*. 45:109-113 (2011).
18. Clark GT, Haynes JJ, Bayliss MA, Burrows L. Utilization of DBS within drug discovery: development of a serial microsampling pharmacokinetic study in mice. *Bioanalysis*. 2:1477-1488 (2010).
19. Turpin PE, Burnett JE, Goodwin L, Foster A, Barfield M. Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories. *Bioanalysis.* 2:1489-1499 (2010).
20. Crawford E, Gordon J, Wu JT, Musselman B, Liu R, Yu S. Direct analysis in real time coupled with dried spot sampling for bioanalysis in a drug-discovery setting. *Bioanalysis*. 3:1217-1226 (2011).
21. Spooner N, Lad R, Barfield M. Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method. *Anal Chem*. 81:1557-1563 (2009).
22. Denniff P, Spooner N. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis*. 2:1385-1395 (2010a).
23. Denniff P, Spooner N. Effect of storage conditions on the weight and appearance of dried blood spot samples on various cellulose-based substrates. *Bioanalysis*. 2:1817-1822 (2010b).
24. Barfield M, Wheller R. Use of Dried Plasma Spots in the Determination of Pharmacokinetics in Clinical Studies: Validation of a Quantitative Bioanalytical Method. *Anal Chem*. 83:118-124 (2011).
25. Barfield M, Ahmad S, Busz M. GlaxoSmithKline's experience of incurred sample reanalysis for dried blood spot samples. *Bioanalysis*. 3:1025-1030 (2011).
26. Timmerman P, White S, Globig S, Ludtke S, Brunet L, Smeraglia J. EBF recommendation on the validation of bioanalytical methods for dried blood spots. *Bioanalysis*. 3:1567-1575 (2011).
27. Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis.* 2:1791-1796 (2010).
28. Mohammed BS, Cameron GA, Cameron L, Hawksworth GH, Helms PJ, McLay JS. Can finger-prick sampling replace venous sampling to determine the pharmacokinetic profile of oral paracetamol? *Br J Clin Pharmacol*. 70:52-56 (2010).
29. Déglon J, Thomas A, Daali Y, Lauer E, Samer C, Desmeules J, Dayer P, Mangin P, Staub C. Automated system for on-line desorption of dried blood spots applied to LC/MS/MS pharmacokinetic study of flurbiprofen and its metabolite. *J Pharm Biomed Anal*. 54:359-367 (2011a).
30. Déglon J, Thomas A, Mangin P, Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal Bioanal Chem*. 402(8):2485-2498 (2012).

31. Abu-Rabie P. Direct analysis of DBS: emerging and desirable technologies. *Bioanalysis*. 3:1675-1678 (2011).
32. Schütz H, Gotta JC, Erdmann F, Risse M, Weiler G. Simultaneous screening and detection of drugs in small blood samples and bloodstains. *Forensic Sci Int*. 126:191-196 (2002).
33. Fuller DC, Pisana P. A Preliminary Investigation into Retrospective Calculation of In-Vivo Drug Concentrations in Dried Crime Scene Blood. Proceedings of the 39th Annual Meeting of the Society of Forensic Toxicologists (SOFT). Oklahoma City, USA (2009).
34. Winkler M, Kaufmann E, Thoma D, Thierauf A, Weinmann W, Skopp G, Alt A. Detection of ethyl glucuronide in blood spotted on different surfaces. *Forensic Sci Int.* 210:243-246 (2011).
35. Henderson LO, Powell MK, Hannon WH, Miller BB, Martin ML, Hanzlick RL, Vroon D, Sexson WR. Radioimmunoassay screening of dried blood spot materials for benzoylecgonine. *J Anal Toxicol.* 17:42-47 (1993).
36. Sosnoff CS, Ann Q, Bernert JT, Jr., Powell MK, Miller BB, Henderson LO, Hannon WH, Fernhoff P, Sampson EJ. Analysis of benzoylecgonine in dried blood spots by liquid chromatography--atmospheric pressure chemical ionization tandem mass spectrometry. *J Anal Toxicol.* 20:179-184 (1996).
37. Henderson LO, Powell MK, Hannon WH, Bernert JT, Jr., Pass KA, Fernhoff P, Ferre CD, Martin L, Franko E, Rochat RW, Brantley MD, Sampson E. An evaluation of the use of dried blood spots from newborn screening for monitoring the prevalence of cocaine use among childbearing women. *Biochem Mol Med*. 61:143-151 (1997).
38. Alfazil AA, Anderson RA. Stability of benzodiazepines and cocaine in blood spots stored on filter paper. *J Anal Toxicol.* 32:511-515 (2008).
39. Garcia Boy R, Henseler J, Mattern R, Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. *Ther Drug Monit.* 30:733-739 (2008).
40. Moll V, Clavijo C, Cohen M, Christians U, Galinkin J. A Analytical Method To Determine Ketamine and Norketamine Levels in Dried Blood Spots. Proceedings of the Annual Meeting of the American Society of Anesthesiologists (ASA). New Orleans, LA (2009).
41. Clavijo CF, Thomas JJ, Hammer GB, Christians U, Galinkin JL. A sensitive LC-MS/MS Assay for Quantification of Methadone and Its Metabolites in Dried Blood Spots. Proceedings of the Annual Meeting of the American Society of Anesthesiologists (ASA). San Diego, CA (2010).
42. Havard G, Théberge M-C, Boudreau N, Lévesque A, Massé R. Development and Validation of a Dried Blood Spot Assay for the Determination of Midazolam in Human Whole Blood by LCMSMS. Proceedings of the American Association of Pharmaceutical Scientists (AAPS) - Pharmaceutical Sciences World Congress. New Orleans, LA (2010).

43. Ingels AS, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal Bioanal Chem.* 398:2173-2182 (2010).
44. Marin LJ, Tyrrel-Pawlowic CA, Moroney EC, Coopersmith BI, Shet MS. Comparative analysis of buprenorphine and oxycodone in human dried blood spots (DBS) using LC/MS/MS. Proceedings of the American Association of Pharmaceutical Scientists (AAPS) - Pharmaceutical Sciences World Congress. New Orleans, LA (2010).
45. Mercolini L, Mandrioli R, Gerra G, Raggi MA. Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection: a novel test for cocaine and alcohol intake. *J Chromatogr A.* 1217:7242-7248 (2010).
46. Thomas A, Déglon J, Steimer T, Mangin P, Daali Y, Staub C. On-line desorption of dried blood spots coupled to hydrophilic interaction/reversed-phase LC/MS/MS system for the simultaneous analysis of drugs and their polar metabolites. *J Sep Sci*. 33:873-879 (2010).
47. Clavijo CF, Hoffman KL, Thomas JJ, Carvalho B, Chu LF, Drover DR, Hammer GB, Christians U, Galinkin JL. A sensitive assay for the quantification of morphine and its active metabolites in human plasma and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem.* 400:715-728 (2011a).
48. Clavijo CF, Thomas JJ, Cromie M, Schniedewind B, Hoffman KL, Christians U, Galinkin JL. A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children. *J Sep Sci.* 34(24):3568-3577 (2011b).
49. Hudson W, Yong B, Boguszewski P. Analysis of Clozapine, Nortriptyline, Paroxetine and Zolpidem Using Dried Blood Spots. [Online] Available at: http://www.chem.agilent.com/Library/applications/5990-8033EN.pdf. Accessed on November, 25th, 2011 (2011).
50. Ingels AS, De Paepe P, Anseeuw K, Van Sassenbroeck DK, Neels H, Lambert WE, Stove CP. Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis.* 3:2271-2281 (2011).
51. Jantos R, Skopp G. Comparison of drug analysis in whole blood and dried blood spots. *Toxichem Krimtech.* 78:268-275 (2011).
52. Jantos R, Schumacher M, Skopp G. Comparison of Opioid Analysis in Whole Blood and Dried Blood Spots. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA (2011a).
53. Jantos R, Veldstra JL, Mattern R, Brookhuis KA, Skopp G. Analysis of 3,4-methylenedioxymetamphetamine: whole blood versus dried blood spots. *J Anal Toxicol.* 35:269-273 (2011b).

54. Langel K, Uusivirta H, Ariniemi K, Lillsunde P. Analysis of Drugs of Abuse by GC-MS in Dried Blood Spot Sample Matrix. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA (2011).
55. Lauer E, Déglon J, Versace F, Thomas A, Mangin P, Staub C. Target Screening of Drugs from Dried Blood Spot Samples Based on LC-MS/MS and On-Line Desorption. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA (2011).
56. Saussereau E, Lacroix C, Gaulier JM, Goullé JP. On-line liquid chromatography/tandem mass spectrometry simultaneous determination of opiates, cocainics and amphetamines in dried blood spots. *J Chromatogr B*. 885-886:1-7 (2012).
57. Ambach L, Hernández Redondo A, König S, Weinmann W. Rapid and simple LC-MS/MS screening of 64 novel psychoactive substances using dried blood spots. *Drug Test Anal*. Doi 10.1002/dta.1505 (2013). Epub ahead of print.
58. Skopp G. Blood spot analysis. Joint Meeting of the ICADTS and TIAFT. Seattle, WA (2007).
59. Thomas A, Geyer H, Schänzer W, Crone C, Kellmann M, Moehring T, Thevis M. Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem*. 403:1279-1289 (2012).
60. Déglon J, Versace, F, Lauer, E, Widmer, C, Mangin, P, Thomas, A, Staub C. Rapid LC-MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. *Bioanalysis*. 4:1337-1350 (2012b).
61. Jantos R, Vermeeren A, Sabljic D, Ramaekers JG, Skopp G. Degradation of zopiclone during storage of spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med*. 127(1):69-76 (2012).
62. Addolorata Saracino M, Marcheselli C, Somaini L, Pieri MC, Gerra G, Ferranti A, Raggi MA. A novel test using dried blood spots for the chromatographic assay of methadone. *Anal Bioanal Chem*. 404:503–511 (2012).
63. Mommers J, Mengerik Y, Ritzen E, Weusten J, van der Heijden J, van der Wal S. Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. *Anal Chem Acta*. 774:26-32 (2013).
64. Mercolini L, Mandrioli R, Sorella V, Somaini L, Giocondi D, Serpelloni G, Raggi MA. Dried blood spots: liquid chromatography-mass spectrometry analysis of Δ9-tetrahydrocannabinol and its main metabolites. *J Chromatogr A*. 1271(1):33-40 (2013).
65. Murphy SE, Wickham KM, Lindgren BR, Spector LG, Joseph A. Cotinine and *trans* 3'-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. *J Expo Sci Environ Epidemiol*. 23(5):513-518 (2013).
66. Hernández Redondo A, Schroeck A, Kneubuehl B, Weinmann W. Determination of ethyl glucuronide and ethyl sulfate from dried blood spots. *Int J Legal Med*. 127(4):769-75 (2013).

67. Faller A, Richter B, Kluge M, Koenig, P, Seitz HK, Skopp, G. Stability of phosphatidylethanol species in spiked and authentic whole blood and matching dried blood spots. *Int J Legal Med*. 127(3):603-610 (2013).
68. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L. Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. Mol Genet Metab. 109(3):255-259 (2013).
69. Faller A, Richter B, Kluge M, Koenig P, Seitz HK, Thierauf A, Gnann H, Winkler M, Mattern R, Skopp G. LC-MS/MS analysis of phosphatidylethanol in dried blood spots versus conventional blood specimens. *Anal Bioanal Chem.* 401:1163-1166 (2011).
70. Jones J, Jones M, Plate C, Lewis D. The detection of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol in human dried blood spots. *Anal Methods-Uk.* 3:1101-1106 (2011).
71. Hernàndez Redondo AH, Weinmann W, König S. Detection of Ethylglucuronide and Ethylsulfate in Dried Blood Spots. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA (2011).
72. Trudeau S, Mineau P, Cartier GS, Fitzgerald G, Wilson L, Wheler C, Knopper LD. Using dried blood spots stored on filter paper to measure cholinesterase activity in wild avian species. *Biomarkers*. 12:145-154 (2007).
73. Wood DM, Nicolaou M, Dargan PI. Epidemiology of recreational drug toxicity in a nightclub environment. *Subst Use Misuse.* 44:1495-1502 (2009).
74. DRUID. DRUID (Driving under the Influence of Drugs, Alcohol and Medicines), Work Package 1: Experimental Studies, Effects of stimulant drugs on actual and simulated driving. [Online] Available at:http://www.druidproject.eu/cln_031/nn_107534/sid_7A536E257F91FDEEACFADC69D72378AA/nsc_true/Druid/EN/deliverales-list/deliverables-list-node.html?nnn=true. Accessed on September 8th, 2011 (2011).
75. Bosker WM, Huestis MA. Oral fluid testing for drugs of abuse. *Clin Chem.* 55:1910-1931 (2009).
76. Huestis MA, Verstraete A, Kwong TC, Morland J, Vincent MJ, de la Torre R. Oral fluid testing: promises and pitfalls. *Clin Chem.* 57:805-810 (2011).
77. SCDHEC. Transporting and Shipping Infectious Substances A and B. [Online] Available at: http://www.scdhec.gov/health/lab/docs/labeling.pdf. Accessed on November 23rd, 2011 (2011).
78. Johannessen A. Dried blood spots in HIV monitoring: applications in resource-limited settings. *Bioanalysis.* 2:1893-1908 (2010).
79. Bowen CL, Hemberger MD, Kehler JR, Evans CA. Utility of dried blood spot sampling and storage for increased stability of photosensitive compounds. *Bioanalysis.* 2:1823-1828 (2010).
80. D'Arienzo CJ, Ji QC, Discenza L, Cornelius G, Hynes J, Cornelius L, Santella JB, Olah T. DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. *Bioanalysis.* 2:1415-1422 (2010).

81. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci Int.* 161:158-162 (2006).
82. Zörntlein SW, Kopp A, Becker J, Kaufmann TJ, Rohrich J, Urban R. In vitro production of GHB in blood and serum samples under various storage conditions. *Forensic Sci Int.* 214(1-3):113-117 (2012).
83. Aradottir S, Moller K, Alling C. Phosphatidylethanol formation and degradation in human and rat blood. *Alcohol Alcsm.* 39:8-13 (2004).
84. DuBey IS, Caplan YH. The storage of forensic urine drug specimens as dry stains: recovery and stability. *J Forensic Sci.* 41:845-850 (1996).
85. Spector LG, Hecht SS, Ognjanovic S, Carmella SG, Ross JA. Detection of cotinine in newborn dried blood spots. *Cancer Epidemiol Biomarkers Prev.* 16:1902-1905 (2007).
86. Funk WE, Waidyanatha S, Chaing SH, Rappaport SM. Hemoglobin adducts of benzene oxide in neonatal and adult dried blood spots. *Cancer Epidemiol Biomarkers Prev.* 17:1896-1901 (2008).
87. Dua VK, Pant CS, Sharma VP, Pathak GK. Determination of HCH and DDT in finger-prick whole blood dried on filter paper and its field application for monitoring concentrations in blood. *Bull Environ Contam Toxicol.* 56:50-57 (1996).
88. Burse VW, DeGuzman MR, Korver MP, Najam AR, Williams CC, Hannon WH, Therrell BL. Preliminary investigation of the use of dried-blood spots for the assessment of in utero exposure to environmental pollutants. *Biochem Mol Med.* 61:236-239 (1997).
89. Shlosberg A, Wu Q, Rumbeiha WK, Lehner A, Cuneah O, King R, Hatzofe O, Kannan K, Johnson M. Examination of Eurasian Griffon Vultures (Gyps fulvus fulvus) in Israel for exposure to environmental toxicants using dried blood spots. *Arch Environ Contam Toxicol.* 62(3):502-511 (2012).
90. Spliethoff HM, Tao L, Shaver SM, Aldous KM, Pass KA, Kannan K, Eadon GA. Use of Newborn Screening Program blood spots for exposure assessment: Declining levels of perfluorinated compounds in New York State infants. *Environ Sci Technol.* 42:5361-5367 (2008).
91. Kato K, Wanigatunga AA, Needham LL, Calafat AM. Analysis of blood spots for polyfluoroalkyl chemicals. *Anal Chim Acta.* 656:51-55 (2009).
92. Ma W, Kannan K, Wu Q, Bell EM, Druschel CM, Caggana M, Aldous KM. Analysis of polyfluoroalkyl substances and bisphenol A in dried blood spots by liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem*. 405:4127-4138 (2013a).
93. Lu D, Wang D, Ip HSS, Barley F, Ramage B, She J. Measurements of polybrominated diphenyl ethers and polychlorinated biphenyls in a single drop of blood. *J Chrom B*. 891-892:36-43 (2012).
94. Ma W, Yun SH, Bell E, Druschel C, Caggana M, Aldous K, Louis GB, Kannan K. Temporal Trends of Polybrominated Diphenyl Ethers (PBDEs) in the Blood of Newborns from New York State during 1997 through 2011: Analysis of Dried Blood Spots from the Newborn Screening Program. *Environ Sci Technol*. DOI: 10.1021/es401857v (2013b). *epub ahead of print*.

95. Otero-Santos SM, Delinsky AD, Valentin-Blasini L, Schiffer J, Blount BC. Analysis of perchlorate in dried blood spots using ion chromatography and tandem mass spectrometry. *Anal Chem.* 81:1931-1936 (2009).
96. Leonard S, Moshin J, Wittrig R, Serreiber A. Quantitation of Bisphenol A in Dried Blood Spots using liquid chromatography tandem mass spectrometry. Proceedings of the American Association for Clinical Chemistry (AACC) Annual Meeting. Atlanta, GA (2011).
97. Augustinsson KB, Heimburger G. The determination of cholinesterase activity in blood samples absorbed on filter paper. *Acta Physiol Scand.* 30:45-54 (1953).
98. Heilbronn E. An electrometric method for the determination of cholinesterase activity. III. Cholinesterase activity in blood spots on filter paper. *Scand J Clin Lab Invest.* 5:308-311 (1953).
99. Augustinsson KB, Holmstedt B. Determination of cholinesterase in blood samples dried on filter-paper and its practical application. *Scand J Clin Lab Invest.* 17:573-583 (1965).
100. Holmstedt B, Oudart JL. Determination of plasma cholinesterase activity by means of a paper test. *Bull Soc Pathol Exot Filiales.* 59:411-426 (1966).
101. Collombel C, Perrot L. Determination of cholinesterase activity in whole blood obtained by capillary puncture and deposited on filter paper. Application to the detection of poisoning by organophosphorus insecticides. *Eur J Toxicol.* 3:368-372 (1970).
102. Oudart JL, Holmstedt B. Determination of plasma cholinesterase activity by means of a test paper and its use in the field. *Arch Toxikol.* 27:1-12 (1970).
103. Augustinsson KB, Eriksson H, Faijersson Y. A new approach to determining cholinesterase activities in samples of whole blood. *Clin Chim Acta*. 89:239-252 (1978).
104. Eriksson H, Faijersson Y. A reliable way of estimating cholinesterases from whole blood in the presence of anti-cholinesterases. *Clin Chim Acta.* 100:165-171 (1980).
105. Rhyanen R, Liesivuori J, Narhi M, Puhakainen E, Hanninen O. Blood cholinesterase activities of flower garden workers after exposure to organophosphates. *Bull Environ Contam Toxicol.* 32:251-258 (1984).
106. Hilborn ED, Padilla S. A dried blood spot method to evaluate cholinesterase activity in young children. *Arch Environ Health.* 59:467-470 (2004).
107. Quandt SA, Chen H, Grzywacz JG, Vallejos QM, Galvan L, Arcury TA. Cholinesterase depression and its association with pesticide exposure across the agricultural season among Latino farmworkers in North Carolina. *Environ Health Perspect.* 118:635-639 (2010).
108. Woodruff TJ, Zota AR, Schwartz JM. Environmental chemicals in pregnant women in the United States: NHANES 2003-2004. *Environ Health Perspect.* 119:878-885 (2011).
109. Langer EK, Johnson KJ, Shafer MM, Gorski P, Overdier J, Musselman J, Ross JA. Characterization of the elemental composition of newborn blood spots using sector-field inductively coupled plasma-mass spectrometry. *J Expo Sci Environ Epidemiol.* 21:355-364 (2010).

110. Shlosberg A, Rumbeiha WK, Lublin A, Kannan K. A database of avian blood spot examinations for exposure of wild birds to environmental toxicants: the DABSE biomonitoring project. *J Environ Monit.* 13:1547-1558 (2011).
111. Twiner MJ, Fire S, Schwacke L, Davidson L, Wang Z, Morton S, Roth S, Balmer B, Rowles TK, Wells RS. Concurrent exposure of bottlenose dolphins (Tursiops truncatus) to multiple algal toxins in Sarasota Bay, Florida, USA. *PLoS One.* 6:e17394 (2011).
112. Fairey ER, Shuart NG, Busman M, Moeller PD, Ramsdell JS. Biomonitoring brevetoxin exposure in mammals using blood collection cards. *Environ Health Perspect.* 109:717-720 (2001).
113. Woofter R, Dechraoui MY, Garthwaite I, Towers NR, Gordon CJ, Cordova J, Ramsdell JS. Measurement of brevetoxin levels by radioimmunoassay of blood collection cards after acute, long-term, and low-dose exposure in mice. *Environ Health Perspect.* 111:1595-1600 (2003).
114. Woofter RT, Brendtro K, Ramsdell JS. Uptake and elimination of brevetoxin in blood of striped mullet (Mugil cephalus) after aqueous exposure to Karenia brevis. *Environ Health Perspect.* 113:11-16 (2005).
115. Schwacke LH, Twiner MJ, De Guise S, Balmer BC, Wells RS, Townsend FI, Rotstein DC, Varela RA, Hansen LJ, Zolman ES, Spradlin TR, Levin M, Leibrecht H, Wang Z, Rowles TK. Eosinophilia and biotoxin exposure in bottlenose dolphins (Tursiops truncatus) from a coastal area impacted by repeated mortality events. *Environ Res.* 110:548-555 (2010).
116. Maucher JM, Ramsdell JS. Ultrasensitive detection of domoic acid in mouse blood by competitive ELISA using blood collection cards. *Toxicon.* 45:607-613 (2005).
117. Maucher JM, Briggs L, Podmore C, Ramsdell JS. Optimization of blood collection card method/enzyme-linked immunoassay for monitoring exposure of bottlenose dolphin to brevetoxin-producing red tides. *Environ Sci Technol.* 41:563-567 (2007).
118. Wang DZ. Neurotoxins from marine dinoflagellates: a brief review. *Mar Drugs.* 6:349-371 (2008).
119. Bottein Dechraoui MY, Wang Z, Turquet J, Chinain M, Darius T, Cruchet P, Radwan FF, Dickey RW, Ramsdell JS. Biomonitoring of ciguatoxin exposure in mice using blood collection cards. *Toxicon.* 46:243-251 (2005).
120. Verebey K, Eng YM, Davidow B, Ramon A. Rapid, sensitive micro blood lead analysis: a mass screening technique for lead poisoning. *J Anal Toxicol.* 15:237-240 (1991).
121. Resano M, Rello L, Garcia-Ruiz E, Belarra MA. Minimally-invasive filter paper test in combination with solid sampling-graphite furnace atomic absorption spectrometry for Pb determination in whole blood. *J Anal At Spectrom.* 22:1250-1259 (2007).
122. Cizdziel JV. Determination of lead in blood by laser ablation ICP-TOF-MS analysis of blood spotted and dried on filter paper: a feasibility study. *Anal Bioanal Chem.* 388:603-611 (2007).

123. Chaudhuri SN, Butala SJ, Ball RW, Braniff CT. Pilot study for utilization of dried blood spots for screening of lead, mercury and cadmium in newborns. *J Expo Sci Environ Epidemiol.* 19:298-316 (2009).
124. Hsieh HF, Chang WS, Hsieh YK, Wang CF. Using dried-droplet laser ablation inductively coupled plasma mass spectrometry to quantify multiple elements in whole blood. *Anal Chim Acta.* 699:6-10 (2011).
125. Shen XM, Zhang YW, Wu SH, Yan CH, Ying JM, Li RQ. Applicability of a filter paper method to measure blood lead levels in large populations of Chinese children. *Clin Chim Acta.* 328:99-104 (2003).
126. American Academy of Pediatrics; Committee on Environmental Health. Lead exposure in children: prevention, detection, and management. *Pediatrics.* 116:1036-1046 (2005).
127. Chandran L, Cataldo R. Lead poisoning: basics and new developments. *Pediatr Rev.* 31:399-405; quiz 406 (2010).
128. Delves HT. A micro-sampling method for the rapid determination of lead in blood by atomic-absorption spectrophotometry. *Analyst.* 95:431-438 (1970).
129. Cernik AA, Sayers MH. Determination of lead in capillary blood using a paper punched disc atomic absorption technique. Application to the supervision of lead workers. *Br J Ind Med.* 28:392-398 (1971).
130. Stanton NV, Maney JM, Jones R. Evaluation of filter paper blood lead methods: results of a pilot proficiency testing program. *Clin Chem.* 45:2229-2235 (1999).
131. Moyer TP, Nixon DN, Ash KO. Filter paper lead testing. *Clin Chem.* 45:2055-2056 (1999).
132. Verebey K. Filter paper-collected blood lead testing in children. *Clin Chem.* 46:1024-1028 (2000).
133. Moyer TP, Nixon DE, Ash OK. Filter paper-collected blood lead testing in children - Response. *Clin Chem.* 46:1026-1028 (2000).
134. Stanton N, Maney J, Jones R. More on filter paper lead testing. *Clin Chem.* 46:1028-1029 (2000).
135. El-Hajjar DF, Swanson KH, Landmark JD, Stickle DF. Validation of use of annular once-punched filter paper bloodspot samples for repeat lead testing. *Clin Chim Acta.* 377:179-184 (2007).
136. Shen X, Rosen JF, Guo D, Wu S. Childhood lead poisoning in China. *Sci Total Environ.* 181:101-109 (1996).
137. Ahamed M, Siddiqui MK. Environmental lead toxicity and nutritional factors. *Clin Nutr.* 26:400-408 (2007).
138. Zhang SM, Dai YH, Xie XH, Fan ZY, Tan ZW, Zhang YF. Surveillance of childhood blood lead levels in 14 cities of China in 2004-2006. *Biomed Environ Sci.* 22:288-296 (2009).
139. Peck HR, Timko DM, Landmark JD, Stickle DF. A survey of apparent blood volumes and sample geometries among filter paper bloodspot samples submitted for lead screening. *Clin Chim Acta*. 400:103-106 (2009).

140. Lombeck I, Papadaki-Papandreou O, Kamiri F, Laryea MD, Jiang YF, Leitzmann P. A screening method for the evaluation of selenium status. *J Trace Elem Electrolytes Health Dis.* 3:175-178 (1989).
141. Goullé JP, Saussereau E, Mahieu L, Bouige D, Guerbet M, Lacroix C. Le profil métallique: un nouveau concept médical. *Rev Med Interne.* 31:128-134 (2010).

CHAPTER II.B
DERIVATIZATION TECHNIQUES IN DBS ANALYSIS

Based on

Derivatization techniques in dried blood spot analysis. In "Dried Blood Spots: Applications and Techniques". Ingels ASME, Sadones N, De Kesel PMM, Lambert WE, Stove CP. Eds. Li W and Lee M, John Wiley & Sons, Chapter 27, *in press*.

II.B.1 INTRODUCTION

The dried blood spot (DBS) sampling technique has several advantages over a venepuncture, making it a cost-effective choice for the collection, transport and storage of blood samples. Inherent to DBS sampling is the small sample volume available, ranging from 5 to 100 µl, compared to 1 ml or more obtained by venepuncture. Although this may represent an advantage in case of sampling patients with restricted or limited venous access, such as neonates and children, these small amounts may impose an analytical challenge and require efficient sample treatment, as well as sensitive detection [1-4]. To achieve adequate method sensitivity for the analysis of different pharmaceutical compounds or biomarkers in DBS, even at lower concentration levels, the majority of DBS applications use tandem mass spectrometry (MS/MS) coupled to liquid chromatography (LC). Other analytical techniques such as direct MS/MS, LC coupled to fluorescence (LC-FLUO) or ultraviolet detection (LC-UV), or gas chromatography coupled to MS (GC-MS) or tandem MS (GC-MS/MS) have been demonstrated to be suitable alternatives [5-7]. Additionally, to achieve the required method sensitivity, DBS analysis may involve derivatization. This may lead to an improvement of the chromatographic properties of the analytes of interest, which consequently may also influence method sensitivity by enhancing volatility, separation efficiency and/or selectivity [8]. Derivatization is primarily known as a technique extending the molecular application range of GC [9]. During sample work-up of DBS GC-MS (/MS) applications, derivatization reactions as silylation, alkylation and/or acetylation have been performed [5,10,11]. Also LC-UV or LC-FLUO applications may integrate a derivatization step during DBS analysis to improve detection sensitivity and selectivity by enhancing the UV properties or the fluorescence yield of the target analytes, respectively [6,12,13].

In contrast, derivatization is less commonly used for LC-MS/MS analysis, especially because omission of derivatization in an analysis is recognized as a major advantage, but also because variation and artifacts can be introduced [14,15]. However, the integration of derivatization techniques could enhance the capabilities of certain MS/MS-based applications and may give rise to several advantages such as improved chromatography and improved mass spectrometric properties (e.g., ionization efficiency, *m/z*) of the target compounds [15]. For example, the electrospray ionization (ESI) yield of neutral ketosteroids may be low as they lack a functional group that is easily ionized under normal conditions. This limitation can be overcome by introducing a chargeable moiety. In this way, derivatization of 17-hydroxyprogesterone to its

positively charged hydrazone before LC-ESI-MS/MS analysis, resulted in a 10-fold gain in sensitivity [16,17].

Although, in theory, higher ionization yield and better selectivity are expected for small molecules or neutral compounds when using derivatization, it may be important to evaluate the differences between derivatization and non-derivatization procedures on an analyte-per-analyte basis prior to selecting the most suitable sample work-up protocol. This point was demonstrated by De Jesus and co-workers, comparing the ionization efficiency for acylcarnitines and amino acids (AA) in derivatized (butyl ester) and non-derivatized forms (free acid) [18]. The authors found that for the majority of the selected compounds, minor differences in quantitative results were observed between both methods, while mass spectrometric responses varied from more intense without derivatization, over being similar, to less than 66% of the mass spectrometric ion counts obtained by derivatization. Moreover, without derivatization the method may be less selective, not capable to differentiate isobaric acylcarnitines.

Different traditional derivatizing reagents are available for GC-MS, LC-FLUO and -UV based procedures, and some of these have been applied in (LC-) MS/MS applications. However, for the latter, limitations may be encountered such as suboptimal ionization efficiency and product ion yield. Furthermore, different LC behaviors are expected for the derivatized compounds. Therefore, efforts have recently been made to design derivatives specifically for (LC-) MS/MS based approaches [15,16,19]. Examples that illustrate the advantages gained as a result of derivatizing the target compounds in DBS are given below. However, as it is beyond the scope of this chapter to provide an exhaustive overview of current derivatization techniques and reagents utilized in GC, LC and (LC-) MS/MS methods, we would like to refer to comprehensive reviews on this subject [8,9,15,16,19,20-22].

II.B.2 Overview of derivatization techniques in DBS analysis

The aim of this chapter is to present an overview of DBS methods utilizing derivatization published since 1990 up till now. Generally, formation of derivatives can be carried out during sample work-up (pre-column) or post-column before the column eluate enters the detection system [23]. A few DBS methods reported in the eighties opted for such a post-column derivatization technique in combination with LC–FLUO, mainly to avoid instability of the derivatives during sample work-up

and separation [24,25]. By mixing column eluates on-line with the derivatizing reagents, the derivatized target compounds are directly detected, but as these are also diluted this resulted in sensitivity loss that needs to be compensated for by the gain in sensitivity due to derivatization [26]. Although considerable improvements in method sensitivity and analysis time have been made in comparison to the original procedures, only a minority of recently reported DBS methods uses this derivatization technique [27]. Hence, contribution of the post-column derivatization technique in DBS analysis has been considered as too limited, and consequently, beyond the scope of this chapter.

The focus of this overview lies on derivatization techniques utilized during sample preparation. Therefore, we made a classification based upon the DBS sample work-up procedure. The first group of methods has in common that the conducted DBS sample treatment is considered to be the 'general' procedure (Table II.B.1, Fig. II.B.1: general procedure). Tables II.B.2 to II.B.4 summarize selected methods with modifications to this 'general' procedure (Fig. II.B.1: modified sample work-up procedure 1-3). The latter include direct derivatization, a procedure in which (extracting and) derivatizing solutions are applied in one single step to the DBS.

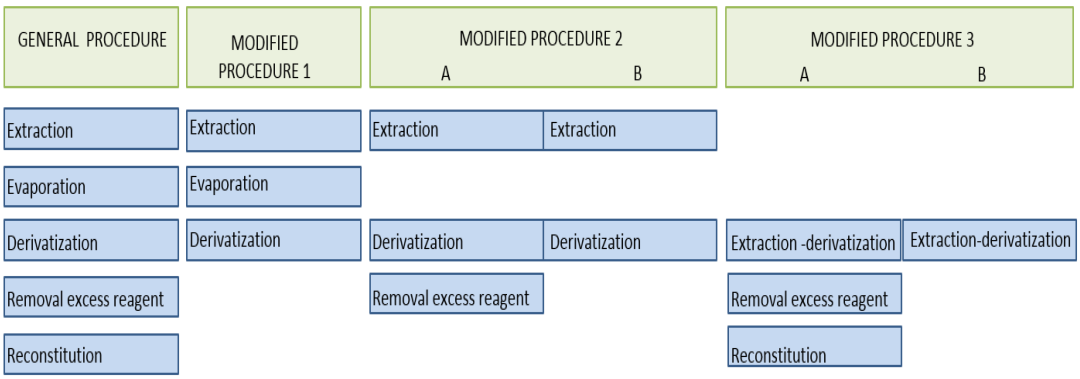


Fig. II.B.1 Schematic overview of the general and modified sample work-up procedures in DBS analysis including derivatization

Important factors that contribute to the choice for a certain derivatization DBS sample work-up procedure are the choice of derivatizing reagent and the circumstances required to form a stable

derivative in a quantitative way. The choice of derivatization reagent depends on the physical and chemical properties of the target analytes and on the instrument characteristics. The reaction yield can be influenced by the type and amount of derivatizing reagent, the pH, temperature and time needed to complete the reaction. In addition, some reagents require an aprotic environment for the reaction to occur while others react well in aqueous media. Furthermore, as not all reagents can be injected directly into an analytical system, excess derivatization reagent may need to be removed prior to analysis [8,15,28].

II.B.2.1 General DBS sample work-up procedure including derivatization

An overview of selected procedures that apply the 'general' procedure is shown in Table II.B.1. These procedures have been widely applied in metabolic screening of newborn DBS and in follow-up monitoring of symptomatic patients [29]. Thereby, as shown in Table II.B.1, various assay methods such as (LC-) MS/MS and GC-MS and derivatization procedures have been utilized to achieve required method sensitivity and/or selectivity. All selected methods follow a similar sample work-up (Fig. II.B.1: general procedure) and start with elution of the analytes of interest from the DBS, sometimes followed by an extra purification step such as solid-phase extraction (SPE) in order to increase analytical column lifetime and reduce MS cleaning [30,31]. Subsequently, (an aliquot of) the extract is transferred and evaporated under a stream of nitrogen before adding the derivatization reagent(s). Then, after completion of the derivatization, the excess reagent is removed by evaporation under a stream of nitrogen, followed by reconstitution of the derivatized extract prior to injection.

In an LC-MS/MS method developed to determine 17-hydroxyprogesterone and 17-hydroxypregnolone in DBS, the target compounds were derivatized according to this general procedure to enhance ionization during ESI-MS/MS [30]. In addition, direct MS/MS, without chromatographic separation, has become a well-established technique for the quantitative determination of several biomarkers in DBS after derivatization. Corresponding butylesters are prepared prior to analysis, in order to enhance sensitivity and to reduce potential background interferences by increasing *m/z* values as a result of mass gain. This procedure, first reported in the nineties by Chace *et al*. [32-34], has replaced historically used newborn screening tests and has evolved to a single-run analysis using fully automated ESI MS/MS, detecting over 65 metabolites and/or specific markers for disorders in amino acid, fatty acid or organic acid metabolism [35,36].

Table II.B.1 Selected examples of DBS methods using the 'general' sample work-up procedure

Assay method	Type of derivatization	Analyte(s) of interest	Application	Selected references
LC-MS/MS	Hydrazone complex formation	17-OH-progesterone 17-OH-pregnolone	NBS	Higashi et al.; 2008 [30] Lai et al.; 2001 [17]
	Alkylation: butylesterification	Guanidinoacetate creatine	NBS	Bodamer et al.; 2001 [44]
	Diels Alder	25-OH-vitamin D_3 25-OH-vitamin D_2	NBS	Eyles et al.; 2009 [37]
	Diels Alder - Acetylation (2-step)	25-OH-vitamin D_3 3-epi-25-OH-vitamin D_3	NBS	Higashi et al.; 2011 [31]
MS/MS	Alkylation: butylesterification	AA (Acyl)carnitine(s) Guanidinoacetate creatine	NBS	Jebrail et al., 2012 [45]; Chace et al., 1993 [32]; Chace et al., 1995 [33]; Chace et al., 1996 [34]; Naylor and Chace, 1999 [35]; Chace et al., 2009 [46]; Turgeon et al., 2008 [47]; Carducci et al., 2006 [29]; Fingerhut et al., 2001 [48]
GC-MS	Alkylation: butylesterification-Acetylation (2-step)	AA	NBS	Deng et al., 2002b [39]; Deng and Deng, 2003 [40]
	Silylation	AA	NBS	Shen et al., 2006 [10]; Deng et al., 2002a [38]; Deng et al., 2005b [41]; Deng et al., 2005c [42]

AA: amino acids; GC: gas chromatography; LC: liquid chromatography; MS/MS: tandem mass spectrometry; MS: mass spectrometry; NBS: newborn screening

Under some conditions, a 2-step derivatization reaction that involves the sequential application of non-compatible derivatization reagents may be required to obtain suitable derivatives. The usefulness of including a 2-step reaction is illustrated by the following example. Eyles *et al*. [37] and Higashi *et al*. [31] both developed an LC-MS/MS method for the determination of 25-OH-vitamin D_3 in DBS to diagnose vitamin D deficiency. In the first method, 25-OH-vitamin D_2 and D_3 were derivatized using a single derivatization reaction to increase their ESI-MS/MS response. In addition, to separate the target analyte from a potentially interfering epimer (3-epi-25-OH-vitamin D_3) during LC, Higashi *et al*. [31] performed an additional derivatization reaction, thereby enhancing method selectivity and avoiding overestimation of the vitamin D_3 level.

Although (LC-) MS/MS has been generally recognized as a powerful tool for newborn screening (NBS), it can be too expensive in some circumstances. Then, a possible and suitable alternative is GC-MS, known to be a relatively inexpensive, simple, but sensitive technique. For the determination of different AA in newborn DBS using GC-MS, previously reported 2-step derivatization sample work-up procedures using alkylation and acetylation have been improved by the use of single-step silylation, hence contributing to simplified sample work-up procedures [38-40,10]. Moreover, instead of classical silylation, microwave-assisted-silylation has been successfully applied to determine AA in DBS. Heating the sample mixture in a sealed vessel by microwave energy resulted in a strong reduction of the time required for energy transfer, and, consequently, in faster completion of derivatization processes [41-43].

II.B.2.2 MODIFICATIONS

Several modifications have been made to the above mentioned 'general' procedure, mainly to minimize and simplify the DBS sample preparation. Reported modified sample work-up procedures can be classified into three groups. Procedures in the first group only differ from the 'general' procedure in that the derivatized extract is not dried and reconstituted, but is directly injected into the analytical system (Table II.B.2, Fig. II.B.1: modified sample work-up procedure 1). In the second modified sample work-up, the evaporation step between the extraction and derivatization is omitted (Fig. II.B.1: modified sample work-up procedure 2). Furthermore, once derivatized, an aliquot of the sample mixture can be injected into the analytical system either after removal of the excess reagent (Table II.B.3a) or directly (Table II.B.3b). The third group achieves a simplified sample work-up by using a direct derivatization technique, meaning that DBS extraction and derivatization of the extracted compounds is performed in one single step (Fig. II.B.1: modified sample work-up procedure 3). To this end, DBS can be exposed to extraction and derivatization reagents simultaneously, or only to the latter, leading to "on spot" derivatization without the use of any extraction solvent. Similar to modified sample work-up procedure 2, the final sample mixture can be injected either after removal of the excess reagent (Table II.B.4a) or directly after derivatization (Table II.B.4b). In the following subsections, these modified sample work-up procedures are further explained and illustrated by several examples.

II.B.2.2.1 Modified sample work-up procedure 1: Extraction-evaporation-derivatization

A first modification of the 'general' sample treatment consists of extraction of a DBS, derivatization of the dried extraction residue and direct injection of an aliquot of the derivatized extract (Fig. II.B.1: modified sample work-up procedure 1). Removal of the excess derivatizing reagent is no longer required if it can be injected directly without causing contamination or chemical damage of the column or detection system. In Table II.B.2, selected examples of DBS methods using this sample work-up procedure are shown.

Table II.B.2 Selected examples of DBS methods using the modified sample work-up procedure 1: Extraction-evaporation-derivatization

Assay method	Type of derivatization	Analyte(s) of interest	Application	Selected references
GC-MS	Silylation	Metabolites (for profiling) 23 drugs of abuse Free fatty acid (cis-4-decenoic acid) 17-OH-progesterone	MP TOX NBS	Kong et al., 2011 [49]; Langel et al., 2011 [50]; Heales and Leonard, 1992 [51]; Deng et al., 2005a [52]
	Silylation – Acetylation (2-step): Formation of TMS-TFA- derivatives	AA Organic acids Glycines	NBS (DPS)	Yoon, 2007 [57]
LC-FLUO	Intramolecular excimer fluorescence derivatization using pyrene reagent	Methylmalonic acid	NBS	Al-dirbashi et al.; 2005 [13]
LC-UV	Formation of AQC-derivatives	AA	NBS	Swenson and McWhinney, 2009 [58]
LC-MS/MS	Alkylation: butylesterification – hydrazone formation (2-step)	Succinylacetone	NBS	Al-Dirbashi et al., 2006 [55]; Al-Dirbashi et al., 2008 [56]

AA: amino acids; AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; DPS: dried plasma spots; FLUO: fluorescence; GC: gas chromatography; LC: liquid chromatography; MP: metabolomic profiling; MS/MS: tandem mass spectrometry; MS: mass spectrometry; NBS: newborn screening; TFA-: trifluoroacyl-; TMS-: trimethylsilyl-; TOX: toxicology; UV: ultraviolet

In this context, a suitable strategy for GC-MS applications is silylation. Using silylating reagents N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA), specific metabolites (for metabolomic profiling), 23 drugs of abuse, a free fatty acid (cis-4-decenoic acid) and 17-hydroxyprogesterone were successfully determined in DBS, the latter following microwave-assisted-silylation [49-52].

Several reagents for pre-column derivatization of nonfluorescent compounds to their fluorescent derivatives prior to reversed-phase high-performance liquid chromatography (RP-HPLC) separation are also suitable for direct injection. In addition, these reagents can mostly be applied directly to the extraction mixture (see subsection II.B.2.2.2), but in some circumstances the extraction solvent needs to be evaporated as it may influence fluorescence yield and stability. For example, for the determination of methylmalonic acid in DBS, the DBS methanolic extract was dried and reconstituted in water, followed by addition of the pyrene reagent and catalysts in dimethylsulphoxide. This procedure resulted in intense fluorescence of the dipyrene derivative of methylmalonic acid, which, in addition, could clearly be discriminated from that of the monomeric fluorescing compounds, thereby eliminating these interferences [13,53,54].

Dansylhydrazine, originally used as derivatization reagent in LC-FLUO applications, has been successfully applied in DBS LC-MS/MS methods determining succinylacetone (SA), which shows very poor ionization efficiency in ESI-MS [16]. Al-Dirbashi and co-workers developed a 2-step derivatization reaction, as they concluded that applying dansylhydrazine alone to the DBS residue resulted in a mono-dansylhydrazone derivative with unfavorable chromatographic properties. Thus, to improve the chromatographic properties such as acceptable retention and less peak tailing and to increase ionization efficiency, SA was butylated prior to dansylation. Between the derivatization processes, the butylated extract was dried and after allowing the second reaction to occur, an aliquot of the resulting mixture was subjected to high-performance LC (HPLC)- or ultra-high-performance LC (UHPLC)-MS/MS analysis [55,56].

II.B.2.2.2 Modified sample work-up procedure 2: Two-step extraction-derivatization

GC and LC applications have been reported where the reagents are applied to the (aqueous) extraction mixture without prior evaporation (Fig. II.B.1: modified sample work-up procedure 2). Derivatization reagents that react under the conditions of the extraction solvent are suitable candidates for this purpose. Moreover, some reagents have the additional advantage of reacting fast without heating. Table II.B.3 shows selected examples of DBS methods using this modified sample work-up procedure, divided into procedures where the excess reagent is removed prior to injection (Table II.B.3a) and procedures where the final mixture is injected directly into the analytical system (Table II.B.3b).

Table II.B.3 Selected examples of DBS methods using the modified sample work-up procedure 2:
Two-step extraction and derivatization, followed by removal of the excess reagent (a) or by direct injection (b)

(a)

Assay method	Type of derivatization	Analyte(s) of interest	Application	Selected references
GC-MS	Alkylation: alkyl chloroformation	AA	NBS	Kawana et al., 2010 [59]; Deng et al., 2004 [60]
LC-UV	Formation of phenylthiocarbamyl-derivatives	AA	NBS	Dale et al., 2003 [12]

AA: amino acids; GC: gas chromatography; LC: liquid chromatography; MS: mass spectrometry; NBS: newborn screening; UV: ultra-violet

(**b)**

Assay method	Type of derivatization	Analyte(s) of interest	Application	Selected references
LC-FLUO	OPA[1] Formation of isoindolic derivatives	AA Aminoglycoside antibiotics	NBS TDM Preclinical studies	Kand'ar et al.. 2009 [61]; Moretti et al., 1990 [62]; Lundsjo et al., 1990 [64]; Vollmer et al., 1990 [66]
	Benzoin Formation of 2-substituted amino-4,5-diphenylimidazoles	GAA	NBS	Carducci et al., 2001 [26]
	OPD[2] Formation of 2-quinoxalinol derivatives	α-keto acids	NBS	Kand'ar et al., 2009 [61]
LC-MS/MS	Formation of PFB[3]-derivatives	Seven-carbon sugars	NBS	Wamelinck et al., 2011 [65]

[1]OPA: O-phtalaldehyde; [2]OPD: O-phenylenediamine; [3]PFB-derivatives: O-2,3,4,5,6-pentafluorobenzyl-derivatives

AA: amino acids; FLUO: fluorescence; GAA: guanidinoacetic acid; LC: liquid chromatography; MS/MS: tandem mass spectrometry; NBS: newborn screening, TDM: therapeutic drug monitoring

When using GC, the injection of excess reagent but also of residual water into the GC system is preferentially avoided (Table II.B.3a). This can be achieved by extracting the derivatized target analytes from the (aqueous) reaction mixture. A GC-MS method developed for the determination of several AA in DBS after "in situ" derivatization with propyl chloroformate illustrates this principle. As water does not interfere with alkyl chloroformation, the chloroformate can be added directly to the eluate of an SPE step, isolating AA from a DBS extract. After completion of the reaction within 1 minute at room temperature, the resulting derivatives are isolated by liquid-

liquid extraction (LLE) using a water-immiscible organic solvent, of which an aliquot was injected [59]. In another method, isobutyl chloroformate derivatized AA were extracted from an aqueous reaction mixture by solid-phase micro extraction (SPME), followed by desorption of the fiber into the GC-MS injector [60].

Likewise, excess derivatizing reagents enabling LC-UV detection may be removed by additional purification steps to avoid interference during analysis (Table II.B.3a). Dale *et al*. (2003) [12] used this approach for the determination of phenylalanine and tyrosine in DBS. Following extraction and the generation of phenylthiocarbamylderivatives, the extracts were dried and reconstituted in 0.1 M sodium acetate buffer. The excess reagent still present in the final aqueous mixture was trapped in methylene dichloride along with other possibly interfering compounds prior to analysis of the clear aqueous phase by reversed-phase HPLC with UV detection.

Similar procedures have been reported in combination with LC-FLUO or LC-MS/MS methods, having the advantage that an aliquot of the final reaction mixture can be injected directly (Table II.B.3b). For example, pre-column derivatization of several AA, guanidinoacetic acid (GAA) and α-keto acids has been performed in order to achieve highly sensitive and selective LC-FLUO methods [61,26,62]. A way to achieve reproducible results and avoid errors from instability of the derivative when applying certain derivatizing reagents pre- rather than post-column, is controlling the time between derivatization and injection by an on-line derivatization technique [61,63,64]. On-line derivatization can be achieved by derivatizing in the autosampler, as has been demonstrated for several DBS-extracted AA, derivatized with o-phthaldialdehyde immediately prior to separation [61,64]. An LC-MS/MS example is the determination of the derivatized seven-carbon sugar sedoheptulose, which proved to be substantially more sensitive, with no interferences of other sugars possibly present in the DBS. Additionally, as retention was increased due to derivatization, simple reversed-phase chromatography using a common mobile phase composition could be performed [65].

II.B.2.2.3 Modified sample work-up procedure 3: Direct derivatization techniques

The most convenient approach for derivatizing analytes from DBS is direct derivatization. This approach has been reported for the analysis of conventional matrices such as blood, plasma and serum and of aqueous matrices such as drinks. In those cases, an aliquot of matrix (20-50 µl) is directly derivatized by adding an excess of derivatization reagent (up to 1 ml), thereby omitting the extraction step [67-69]. Implementing this principle in DBS analysis, extracting and derivatizing solutions could be applied simultaneously to the DBS or even only the derivatizing reagent(s) could be added without use of any extraction solvent (Fig. II.B.1: modified sample work-up procedure 3). We coined the latter approach "on spot" derivatization. Table II.B.4 shows selected examples of DBS methods using direct derivatization techniques, followed by removal of the excess reagent before injection (Table II.B.4a) or by direct injection (Table II.B.4b).

Depending on the type of derivatization reagents used, a proper extraction solvent should be selected, which still allows the reaction to occur and results in acceptable extraction recovery of the (derivatized) target analyte. For example, for the determination of antidepressants in DBS, a method was developed combining a fast and sensitive GC-MS/MS technique with direct derivatization of the DBS. Fluorinated agents were chosen to derivatize the analytes, increasing electro-affinity which was important for MS/MS detection operating in negative chemical ionization (NCI) mode (this mode being highly selective and sensitive for compounds with high electron-affinity). Although the use of methanol and water as extraction solvent resulted in higher extraction recovery of fluoxetine, norfluoxetine, reboxetine and paroxetine, the authors selected an aprotic organic solvent (butyl chloride), as this could be applied simultaneously with the fluorinating agent, simplifying sample work-up and still resulting in the required method sensitivity [5].

In some circumstances, adequate sensitivity and selectivity can be achieved by applying the derivatization reagent (mixture) directly "on spot", without the use of additional (co-) extracting solvent. In this scenario, it is assumed that the reagent functions both as extracting and derivatizing agent, as no other solutions or solvents are added to the DBS. To our knowledge, up-to-date this approach of "on spot" derivatization has only been applied for the GC-MS-based determination of gamma-hydroxybutyric acid (GHB), a notorious club and date-rape drug, in DBS

[11,70]. More specifically, GHB was derivatized by adding a mixture of trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB-OH) directly "on spot". Although only a small volume of whole blood was analyzed (a 6-mm diameter punch from a DBS, corresponding to approximately 10 µl), adequate sensitivity was achieved [70].

Table II.B.4 Selected examples of DBS methods using the modified sample work-up procedure 3: Direct derivatization, followed by removal of the excess reagent (a) or by direct injection (b)

(a)

Assay method	Type of derivatization	Analyte(s) of interest	Application	Selected references
GC-MS/MS	Acetylation	Antidepressants	TDM	Déglon et al., 2010 [5]
GC-MS	On spot alkylation and acetylation	GHB	TOX	Ingels et al., 2010 [11] ; Ingels et al., 2011 [70]
	Transmethylation	Free fatty acids	NBS	Kimura et al., 2002 [71]; Morton and Kelley, 1990 [76]
MS/MS	Formation of 1-phenyl-3-methyl pyrazolone derivatives	Oligosaccharides	MD	Ramsay et al., 2003 [73]; Rozaklis et al., 2002 [72]

GC: gas chromatography; GHB: gamma-hydroxybutyric acid; MD: metabolic disorder; MS/MS: tandem mass spectrometry direct injection; MS: mass spectrometry; NBS: newborn screening; TDM: therapeutic drug monitoring; TOX: toxicology

(b)

Assay method	Type of derivatization	Analyte(s) of interest	Application	Selected references
LC-MS/MS	Formation of DAABD-AE-derivatives	3-OH-glutaric acid Glutaric acid	NBS (DUS)	Al-Dirbashi et al., 2011 [74]
MS/MS	Hydrazone formation	Succinylacetone	NBS	Allard et al., 2004 [75]

DAABD-AE: 4-2-(N,N-dimethylamino)ethylaminosulfonyl-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; DUS: dried urine spots; LC: liquid chromatography; MS/MS: tandem mass spectrometry direct injection; NBS: newborn screening

Although in the above-mentioned GC-methods, removal of the excess reagent was achieved by simple evaporation, in some circumstances, additional LLE and/or SPE is required in order to isolate the derivatized analyte and/or remove the excess reagent before injection (Table II.B.4a). For example, after a single step transmethylation of free fatty acids present in DBS, the reaction was halted by adding 6% potassium carbonate and the derivatives were extracted in hexane, of which an aliquot was injected into the GC-MS [71]. In another example, the excess reagent used to derivatize oligosaccharides in DBS with a single step extraction/derivatization procedure was trapped in chloroform. The remaining aqueous layer was subjected to SPE, including wash steps to further remove any unincorporated reagent, and finally, the eluate containing the derivatives was dried and reconstituted before injection into the ESI-MS/MS system [72,73].

As complicated procedures to remove excess reagent extend the sample work-up associated with a simplified extraction/derivatization procedure, direct derivatization techniques in which an aliquot of the supernatant can be analyzed directly are even more practical (Table II.B.4b). Since in this scenario the complete DBS sample preparation has been reduced to a single step, sample throughput is consequently only limited by the time required for the derivatization reaction to occur. To illustrate this, sample work-up of an LC-MS/MS method determining 3-hydroxy-glutaric acid and glutaric acid in dried urine spots (DUS) was completed within 45 min since the direct derivatization step required 45 min at 60°C to obtain maximum derivatization yield. Here, internal standard solution and derivatizing reagents were added successively to the dried spot, hence eliminating a separate extraction step, and an aliquot of the final derivatized solution was analyzed directly after the reaction had been stopped. Derivatization was required to enhance ionization and fragmentation of the target compounds [74].

Special considerations will need to be taken into account when using direct derivatization techniques. This is because the derivatization yield reflects both elution of the target compounds from the DBS filter paper as well as the formation of the derivatives. An example is the simultaneous extraction of SA from DBS and production of SA derivatives by the use of a single step derivatization using a hydrazine-containing solution. The simultaneous extraction is assumed to occur via cleavage of covalently linked SA-protein adducts by hydrazine and results in extraction of SA as a hydrazone derivative. Moreover, although it might be possible to obtain a lower limit of quantification by including a purification step before MS/MS analysis and an additional

derivatization step (see subsection II.B.2.2.1), the main advantages of the method are simplicity, the requirement of only minimal technical time and the provision of enough sensitivity to detect positive cases [75].

Another point to consider with direct derivatization techniques is that the filter paper is derivatized as well. Normally after extraction, an aliquot of the solvent is transferred in order to evaporate, filter or derivatize the extraction solvent containing the extracted compounds. Since the reagents are now applied directly to the filter paper present in the test tube, it might be relevant to check for interferences as a result of derivatizing the blank filter paper. In the case of derivatizing the blank filter paper used for applying whole blood from possible GHB-intoxicated patients, a small interference was seen at the retention time of GHB (< 20 % of the LLOQ) [11].

II.B.3 CONCLUSION AND FUTURE PERSPECTIVES

To conclude, DBS analysis has proven useful in a wide range of applications, because of the typical benefits associated with this sampling technique. Although LC-MS/MS is commonly used for DBS analysis, other techniques such as GC-MS (/MS) may also play a role in the determination of drugs or biomarkers in DBS. To determine trace levels of low molecular weight compounds in DBS, a derivatization step during DBS sample work-up may be necessary or may help to achieve adequate method sensitivity. Dependent on the target compounds and the derivatizing reagents, different procedures are possible as shown in Fig. II.B.1. Moreover, a single DBS can be subjected to a combination of sample work-up procedures including different derivatization techniques to extend the range of target compounds of a DBS method. For example, to include SA, most favorably extracted as hydrazone-derivative (see subsection II.B.2.2.3), in a method determining amino acids (AA) and acylcarnitines (AC) in newborn DBS, combinations of the 'general' procedure (for AA and AC) and the direct derivatization technique (for SA) have been described [46,47,77].

Although incorporating a derivatization procedure in DBS sample work-up may significantly increase method sensitivity, this additional step may be experienced as laborious and tedious (e.g., requiring more technical time). Hence, replacement of manual preparation by automated techniques but also implementation of procedures using simpler sample work-up such as direct derivatization, may be less time-consuming and lead to increased sample throughput.

Thus, the introduction of automated techniques may result in high-throughput DBS methods including derivatization, as illustrated by a recently developed fully automated sample preparation technique using a digital microfluidic method including extraction and derivatization of several AA in DBS by in-line or off-line nano-ESI-MS/MS [45]. In addition, modified sample work-up procedures such as direct derivatization techniques, using a single step extraction and derivatization or "on spot" derivatization, may lead to a less time-consuming and less laborious sample work-up. These procedures may be considered in cases where a derivatization step is needed, to ensure a minimal, economic and fast sample treatment.

Consequently, a similar workload may be achieved as with classical DBS analyses which only use extraction and purification procedures, but with additional gain in method sensitivity and selectivity due to derivatization. Hence, the application range of DBS analysis may be extended. Automated techniques are being developed such as paper-spray ionization MS/MS analysis with on-line derivatization, implemented by preloading reagents onto the paper or using solutions containing the derivatizing reagents. This approach was already successfully applied to detect cholesterol in dried serum spots via formation of a characteristic fragment ion, with enhanced MS/MS sensitivity and selectivity [78]. Furthermore, we envisage that direct derivatization approaches may gain importance in the future, not only in GC, but also in LC-MS/MS and other MS(/MS)-based approaches, where they could be combined with on-line elution, or could even precede direct desorption from the DBS (e.g., following application of a derivatizing reagent sprayed on the DBS).

REFERENCES

1. McDade TW, Williams S, Snodgrass JJ. What a Drop Can Do: Dried Blood Spots as a Minimally Invasive Method for Integrating Biomarkers into Population-Based Research. *Demography.* 44:899-925 (2007).
2. Garcia Boy R, Henseler J, Mattern R, Skopp G. Determination of Morphine and 6-Acetylmorphine in Blood with Use of Dried Blood Spots. *Ther Drug Monit*. 30:733-39 (2008).
3. Edelbroek PM, van der Heijden J, Stolk LM. Dried Blood Spot Methods in Therapeutic Drug Monitoring: Methods, Assays, and Pitfalls. *Ther Drug Monit.* 31:327-36 (2009).
4. Li WK, Tse FLS. Dried Blood Spot Sampling in Combination with LC-MS/MS for Quantitative Analysis of Small Molecules. *Biomed Chromatogr.* 24:49-65 (2010).
5. Déglon J, Lauer E, Thomas A, Mangin P, Staub C. Use of the Dried Blood Spot Sampling Process Coupled with Fast Gas Chromatography and Negative-Ion Chemical Ionization Tandem Mass Spectrometry: Application to Fluoxetine, Norfluoxetine, Reboxetine, and Paroxetine Analysis. *Anal Bioanal Chem.* 396:2523-32 (2010).
6. Tanna S, Lawson G. Analytical Methods Used in Conjunction with Dried Blood Spots. *Anal Meth.* 3:1709-1718 (2011).
7. Stove CP, Ingels ASME, De Kesel PMM, Lambert WE. Dried Blood Spots in Toxicology: From the Cradle to the Grave? *Crit Rev Tox*. 42(3):230-43 (2012).
8. Lin DL, Wang SM, Wu CH, Chen BG, Lu RH. Chemical Derivatization for the Analysis of Drugs by GC-MS - a Conceptual Review. *J Food Drug Anal.* 16: 1-10 (2008).
9. Kaal E, Janssen HG. Extending the Molecular Application Range of Gas Chromatography. *J Chromatogr A.* 1184: 43-60 (2008).
10. Shen X, Deng C, Wang B, Dong L. Quantification of Trimethylsilyl Derivatives of Amino Acid Disease Biomarkers in Neonatal Blood Samples by Gas Chromatography-Mass Spectrometry. *Anal Bioanal Chem.* 384: 931-8 (2006).
11. Ingels ASME, Lambert WE, Stove CP. Determination of Gamma-Hydroxybutyric Acid in Dried Blood Spots Using a Simple GC-MS Method with Direct "On Spot" Derivatization. *Anal Bioanal Chem.* 398: 2173-82 (2010).
12. Dale Y, Mackey V, Mushi R, Nyanda A, Maleque M, Ike J. Simultaneous Measurement of Phenylalanine and Tyrosine in Phenylketonuric Plasma and Dried Blood by High-Performance Liquid Chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci.* 788: 1-8 (2003).
13. Al-Dirbashi OY, Jacob M, Al-Hassnan Z, El-Badaoui F, Rashed MS. Diagnosis of Methylmalonic Acidemia from Dried Blood Spots by HPLC and Intramolecular-Excimer Fluorescence Derivatization. *Clin Chem.* 51: 235-7 (2005).
14. Wuyts B, Stove V, Goossens L. Critical Sample Pretreatment in Monitoring Dried Blood Spot Citrulline. *Clin Chim Acta.* 386: 105-09 (2007).

15. Xu F, Zou L, Liu Y, Zhang Z, Ong CN. Enhancement of the Capabilities of Liquid Chromatography-Mass Spectrometry with Derivatization: General Principles and Applications. *Mass Spectrom Rev*. 30: 1143-72 (2011).
16. Santa T. Derivatization Reagents in Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry. *Biomed Chromatogr.* 25: 1-10 (2011).
17. Lai CC, Tsai CH, Tsai FJ, Lee CC, Lin WD. Rapid Monitoring Assay of Congenital Adrenal Hyperplasia with Microbore High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry from Dried Blood Spots. *Rapid Commun Mass Spectrom.* 15: 2145-51 (2001).
18. De Jesus VR, Chace DH, Lim TH, Mei JV, Hannon WH. Comparison of Amino Acids and Acylcarnitines Assay Methods Used in Newborn Screening Assays by Tandem Mass Spectrometry. *Clin Chim Acta.* 411: 684-9 (2010).
19. Niwa M. Chemical Derivatization as a Tool for Optimizing MS Response in Sensitive LC-MS/MS Bioanalysis and Its Role in Pharmacokinetic Studies. *Bioanalysis.* 4: 213-20 (2012).
20. Knapp DR. Handbook of Analytical Derivatization Reactions. John Wiley and sons, New York, Chichester, Brisbane, Toronto. (1979).
21. Segura J, Ventura R, Jurado C. Derivatization Procedures for Gas Chromatographic-Mass Spectrometric Determination of Xenobiotics in Biological Samples, with Special Attention to Drugs of Abuse and Doping Agents. *J Chromatogr B Biomed Sci Appl.* 713: 61-90 (1998).
22. Todoroki K, Yoshida H, Hayama T, Itoyama M, Nohta H, Yamaguchi M. Highly Sensitive and Selective Derivatization-LC Method for Biomolecules Based on Fluorescence Interactions and Fluorous Separations. *J Chromatogr B Analyt Technol Biomed Life Sci*. 879: 1325-37 (2011).
23. Moffat AC, Osselton MD, Widdop B. Clarke's Analysis of Drugs and Poisons, Third Edition. *Pharmaceutical Press, London*. (2004).
24. Hayashi T, Tsuchiya H, Naruse H. Reversed-Phase Ion-Pair Chromatography of Amino Acids. Application to the Determination of Amino Acids in Plasma Samples and Dried Blood on Filter Papers. *J Chromatogr.* 274: 318-24 (1983).
25. Roesel RA, Blankenship PR, Hommes FA. HPLC Assay of Phenylalanine and Tyrosine in Blood Spots on Filter Paper. *Clin Chim Acta.* 156: 91-6 (1986).
26. Carducci C, Birarelli M, Santagata P, Leuzzi V, Antonozzi I. Automated High-Performance Liquid Chromatographic Method for the Determination of Guanidinoacetic Acid in Dried Blood Spots: A Tool for Early Diagnosis of Guanidinoacetate Methyltransferase Deficiency. *J Chromatogr B Biomed Sci Appl.* 755: 343-8 (2001).
27. Abdulrazzaq YM, Ibrahim A. Determination of Amino Acids by Ion-Exchange Chromatography on Filter Paper Spotted Blood Samples Stored at Different Temperatures and for Different Periods: Comparison with Capillary and Venous Blood. *Clin Biochem.* 34: 399-406 (2001).

28. Rood D. A Practical Guide to the Care, Maintenance, and Troubleshooting of Capillary Gas Chromatographic Systems. Wiley-VCH, Weinheim, New York, Chichester, Brisbane, Singapore, Toronto. (1999).
29. Carducci C, Santagata S, Leuzzi V, Artiola C, Giovanniello T, Battini R, Antonozzi I. Quantitative Determination of Guanidinoacetate and Creatine in Dried Blood Spot by Flow Injection Analysis-Electrospray Tandem Mass Spectrometry. *Clin Chim Acta*. 364:180-7 (2006).
30. Higashi T, Nishio T, Uchida S, Shimada K, Fukushi M, Maeda M. Simultaneous Determination of 17alpha-Hydroxypregnenolone and 17alpha-Hydroxyprogesterone in Dried Blood Spots from Low Birth Weight Infants Using LC-MS/MS. *J Pharm Biomed Anal*. 48:177-82 (2008).
31. Higashi T, Suzuki M, Hanai J, Inagaki S, Min JZ, Shimada K, Toyo'oka T. A Specific LC/ESI-MS/MS Method for Determination of 25-Hydroxyvitamin D3 in Neonatal Dried Blood Spots Containing a Potential Interfering Metabolite, 3-Epi-25-Hydroxyvitamin D3. *J Sep Sci.* 34:725-32 (2011).
32. Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF. Rapid Diagnosis of Phenylketonuria by Quantitative Analysis for Phenylalanine and Tyrosine in Neonatal Blood Spots by Tandem Mass Spectrometry. *Clin Chem.* 39:66-71 (1993).
33. Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW. Rapid Diagnosis of Maple Syrup Urine Disease in Blood Spots from Newborns by Tandem Mass Spectrometry. *Clin Chem.* 41:62-8 (1995).
34. Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL. Rapid Diagnosis of Homocystinuria and Other Hypermethioninemias from Newborns' Blood Spots by Tandem Mass Spectrometry. *Clin Chem*. 42:349-55 (1996).
35. Naylor EW, Chace DH. Automated Tandem Mass Spectrometry for Mass Newborn Screening for Disorders in Fatty Acid, Organic Acid, and Amino Acid Metabolism. *J Child Neurol*. 14(Suppl 1):S4-8 (1999).
36. Chace DH. Mass Spectrometry in Newborn and Metabolic Screening: Historical Perspective and Future Directions. *J Mass Spectrom.* 44:163-70 (2009).
37. Eyles D, Anderson C, Ko P, Jones A, Thomas A, Burne T, Mortensen PB, Norgaard-Pedersen B, Hougaard DM, McGrath J. A Sensitive LC/MS/MS Assay of 25OH Vitamin D3 and 25OH Vitamin D2 in Dried Blood Spots. *Clin Chim Acta.* 403:145-51 (2009).
38. Deng C, Deng Y, Wang B, Yang X. Gas Chromatography-Mass Spectrometry Method for Determination of Phenylalanine and Tyrosine in Neonatal Blood Spots. *J Chromatogr B Analyt Technol Biomed Life Sci.* 780:407-13 (2002a).
39. Deng C, Shang C, Hu Y, Zhang X. Rapid Diagnosis of Phenylketonuria and Other Aminoacidemias by Quantitative Analysis of Amino Acids in Neonatal Blood Spots by Gas Chromatography-Mass Spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 775:115-20 (2002b).

40. Deng C, Deng Y. Diagnosis of Maple Syrup Urine Disease by Determination of L-Valine, L-Isoleucine, L-Leucine and L-Phenylalanine in Neonatal Blood Spots by Gas Chromatography-Mass Spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 792:261-268 (2003).
41. Deng C, Yin X, Zhang L, Zhang X. Development of Microwave-Assisted Derivatization Followed by Gas Chromatography/Mass Spectrometry for Fast Determination of Amino Acids in Neonatal Blood Samples. *Rapid Commun Mass Spectrom.* 19:2227-2234 (2005b).
42. Deng CH, Wang B, Liu LF. Fast Diagnosis of Neonatal Phenylketonuria by Gas Chromatography-Mass Spectrometry Following Microwave-Assisted Silylation. *Chromatographia.* 62:617-621 (2005c).
43. Soderholm SL, Damm M, Kappe CO. Microwave-Assisted Derivatization Procedures for Gas Chromatography/Mass Spectrometry Analysis. *Mol Divers.* 14:869-888 (2010).
44. Bodamer OA, Bloesch SM, Gregg AR, Stockler-Ipsiroglu S, O'Brien WE. Analysis of Guanidinoacetate and Creatine by Isotope Dilution Electrospray Tandem Mass Spectrometry. *Clin Chim Acta.* 308:173-178 (2001).
45. Jebrail MJ, Yang H, Mudrik JM, Lafreniere NM, McRoberts C, Al-Dirbashi OY, Fisher L, Chakraborty P, Wheeler AR. A Digital Microfluidic Method for Dried Blood Spot Analysis. *Lab Chip.* 11:3218-3224 (2012).
46. Chace DH, Lim T, Hansen CR, De Jesus VR, Hannon WH. Improved MS/MS Analysis of Succinylacetone Extracted from Dried Blood Spots When Combined with Amino Acids and Acylcarnitine Butyl Esters. *Clin Chim Acta.* 407:6-9 (2009).
47. Turgeon C, Magera MJ, Allard P, Tortorelli S, Gavrilov D, Oglesbee D, Raymond K, Rinaldo P, Matern D. Combined Newborn Screening for Succinylacetone, Amino Acids, and Acylcarnitines in Dried Blood Spots. *Clin Chem.* 54:657-664 (2008).
48. Fingerhut R, Roschinger W, Muntau AC, Dame T, Kreischer J, Arnecke R, Superti-Furga A, Troxler H, Liebl B, Olgemoller B, Roscher AA. Hepatic Carnitine Palmitoyltransferase I Deficiency: Acylcarnitine Profiles in Blood Spots are Highly Specific. *Clin Chem.* 47:1763-1768 (2001).
49. Kong ST, Lin HS, Ching J, Ho PC. Evaluation of Dried Blood Spots as Sample Matrix for Gas Chromatography/Mass Spectrometry Based Metabolomic Profiling. *Anal Chem.* 83:4314-4318 (2011).
50. Langel K, Uusivirta H, Ariniemi K, Lillsunde P. Analysis of Drugs of Abuse by GC-MS in Dried Blood Spot Sample Matrix. Presented at the 49th TIAFT meeting. San Francisco, CA, September 25-30. (2011).
51. Heales SJ, Leonard JV. Diagnosis of Medium Chain Acyl Coa Dehydrogenase Deficiency by Measurement of Cis-4-Decenoic Acid in Dried Blood Spots. *Clin Chim Acta.* 209:61-66 (1992).
52. Deng C, Ji J, Zhang L, Zhang X. Diagnosis of Congenital Adrenal Hyperplasia by Rapid Determination of 17alpha-Hydroxyprogesterone in Dried Blood Spots by Gas Chromatography/Mass Spectrometry Following Microwave-Assisted Silylation. *Rapid Commun Mass Spectrom.* 19:2974-2978 (2005a).

53. Nohta H, Satozono H, Koiso K, Yoshida H, Ishida J, Yamaguchi M. Highly Selective Fluorometric Determination of Polyamines Based on Intramolecular Excimer-Forming Derivatization with a Pyrene-Labeling Reagent. *Anal Chem.* 72:4199-4204 (2000).
54. Nohta H, Sonoda J, Yoshida H, Satozono H, Ishida J, Yamaguchi M. Liquid Chromatographic Determination of Dicarboxylic Acids Based on Intramolecular Excimer-Forming Fluorescence Derivatization. *J Chromatogr A.* 1010:37-44 (2003).
55. Al-Dirbashi OY, Rashed MS, Brink HJ, Jakobs C, Filimban N, Al-Ahaidib LY, Jacob M, Al-Sayed MM, Al-Hassnan Z, Faqeih E. Determination of Succinylacetone in Dried Blood Spots and Liquid Urine as a Dansylhydrazone by Liquid Chromatography Tandem Mass Spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 831:274-280 (2006).
56. Al-Dirbashi OY, Rashed MS, Jacob M, Al-Ahaideb LY, Al-Amoudi M, Rahbeeni Z, Al-Sayed MM, Al-Hassnan Z, Al-Owain M, Al-Zeidan H. Improved Method to Determine Succinylacetone in Dried Blood Spots for Diagnosis of Tyrosinemia Type 1 Using UPLC-MS/MS. *Biomed Chromatogr.* 22:1181-1185 (2008).
57. Yoon HR. Two Step Derivatization for the Analyses of Organic, Amino Acids and Glycines on Filter Paper Plasma by GC-MS/SIM. *Arch Pharm Res.* 30:387-395 (2007).
58. Swenson RJ, McWhinney BC. Utilising UPLC to Develop a Less Invasive Method for Monitoring Maple Syrup Urine Disease Patients. Presented at the AACB 47th Annual Scientific Conference, Brisbane, September 14-17. (2009).
59. Kawana S, Nakagawa K, Hasegawa Y, Yamaguchi S. Simple and Rapid Analytical Method for Detection of Amino Acids in Blood Using Blood Spot on Filter Paper, Fast-GC/MS and Isotope Dilution Technique. *J Chromatogr B Analyt Technol Biomed Life Sci.* 878:3113-3118 (2010).
60. Deng C, Li N, Zhang X. Rapid Determination of Amino Acids in Neonatal Blood Samples Based on Derivatization with Isobutyl Chloroformate Followed by Solid-Phase Microextraction and Gas Chromatography/Mass Spectrometry. *Rapid Commun Mass Spectrom.* 18:2558-2564 (2004).
61. Kand'ar R, Zakova P, Jirosova J, Sladka M. Determination of Branched Chain Amino Acids, Methionine, Phenylalanine, Tyrosine and Alpha-Keto Acids in Plasma and Dried Blood Samples Using HPLC with Fluorescence Detection. *Clin Chem Lab Med.* 47:565-572 (2009).
62. Moretti F, Birarelli M, Carducci C, Pontecorvi A, Antonozzi I. Simultaneous High-Performance Liquid Chromatographic Determination of Amino Acids in a Dried Blood Spot as a Neonatal Screening Test. *J Chromatogr.* 511:131-136 (1990).
63. Ziegler F, Le Boucher J, Coudray-Lucas C, Cynober L. Plasma Amino-Acid Determinations by Reversed-Phase HPLC: Improvement of the Orthophthalaldehyde Method and Comparison with Ion Exchange Chromatography. *J Automat Chem.* 14:145-149 (1992).

64. Lundsjo A, Hagelberg S, Palmer K, Lindblad BS. Amino Acid Profiles by HPLC after Filter Paper Sampling: 'Appropriate Technology' for Monitoring of Nutritional Status. *Clin Chim Acta.* 191:201-209 (1990).
65. Wamelink MM, Struys EA, Jansen EE, Blom HJ, Vilboux T, Gahl WA, Komhoff M, Jakobs C, Levtchenko EN. Elevated Concentrations of Sedoheptulose in Bloodspots of Patients with Cystinosis Caused by the 57-Kb Deletion: Implications for Diagnostics and Neonatal Screening. *Mol Genet Metab.* 102:339-342 (2011).
66. Vollmer DW, Jinks DC, Guthrie R. Isocratic Reverse-Phase Liquid-Chromatography Assay for Amino-Acid Metabolic Disorders Using Eluates of Dried Blood Spots. *Anal Biochem.* 189:115-121 (1990).
67. Van hee P, Neels H, De Doncker M, Vrydags N, Schatteman K, Uyttenbroeck K, Hamers N, Himpe D, Lambert W. Analysis of Gamma-Hydroxybutyric Acid, DL-Lactic Acid, Glycolic Acid, Ethylene Glycol and Other Glycols in Body Fluids by a Direct Injection Gas Chromatography-Mass Spectrometry Assay for Wide Use. *Clin Chem Lab Med.* 42:1341-1345 (2004).
68. Sabucedo AJ, Furton KG. Extractionless GC-MS Analysis of Gamma-Hydroxybutyrate and Gamma-Butyrolactone with Trifluoroacetic Anhydride and Heptafluoro-1-Butanol from Aqueous Samples. *J Sep Sci.* 27:703-709 (2004).
69. Meyer MR, Weber AA, Maurer HH. A Validated GC-MS Procedure for Fast, Simple, and Cost-Effective Quantification of Glycols and GHB in Human Plasma and Their Identification in Urine and Plasma Developed for Emergency Toxicology. *Anal Bioanal Chem.* 400:411-414 (2011).
70. Ingels AS, De Paepe P, Anseeuw K, Van Sassenbroeck D, Neels H, Lambert W, Stove CP. Dried Blood Spot Punches for Confirmation of Gamma-Hydroxybutyric Acid Intoxications: Validation of an Optimized GC-MS Procedure. *Bioanalysis.* 3(20):2271-2281 (2011).
71. Kimura M, Yoon HR, Wasant P, Takahashi Y, Yamaguchi S. A Sensitive and Simplified Method to Analyze Free Fatty Acids in Children with Mitochondrial Beta Oxidation Disorders Using Gas Chromatography Mass Spectrometry and Dried Blood Spots. *Clin Chim Acta.* 316:117-121 (2002).
72. Rozaklis T, Ramsay SL, Whitfield PD, Ranieri E, Hopwood JJ, Meikle PJ. Determination of Oligosaccharides in Pompe Disease by Electrospray Ionization Tandem Mass Spectrometry. *Clin Chem.* 48:131-139 (2002).
73. Ramsay SL, Meikle PJ, Hopwood JJ. Determination of Monosaccharides and Disaccharides in Mucopolysaccharidoses Patients by Electrospray Ionisation Mass Spectrometry. *Mol Gen Metab.* 78:193-204 (2003).
74. Al-Dirbashi OY, Kolker S, Ng D, Fisher L, Rupar T, Lepage N, Rashed MS, Santa T, Goodman SI, Geraghty MT, Zschocke J, Christensen E, Hoffmann GF, Chakraborty P. Diagnosis of Glutaric Aciduria Type 1 by Measuring 3-Hydroxyglutaric Acid in Dried Urine Spots by Liquid Chromatography Tandem Mass Spectrometry. *J Inherit Metab Dis.* 34:173-180 (2011).

75. Allard P, Grenier A, Korson MS, Zytkovicz TH. Newborn Screening for Hepatorenal Tyrosinemia by Tandem Mass Spectrometry: Analysis of Succinylacetone Extracted from Dried Blood Spots. *Clin Biochem.* 37:1010-1015 (2004).
76. Morton DH, Kelley RI. Diagnosis of Medium-Chain Acyl-Coenzyme a Dehydrogenase Deficiency in the Neonatal Period by Measurement of Medium-Chain Fatty Acids in Plasma and Filter Paper Blood Samples. *J Pediatr.* 117:439-442 (1990).
77. la Marca G, Malvagia S, Pasquini E, Innocenti M, Fernandez MR, Donati MA, Zammarchi E. The Inclusion of Succinylacetone as Marker for Tyrosinemia Type I in Expanded Newborn Screening Programs. *Rapid Commun Mass Spectrom.* 22:812-818 (2008).
78. Liu J, Wang H, Manicke NE, Lin JM, Cooks RG, Ouyang Z. Development, Characterization, and Application of Paper Spray Ionization. *Anal Chem.* 82:2463-2471 (2011).

CHAPTER II.C
DETERMINATION OF GHB IN DBS USING "ON SPOT" DERIVATIZATION AND GC-MS

Based on

Determination of gamma-hydroxybutyric acid in dried blood spots using "on spot" derivatization and a simple GC-MS method. *Anal Bioanal Chem*. 398:2173-2182 (2010). Ann-Sofie M.E. Ingels, Willy E. Lambert, and Christophe P. Stove. (II.C.2)

Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis*. 3(20):2271-2281 (2011). Ann-Sofie M.E. Ingels, Peter De Paepe, Kurt Anseeuw, Diederik K. Van Sassenbroeck, Hugo Neels, Willy E. Lambert, and Christophe P. Stove. (II.C.3)

Feasibility of Following up Gamma-Hydroxybutyric Acid Concentrations in Sodium Oxybate (Xyrem®)-Treated Narcoleptic Patients Using Dried Blood Spot Sampling at Home. An Exploratory Study. *CNS Drugs*. 27(3):233-237 (2013). Ann-Sofie M.E. Ingels, Katrien B. Hertegonne, Willy E. Lambert and Christophe P. Stove. (II.C.4)

II.C.1 Optimization of the GC-MS method to determine derivatized GHB in plasma and DBS

II.C.1.1 Introduction

As the polarity and the low molecular weight of GHB requires derivatization or conversion to GBL prior to GC analysis, we chose to modify the derivatization procedure reported by Sabucedo *et al*. in 2004 [1]. These authors used a mixture of trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB-OH) to derivatize GHB in aqueous samples (drinks). Fig. II.C.1 shows the reaction scheme of the derivatization reaction.

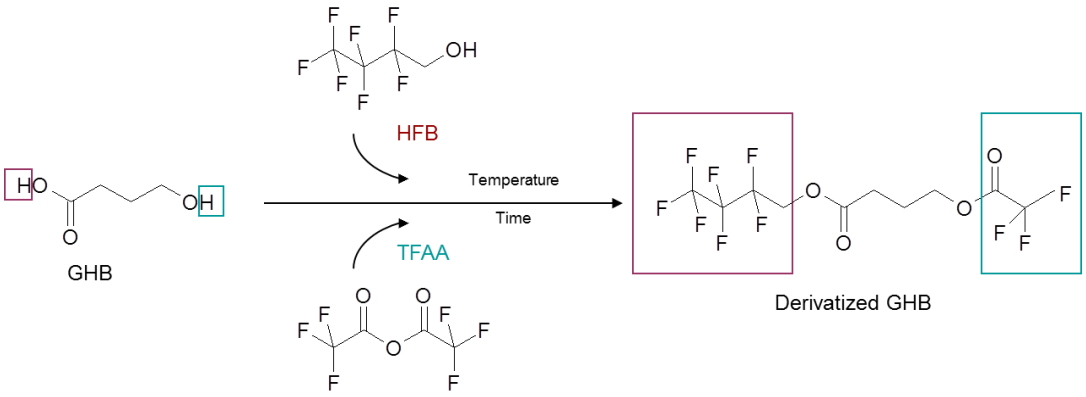


Fig. II.C.1 Reaction scheme of the applied derivatization reaction to derivatize GHB in plasma and DBS

In this section, optimization of the GC-MS method parameters to determine GHB, following derivatization with TFAA/HFB-OH, is described. To this end, 50 µl plasma was spiked with 100 µl of a 40 µg/ml-solution. As we wished to use the final GC-MS method to determine GHB in DBS, the optimal temperature program was selected based on selectivity and peak characteristics of GHB specifically in DBS extracts. We aimed for an optimal resolution within the shortest run time possible. Therefore, GC-MS parameters have been adjusted, in particular sample introduction (1), chromatographic separation (2) and MS detection (3) (Table II.C.1).

Table II.C.1 Overview of the different parameters, those in bold were optimized for the determination of GHB (derivatized with TFAA/HFB-OH)

(1) Sample introduction	(2) Chromatographic separation	(3) MS detection
type of injection: split OR splitless **injection solvent:** hexane, ethyl acetate OR toluene **injection temperature:** 200, 250 OR 300°C **inlet pressure:** 11.8 psi OR 20, 25, 30 psi **purge activation time:** 45, 60, 75, 90 OR 120 sec	type of column: HP-5-MS 30m; 0.25 mm; 0.25 µm carrier gas: helium **flow rate carrier gas:** 0.7;0.9; 1.0; 1.1;1.3; 1.5 ml/min **temperature program:** different temperature programs	transfer line temp.: 300°C ion source temp.: 230°C quadrupole temp.: 150°C **SIM:** m/z specific for GHB derivatized with TFAA/HFB-OH

II.C.1.2 MATERIALS AND METHODS

II.C.1.2.1 Standards, solvents and reagents

GHB (sodium salt) as a 1-mg/ml solution in methanol, as well as a 1-mg/ml solution in methanol of the internal standard (IS) GHB-d6 (sodium salt) were obtained from Sigma-Aldrich (Steinheim, Germany). The derivatization reagents TFAA and HFB-OH were also purchased from Sigma Aldrich. Methanol, toluene, acetonitrile and ethylacetate, all of suprasolve quality suitable for GC analysis, were delivered by Merck (Darmstadt, Germany). Hexane was purchased from Fluka (Bornem, Belgium).

II.C.1.2.2 Preparation of working solutions

Working solutions of GHB were prepared by appropriate dilution of the 1 mg/ml stock solution to obtain 0.1 mg base/ml methanolic solutions. For the IS, a solution of 0.05 mg base/ml was prepared starting from a 1-mg Na-GHB-d6/ml stock solution in methanol. All solutions were stored at -20 °C.

II.C.1.2.3 Sample preparation

GC-MS method parameters to determine GHB following derivatization with TFAA/HFB-OH were optimized using a working solution with a GHB concentration of 40 µg/ml. One hundred µl of this solution was added to 50 µl plasma, and the samples were stored overnight at 4 °C. Prior to GC

analysis, to complete protein precipitation, acetonitrile was added to the plasma samples (2:1 acetonitrile:sample, v:v), followed by a 10-min centrifugation (*1600 x g*; Mistral MSE 200 BRS, Drogenbos, Belgium). The resulting supernatant was transferred and subsequently evaporated under a gentle stream of nitrogen at 40 °C.

As mentioned above, a procedure based on the one reported by Sabucedo *et al*. (2004) [1] to determine GHB in aqueous samples (drinks) has been adjusted to derivatize GHB: 75 µl of a freshly prepared mixture of TFAA and HFB-OH (2:1) was added to the dried residue. Following thorough vortexing, the sample extract was heated for 30 min at 85 °C in a heating block (Lab-Line, Tier, The Netherlands). Then, the derivatized extract was evaporated after cooling down for 10 min. Finally the residue was dissolved in 100 µl of injection solvent.

II.C.1.2.4 GC-MS conditions

Samples were analyzed on an Agilent HP 6890 GC system coupled to a HP 5973 mass-selective detector (Agilent technologies, Avondale, PA, USA). An Agilent Chem Station, version D.02.00 (G1701DA) was used for data acquisition. The HP 7683 split/splitless injector contained a splitless deactivated inlet liner with glass wool. Splitless injections were performed automatically at an injection temperature of 250 °C and a purge time of 2 min. The injection volume was 1 µl. Helium was used as carrier gas at a constant flow rate of 1.0 ml/min. A 30 m x 0.25 mm i.d. x 0.25 µm Agilent HP-5-MS capillary column was used. The temperature program when using hexane as injection solvent was started at 45 °C for 2 min, ramped at 5 °C/min to 110 °C, then raised by 30 °C/min to 300 °C, which was held for 2 more min for optimal column performance and maintenance, resulting in a total run time of 23.3 min (non-optimized).

The transfer line temperature and MS ion source temperature were set at 280 and 230 °C, respectively. MS quadrupole temperature was set at 150 °C and ionization energy of 70 eV was used. The MS, which operated in electron impact ionization (EI) mode, was first used in full-SCAN mode to obtain a total mass spectrum of derivatized GHB (TIC or total ion chromatogram). Next, for GHB and its internal standard GHB-d6, 1 quantifier and 2 qualifier ions were selected for quantification using the MS in selected ion monitoring mode (SIM). A quantifier ion was chosen as the *m/z* value with the highest abundance in the spectrum, while qualifier ions were selected

based on their selectivity for the analyte of interest [2]. Once the MS operated in SIM mode, 100 µl plasma was spiked with 100 µl of a 40 µg/ml GHB solution prior to analysis.

II.C.1.3 METHOD DEVELOPMENT AND DISCUSSION

II.C.1.3.1 Sample introduction

We opted for a splitless injection, which was required to determine trace levels of GHB in biological matrices. A splitless injection consists of 3 subsequent steps (Fig. II.C.2) [3]. First, the liquid sample is injected into the heated liner, while the split outlet is closed. Secondly, the sample will evaporate in the heated injection port and will be transferred onto the column by mixing with the mobile phase. This is called the splitless period, since the split outlet is still closed. Finally, in a third step, the split outlet is opened to remove what is left from the sample.

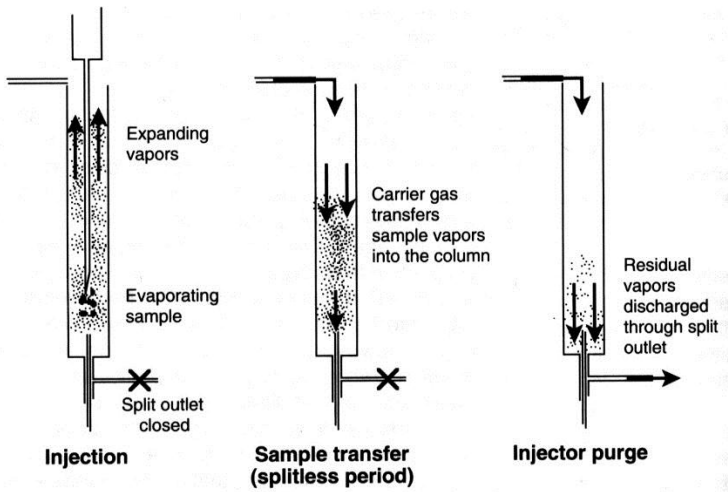


Fig. II.C.2 Principle of splitless injection with opening of the split outlet (Grob, 1993) [3]

II.C.1.3.1.1 Injection solvent

The choice of the injection solvent is based on the start temperature of the GC temperature program and on the best solvation properties for derivatized GHB. In optimum conditions, for low boiling compounds, the temperature should ideally be 20 °C lower than the boiling point of the organic solvent used as injection solvent [3]. This means that for hexane, it should be around 45 °C, for ethylacetate around 60 °C, and for toluene around 85 °C. Toluene was not an option as the

begin temperature of the GC program was too high for derivatized GHB, which eluted too early. We evaluated hexane and ethylacetate, and finally chose ethylacetate, as the highest signals for GHB were observed and the temperature program could start around 60 °C, positively influencing analysis time.

II.C.1.3.1.2 Injection temperature

The injection temperature should be high enough to completely and rapidly evaporate the analytes of interest without degradation [4]. Therefore, injection temperature was varied between 200, 250 and 300 °C (n=3). As shown in Fig. II.C.3, resulting peak areas of GHB were similar with these injection temperatures; therefore, 250 °C was chosen.

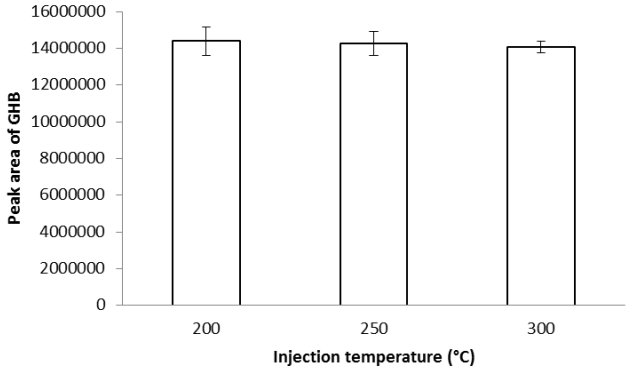


Fig. II.C.3 Influence of injection temperature on GHB signal (mean ± SD, n=3). The dried derivatized extracts were redissolved in 100 µl ethylacetate and the MS operated in SCAN mode.

II.C.1.3.1.3 Purge activation time

The purge activation time is the time point when the split outlet is opened during a splitless injection. If this outlet is opened too early, sample losses will occur; opening of the outlet too late has as a consequence that the solvent peak will be wider and components have more time to degrade in and adsorb onto the liner. The optimal purge activation time depends on the flow rate of the carrier gas. Ideally, the split outlet is opened when approximately 1 to 1.5 liner volumes of carrier gas have passed through the injector [5].

The following equation can be used to calculate the sweep rate or the time when 1 volume of carrier gas has left the injector:

Sweep rate = volume of the liner / F [5]

With volume of the liner (cm^3) = $\pi r^2 L$

F (ml/ min): flow rate

In subsection II.C.1.3.2.2, 1.3 ml/min was chosen as optimal flow rate of helium, while the liner has a length of 8 cm and internal diameter of 0.4 cm. Consequently, the sweep rate was 46 sec. Multiplying it with 1 and 1.5 results in purge activation times of 46 and 70 sec. Therefore, samples were injected using the following purge activation times: 45, 60, 75, 90 and 120 sec (n=3). As shown in Fig. II.C.4, the peak areas are clearly similar for the times tested and 90 sec was chosen since peaks with little or no tailing were seen in the chromatogram with a sufficiently high signal.

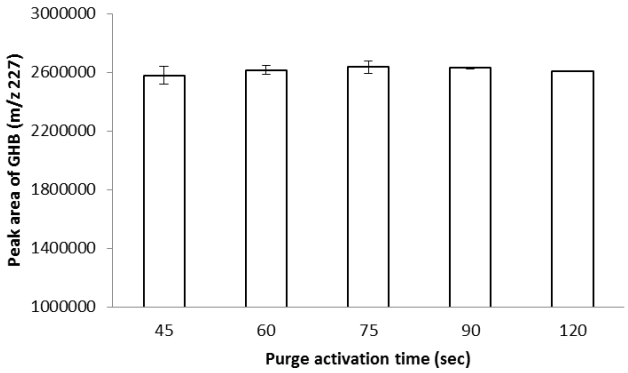


Fig. II.C.4 Influence of varying purge activation times on the peak area of GHB (*m/z* 227; mean ± SD, n=3). The dried derivatized extracts were redissolved in 100 µl ethylacetate.

II.C.1.3.1.4 Pulse time and pulsed splitless injection

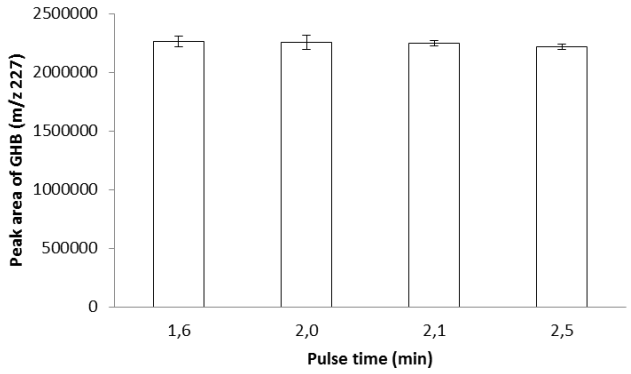
Inlet pressure needs to be high enough to ensure a complete transfer of the sample to the column and to avoid peak broadening. If the inlet pressure is too high, resolution will be decreased. It is possible to increase the inlet pressure during injection, so-called pulse injection, resulting in a narrower initial band on the column and therefore more efficient chromatography. On the other hand, a constant high gas flow during analysis lowers resolution. Therefore, when the injection is

done, the inlet pressure should decrease again to obtain the optimum flow rate to separate the analytes of interest. The time when this pressure is reduced again is called the pulse time and is ideally 0.1 to 0.5 min longer than the purge activation time to prevent the pressure from decreasing while the split line is opened [5].

Since purge activation times of 90 and 120 sec showed little difference in resulting GHB signal and chromatography (Fig. II.C.4), we chose to evaluate 2 pulse times for each purge activation time. Pulse times of 1.6 and 2.0 min were tested for a 90 sec (1.5 min) purge activation time, and 2.1 and 2.5 min for 120 sec (2 min). Fig. II.C.5a shows that there is little difference in mean peak area of GHB for the various pulse times; therefore, we chose to work with 90 sec purge activation time and 2.0 min pulse time.

Starting from these purge activation and pulse times, different inlet pressures were tested: 11.8 psi or the pressure required for 1.3 ml/min flow rate was compared with pulsed splitless injections using 20, 25 or 30 psi during injection. As shown in Fig. II.C.5b, no pulsed splitless injection was required for injection of derivatized GHB. Although at an inlet pressure of 30 psi, GHB eluted earlier, the mean GHB signal was higher when no-pulsed splitless injection was used.

(a)



(b)

Fig. II.C.5

(a) GHB peak area (*m/z* 227; mean ± SD, n=3) in function of the different pulse times tested. The dried derivatized extracts were redissolved in 100 µl ethylacetate.

(b) Comparison of peak area of GHB (*m/z* 227; mean ± SD, n=3) in function of various inlet pressures: non-pulsed (11.8 psi) *vs.* pulsed splitless injection (20, 25, 30 psi).

II.C.1.3.2 Chromatographic separation

II.C.1.3.2.1 Analytical column and temperature program

We chose a capillary column with a stationary phase containing 95 % dimethyl- and 5 % diphenylpolysiloxane, often used in forensics. More specifically, a HP-5-MS column (Agilent, Avondale, USA) of 30 m, 0.25 mm ID, 0.25 µm film thickness was used, with acceptable retention (time) and peak shape for GHB and its internal standard GHB-d6. Furthermore, various temperature programs were tested and the final choice was based on peak shape, run time, injection solvent and selectivity. As mentioned above, DBS extracts were injected and the final, optimized temperature program started at 65 °C for 1.5 min, ramped at 10 °C/min to 110 °C, then at 50 °C/min to 300 °C, which was held for 2 more min.

II.C.1.3.2.2 Mobile phase and flow rate

The type of carrier gas passing through the analytical column with a given velocity influences resolution and retention times. Helium was used as carrier gas, an inert carrier gas typically used in GC. The optimum flow rate of the carrier gas depends on type of column used (length, diameter and film thickness) as well as on the volatility of the analytes of interest. Optimization of this parameter aims for an acceptable resolution in the shortest analysis time possible. The flow rate stands for the volume of gas that flows through the column per time unit and the optimum flow rate is predicted using the linear gas velocity (u). The latter is the velocity by which the carrier gas flows through the column [5].

When using a 30 m x 0.25 mm x 0.25 µm column, the optimum linear velocity is 30 to 40 cm/ sec. These linear velocities correspond to flow rates of 0.88 (0.7) to 1.18 (1.5) ml/ min. In addition, combining GC separation with MS detection limits the flow rate to 1 to 2 ml/ min. So, flow rates varying from 0.7 to 1.5 ml/ min were tested for derivatized GHB (n=3) and Fig. II.C.6 shows the mean peak area of GHB (mean ± SD, n=3) in function of the various flow rates. Besides the peak area, resolution and peak shape were also evaluated. Little differences in peak area were seen, so we chose a 1.3 ml/min flow rate.

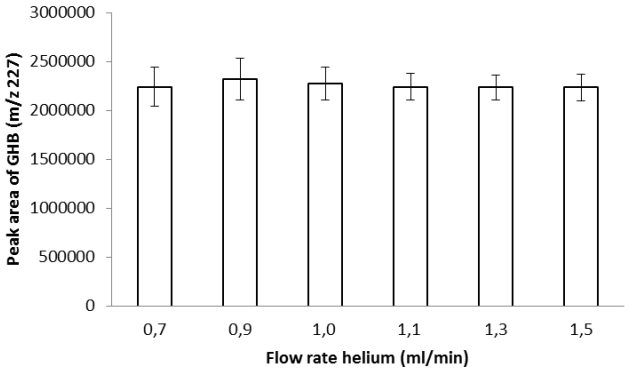


Fig. II.C.6 Peak area of GHB (*m/z* 227; mean ± SD, n=3) in function of various flow rates of the carrier gas helium

II.C.1.3.3 MS detection

II.C.1.3.3.1 Full-scan monitoring

Analyzing a standard solution (with a concentration of 100 µg/ml) of GHB and its internal standard GHB-d6 in full scan (*m/z* varying from 35 to 400) resulted in a chromatogram and for each peak, a mass spectrum could be reconstructed. Fig. II.C.7 gives the resulting mass spectrum for GHB following derivatization with TFAA/HFB-OH (using the non-optimized GC temperature program).

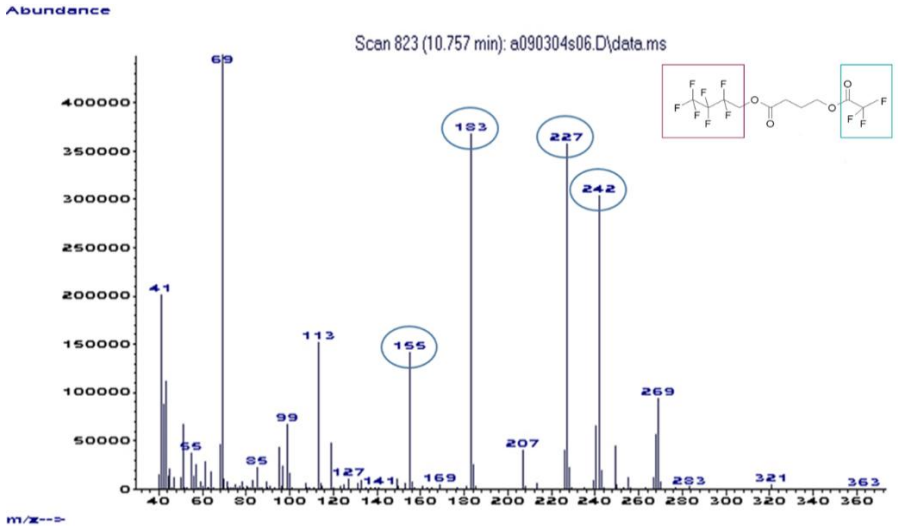


Fig. II.C.7 Full-scan mass spectrum of GHB derivatized with TFAA/HFB-OH

II.C.1.3.3.2 SIM-monitoring

If an MS operates in SIM-mode, one or a selected group of *m/z* ions will reach the detector and be registered. Since the MS spends more time per *m/z* value, SIM-mode results in better detector sensitivity than when the MS operates in scan. Which *m/z* ions should be registered depends on their intensity in the full-scan mass spectrum and on their value. Intensity should be high enough to obtain sufficient sensitivity, while higher *m/z* ions are more selective for the compound of interest, since the chance of occurrence of these *m/z* values in co-eluting analytes decreases when their value increases [6].

From the mass spectra obtained in scan-mode (Fig. II.C.7), 1 quantifier and 3 qualifier ions were selected for GHB and GHB-d6. The quantifier ion will be used for quantification, while the qualifier ions should result with high certainty from fragmentation of the analyte of interest [2]. The following ions were monitored in the SIM-mode: *m/z* 155, 183, <u>227</u> and 242 for derivatized GHB and *m/z* 161, 189, <u>231</u> and 245 for derivatized GHB-d6 (underscored ions represent the quantifier ions, as they had high abundance, the other ions were selected as qualifier ions). Fig. II.C.8 gives the proposed fragments corresponding to *m/z* 155, 183 and 242. Formation of the fragment with *m/z* 242 can be explained by McLafferty rearrangement. The fragmentation leading to *m/z* 227 (and the identity of the resulting fragment) could not be determined. Sabucedo *et al*. [1] proposed the following fragment $[CF_3\text{-}CF_2\text{-}CF_2\text{-}CH_2\text{-}O\text{-}CO]^+$. Although this fragment has indeed a *m/z* of 227, it does not correspond to the *m/z* value of 231 of the corresponding fragment observed by us resulting from the fragmentation of GHB-d6. Fig. II.C.9 shows a representative chromatogram of a DBS extract analyzed with the optimized GC-MS method (using the final temperature program, see II.C.1.3.2.1).

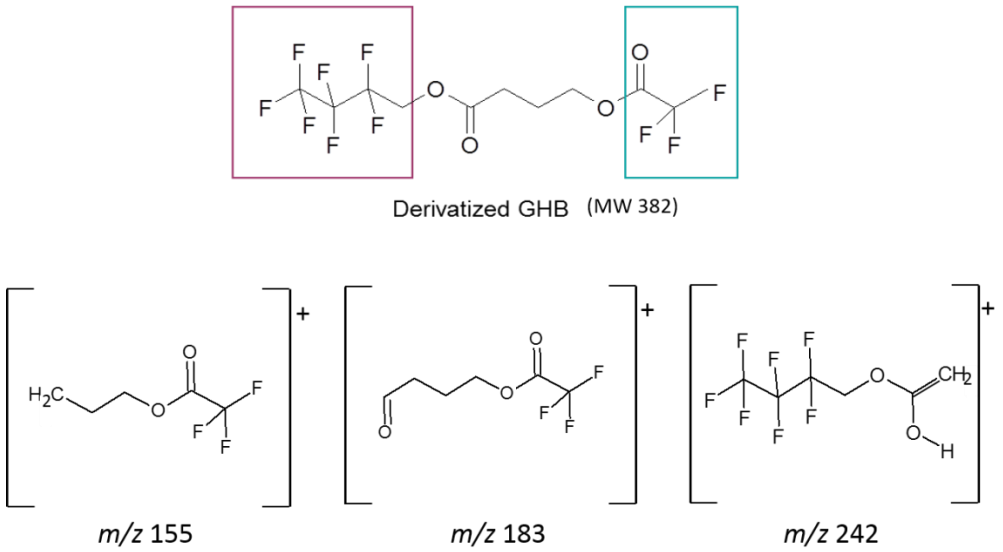


Fig. II.C.8 Proposed fragments of GHB derivatized with TFAA/HFB-OH

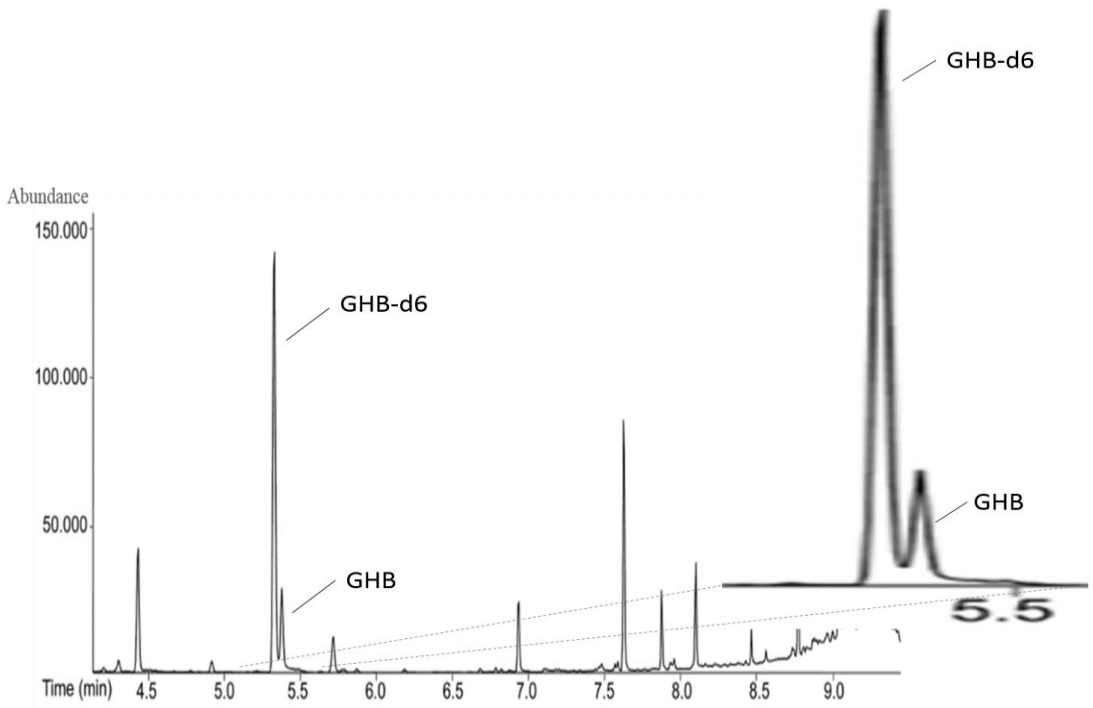


Fig. II.C.9 Representative chromatogram of a 50-µl DBS spiked with GHB at 2 µg/ml and GHB-d6 at 10 µg/ml, analyzed with GC-MS in EI-SIM mode using the optimized GC-MS parameters

II.C.2 DETERMINATION OF GHB IN 50-µL DBS

*(**Based on** Determination of gamma-hydroxybutyric acid in dried blood spots using "on spot" derivatization and a simple GC-MS method. Anal Bioanal Chem. 398:2173-2182 (2010). Ann-Sofie M.E. Ingels, Willy E. Lambert, and Christophe P. Stove.)*

II.C.2.1 INTRODUCTION

Gamma-hydroxybutyric acid (GHB) as well as its precursors gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) are popular as a party or club drug ("liquid ecstasy") and appear occasionally in drug-facilitated sexual assaults (DFSA). This is due to their effects and chemical properties as they are colourless and odourless liquids which can be easily mixed with other liquids [7-10]. GHB is mostly misused in combination with other drugs, such as alcohol, ecstasy (MDMA), cocaine, amphetamines and cannabis [8,9,11]. Most commonly reported effects of GHB abuse are euphoria, increased sexuality, well-being and tranquillity, while an overdose results in seizures, respiratory depression, coma and sometimes even death [11-13]. The range between the desired effects and an overdose is narrow, frequently resulting in (non-) fatal intoxications in humans, as described in several case reports [14-20]. Consequently, the identification and correct quantification of GHB is important in forensic and clinical toxicology. Many previously reported analytical methods detect GHB in different biological matrices and involve the use of gas chromatography (GC) [1,2,21], but also liquid chromatography (LC) [22,23] and capillary zone electrophoresis (CZE) [24]. Also the determination of GHB through headspace solid phase micro extraction (SPME) and dynamic extraction (SPDE) has recently been published [25,26].

However, the determination of GHB in biological matrices remains an analytical challenge for several reasons. First, GHB is a small polar molecule, making its extraction and the direct detection with GC difficult. Hence, two approaches are generally used: besides the conversion of GHB under acidic conditions to GBL, which is more easily extracted from the biological matrix, the carboxyl and hydroxyl group can be derivatized, resulting in a more volatile and less polar compound. As derivatization technique, silylation is mostly used, but also alkylation and acylation have previously been described [1,21]. Furthermore, GHB occurs naturally in blood and urine, so no blank matrix is available and positive samples must be carefully interpreted. To enable the differentiation between exogenously-administered and endogenous GHB, cut-off levels have been proposed by several authors. These are currently set at 4 or 5 µg/ml for blood samples and 6 or 10 µg/ml for

urine samples [27-30]. In addition, once orally ingested, GHB is rapidly metabolized, with a reported plasma half-life of less than one hour. As this limits the detection window, plasma samples must be taken within 6 hours and urine samples within 12 hours (or better both within 6 hours) after ingestion [14].

In this study, we use dried blood spots (DBS) to improve the detection and quantification of GHB by facilitating sample collection. A DBS is capillary whole blood obtained by a finger or heel prick and collected on a filter paper. This sampling technique ensures an easy and rapid collection of a representative sample without specific handling and storage requirements. These advantages as to a venepuncture make it a cost-effective choice for the collection, transport and storage of blood samples [31,32]. The DBS sampling method, originally used in newborn screening for metabolic disorders, is currently applied for the determination of various compounds such as biomarkers and is promoted for therapeutic drug monitoring (TDM). Already several compounds can be detected in DBS, most common are anti-malaria drugs, immunosuppressive drugs, anti-epileptics, antibiotics and anti-diabetics [32]. Inherent to DBS sampling is the small sample volume available, ranging from 10 to 200 µl, comparing to 1 ml or more obtained by a venepuncture. Although this may be an advantage when the collection of larger amounts of whole blood is limited, such as in neonates and children, these small amounts may impose an analytical challenge, requiring an efficient sample pre-treatment and a sensitive detection [33,34].

The determination of GHB in DBS may be interesting in situations where there is a suspicion of illicit use of GHB or one of its precursors, for example in case of driving under the influence of drugs (DUID) or a presumed DFSA. As mentioned above, the short half-life of GHB implies a limited detection window and, consequently, a rapid collection to obtain a representative sample. A delay caused by the need for a venepuncture by medical staff may bring the blood levels of GHB under the established cut-off levels. Moreover, no extraction step is necessary as DBS can be directly derivatized, minimizing the sample preparation and reducing the sample turn-around time. Besides the advantages of a rapid collection, also storage of whole blood samples as DBS may be of interest as this may avoid *in vitro* formation of GHB, which has previously been reported [29,35,36].

Only recently, an LC-MS/MS-based method has been reported to screen for elevated GHB concentrations in DBS obtained from newborns. This method was developed to detect the presence of a rare inherited metabolic disorder, i.e. succinic semi-aldehyde dehydrogenase (SSADH) deficiency [37]. Even though most DBS analyses are commonly performed using LC-MS/MS, we opted to use GC-MS for several reasons. First of all, GC is a common and available technique in forensic laboratories, proven to be suitable for the detection of derivatized GHB with good sensitivity [21]. Secondly, given the low molecular weight of GHB, adequate and sensitive detection following liquid chromatography requires extensive sample pre-treatment, involving an extraction step and/or derivatization to enhance selectivity. The aim of our study was to develop and validate a GC-MS method for the identification and quantification of GHB in DBS, based on a new procedure, involving direct derivatization of GHB "on spot", ensuring a minimal, economic and less time-consuming sample pre-treatment.

II.C.2.2 MATERIALS AND METHODS

II.C.2.2.1 Reagents

II.C.2.2.1.1 Chemicals

GHB (sodium salt) as powder and as a 1-mg/ml solution in methanol, as well as a 1-mg/ml solution in methanol of the internal standard (IS) GHB-d6 (sodium salt) were obtained from LGC standards (Molsheim, France). The derivatization reagents trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB-OH) were purchased from Sigma Aldrich (Steinheim, Germany). Methanol and ethylacetate, both of suprasolve quality suitable for GC analysis, were delivered by Merck (Darmstadt, Germany).

II.C.2.2.1.2 Stock and working solutions

Stock and working solutions of GHB were prepared by dissolving 10 mg of the base in 1 ml of methanol, followed by appropriate dilution to obtain 1 and 0.1 mg base/ml methanolic solutions. Quality controls (QC's) for all analyses were obtained from the commercially available stock solution of 1 mg Na-GHB/ml methanol. For the IS, a solution of 0.05 mg base/ml was prepared starting from a 1-mg Na-GHB-d6/ml stock solution in methanol. All solutions were stored at -20 °C.

II.C.2.2.2 Materials

Protein saver cards number 903, used as sampling paper, were kindly provided by Whatman (ref n° WHA10334885, Dassel, Germany). The automatic lancets for capillary blood collection were purchased from Becton Dickinson (ref n° VAC366594, Franklin Lakes, USA), while the 50-µl precision capillaries were obtained from Servo-Prax (Wesel, Germany) (Fig. II.C.10a). The centrifuges used were a MSE Mistral 2000 (Beun de Ronde Serlabo, Anderlecht, Belgium) and a 5804R Eppendorf (Hamburg, Germany). Evaporation under nitrogen was conducted in a TurboVap LV evaporator from Zymark (Hopkinton, MA, USA).

II.C.2.2.3 DBS sample collection

Two methods are commonly used to obtain capillary whole blood on a filter paper. The drop of blood can be collected directly on the filter paper or with the aid of a precision capillary (Fig. II.C.10a). In this study, we opted to apply a drop with a fixed volume onto the paper, similar to the application with a precision capillary. As the complete drop can be excised instead of punching out a disk from it, a fixed sample volume is analyzed and the effect of hematocrit and sampling technique is minimized [32].

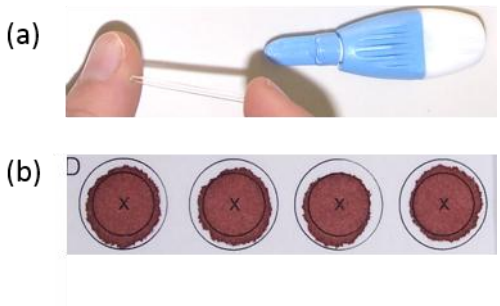


Fig.II.C.10

(a) A 50-µl precision capillary (left) used to collect capillary whole blood obtained by a fingerprick using an automatic lancet (right)

(b) Examples of 50-µl DBS spotted on Whatman 903 filter paper. The inner circle must be enterily filled with blood, the outer circle line was used for excision of the DBS.

In the procedure to obtain a DBS, the hand is first cleaned and held down or warmed for a few minutes. With the help of an automatic lancet, the fingertip is pricked. While the first drop is wiped off with a sterile piece of cloth because of the presence of tissue fluid, the following drops are collected in a 50-µl precision capillary. Then, once completely filled, the entire capillary is placed in the centre of two concentric circles pre-printed on a Whatman 903 filter paper (Fig. II.C.10b). The inner circle (10 mm diameter) must be entirely filled with blood, but blood may not pass the outer circle line (15 mm diameter), which was used for excision of the DBS. Although the blood is spot on just one side, both sides of the filter paper must be coloured. After visual inspection of the DBS, the analyses can start [32].

For method development and validation, we used 50-µl spots of venous whole blood from healthy non-user volunteers with endogenous GHB concentration below the established lower limit of quantification (LLOQ) and preserved for maximum one week at 4 °C. The DBS are dried for minimum 4 hours at ambient temperature and subsequently analyzed or preserved in a sealable plastic bag at room temperature or -20 °C until analysis.

II.C.2.2.4 Optimization of the DBS sample preparation

First, 10 µl of the IS solution was applied to a DBS and left to dry for 15 min. Subsequently, the spot was completely excised following the outer circle line, placed in a test tube and a freshly prepared mixture of TFAA and HFB-OH was added, followed by sonication for 5 min. Sabucedo and Furton [1] described the use of 1 ml of this mixture to derivatize GHB in aqueous samples (drinks) at 85 °C for 30 min, while we desired the derivatization of GHB in a biological matrix. For this purpose, the main critical parameters for derivatization, such as amount of reagent, temperature and time, were thoroughly evaluated. So, different amounts of derivatization mixture were tested, respectively 75, 100 and 125 µl of a TFAA/HFB-OH (2:1) mixture. Also derivatization temperatures varying from room temperature to 60, 70, 85 and 100 °C and derivatization times ranging from 5 to 30 min were tested. For each condition, at least 3 DBS spiked with GHB at a 10 µg/ ml concentration level were analyzed and the resulting absolute peak areas of derivatized GHB were compared.

After the derivatization step, the DBS was cooled down by centrifugation for 5 min at 4 °C and evaporated to dryness under a gentle stream of nitrogen at 25 °C to remove excess of

derivatization reagent. The dried sample was redissolved in 200 µl of ethylacetate, sonicated and centrifuged for 5 min at 1600 *x g*. Eighty-five µl was transferred, centrifuged again and finally 50 µl was transferred to a vial, of which 1 µl was injected into the GC-MS.

II.C.2.2.5 The analytical procedure

Samples were analyzed on an Agilent 6890 GC system coupled to a 5973 mass-selective detector. Splitless injections were performed automatically at an injection temperature of 250 °C, a purge time of 1.5 min and helium was used as carrier gas at a constant flow rate of 1.3 ml/min. A 30 m x 0.25 mm i.d. x 0.25 µm Varian VF-5-MS column (Varian; Middelburg, The Netherlands) was used. The temperature program was started at 60 °C for 1.5 min, ramped at 10 °C/min to 110 °C, then raised by 50 °C/min to 300 °C, which was held for 2 more min for optimal column performance and maintenance, resulting in a total run time of 12.3 min. The transfer line temperature and ion source temperature were set at 280 and 230 °C, respectively. MS quadrupole temperature was set at 150 °C and ionization energy of 70 eV was used. The mass spectrometer operated in the selected ion monitoring (SIM) mode using electron impact ionization (EI) for quantification of GHB and GHB-d6. By running standards in full scan, typical mass spectra were obtained and following ions were monitored in the SIM mode: *m/z* 155, 183, 227 and 242 for derivatized GHB and *m/z* 161, 189, 231 and 245 for derivatized GHB-d6 (underscored ions represent the quantifier ions, as they had the highest abundance, the other ions were selected as qualifier ions).

II.C.2.2.6 Validation

The following criteria were evaluated to validate the method: linearity, precision, accuracy, sensitivity, selectivity and stability [38-40].

II.C.2.2.6.1 Linearity, precision, accuracy and sensitivity

As no blank matrix is available, fresh venous human whole blood withdrawn on EDTA as anti-coagulant was used to prepare the calibration standard solutions. The concentration of endogenous GHB was tested to be below the LOQ of the method (less than 2 µg/ml) and the ratio GHB to GHB-d6 was always lowered with the mean ratio of the zero samples (blank whole blood + IS, in duplicate) measured on each calibration day.

A 5-point calibration curve was constructed six times on six different days. Each day a blank, 2, 10, 25, 50 and 100 µg/ml solution of GHB in whole blood was prepared, as well as the QC solutions at

low (2 µg/ml), medium (10 µg/ml) and high (100 µg/ml) concentration level, covering the whole calibration range. From each solution, 50-µl spots were made, left to dry at room temperature for minimum 4 h, followed by analysis. The resulting data were statistically evaluated and weighted if necessary, based on the sum % relative error (RE) and the % RE plot versus concentration, where % RE is the concentration found lowered with the nominal concentration, divided by the nominal concentration and multiplied by 100. Linearity was assessed by performing the Fisher-test [38,41].

Intra-batch-precision was assessed by replicate analysis of spiked samples (n=5 at low, medium and high concentration level) in a single day, inter-batch-precision was evaluated by determination of spiked samples per concentration on 6 days. Precision was measured by calculating the relative standard deviation (RSD, SD divided by the mean and multiplied by 100 %). Also accuracy was determined for each concentration level, calculated by the percent deviation from the nominal concentration (presented as % bias).

For evaluation of sensitivity, the limit of detection (LOD) and the limits of quantification (LLOQ and ULOQ) were determined. The LOD was estimated as the minimum concentration of GHB with a signal-to-noise ratio equally or larger than 3, so with reliable differentiation of the background noise and in compliance with the identification criteria [41,42]. The lowest and highest concentration of GHB still measured with acceptable precision (RSD less than 20 % for LLOQ, 15 % for ULOQ) and accuracy (80-120 % for LLOQ, 85-115 % for ULOQ) were chosen as the LLOQ and ULOQ, respectively.

Furthermore, possible dilution of the final extract of samples above the ULOQ was investigated. Therefore, human whole blood was spiked at 200 µg/ml (twice the ULOQ) and 50-µl spots were processed as described above (n=2 x 3). Ten µl of the final derivatized extract was diluted to 100 µl with ethylacetate, analyzed by GC-MS and corrected for the dilution factor. The RSD was calculated using one-way-ANOVA as described by Wille *et al*., and needed to be < 15 % [43]. Accuracy needed to be within 85 to 115 % of the nominal value. Carry-over was also tested by injecting the highest concentration level of the calibration curve, followed by 3 blank (ethylacetate) injections.

II.C.2.2.6.2 Selectivity

The possible contribution of the isotopically labelled IS was assessed by analyzing both blank and zero (blank spiked with IS) samples. Furthermore, we investigated the possible interference of structural analogues such as beta-hydroxybutyric acid (BHB), alpha-hydroxybutyric acid (AHB) and GABA and the precursors GBL and 1,4-BD (spiked at 100 µg/ml); as well as the mainly co-ingested club and date-rape drugs (alcohol, cocaine, benzoylecgonine, Δ9-tetra-hydrocannabinol (THC), 11-nor-9-carboxy-THC, ketamine, flunitrazepam, MDMA and amphetamine, spiked at or well above concentrations typically found in abusers). At least six different sources of whole blood were spiked and analyzed, in order to compare the ratio of GHB to GHB-d6 of blank samples, spiked at a known GHB concentration, with that of the samples containing the interferences.

II.C.2.2.6.3 Stability

As the filter paper matrix is expected to stabilize most analytes in DBS, we evaluated the stability of GHB in DBS stored at different conditions [31]. Therefore, three separate solutions in whole blood at both low (5 µg/ml) and high (100 µg/ml) concentration levels were prepared and the resulting spots were preserved in a sealable plastic bag at room temperature for one week, at 4 °C for 24 h and at -20 °C for 14 days. For the first 48 h at room temperature, DBS were analyzed after 2, 4, 8, 12, 24 and 48 h to assess the influence of drying time. The mean percentage found needs to be within 85 to 115 % of the results obtained when analyzed after 4 h of drying, as this is the minimum drying time recommended by the manufacturer and therefore chosen as the reference time point.

To assess the stability of the processed samples, extracts (low and high concentration level, in triplicate) were re-injected after 24 h at room temperature and after storage for minimum one week at -20 °C (per concentration level, in duplicate). The ratio of GHB to GHB-d6 was compared to this of the directly analyzed extract. Stability of stock solutions at two concentration levels (n=3) was assessed over 14 days at -20 °C and after three freeze-thaw cycles.

II.C.2.2.7 Application

The described procedure was applied to blood samples of two young men suspected of GHB/GBL-intake, along with other drugs, to evaluate the routine applicability and the easiness of the method in a laboratory setting. The two young men were found on the street under influence, one was in critical condition, and were brought to a nearby hospital. Blood and urine samples were taken and

sent to our laboratory for analysis of drugs and alcohol. As soon as the blood samples arrived at the laboratory, DBS were prepared and stored until analysis. Along with the DBS analysis, the blood samples were also subjected to routine toxicological analysis, involving screening by immuno- and enzymatic assays and confirmation of positive results by high performance liquid chromatography-diode array detection (HPLC-DAD) and GC-MS.

II.C.2.3 RESULTS AND DISCUSSION

II.C.2.3.1 Optimization of the DBS sample preparation

Inherent to DBS sampling is the small sample volume available, necessitating an efficient sample preparation [33]. Therefore, different procedures were tested and compared to obtain the optimal conditions of extraction and derivatization of GHB, in order to work as sensitive as possible in an easy and time-saving way. First, the derivatization reaction was optimized. The influence of the amount of the TFAA/HFB-OH (2:1) mixture was evaluated. As shown in Fig. II.C.11a, the optimal amount added was 100 µl of the freshly prepared mixture. Then, different derivatization temperatures were evaluated. Fig. II.C.11b shows the resulting GHB peak area (mean ± SD) in function of the derivatization temperature, and 60 °C was chosen as optimum temperature to derivatize GHB to its corresponding derivative. Next, the time needed to perform the reaction was evaluated. Fig. II.C.11c gives the peak area of GHB in function of the derivatization times, and 10 min was chosen.

Optimization of the sample preparation resulted in the set-up of a quick and efficient protocol. Direct derivatization took place by adding 100 µl of a freshly prepared mixture of TFAA and HFB-OH (2:1) to a test tube containing the excised DBS. Then, the test tube was sonicated for 5 min, ensuring the distribution of the derivatization reagent [44] and placed in a heating block at 60 °C for 10 min. After cooling down, the sample was dried under a gentle stream of nitrogen at 25 °C. Next, the DBS was re-dissolved in 200 µl of ethylacetate, sonicated and centrifuged two subsequent times, followed by transfer of an aliquot of the supernatant to a vial, of which 1 µl was injected into the GC-MS. The overall procedure from receipt of a DBS to a quantitative result can be completed in less than 2 h.

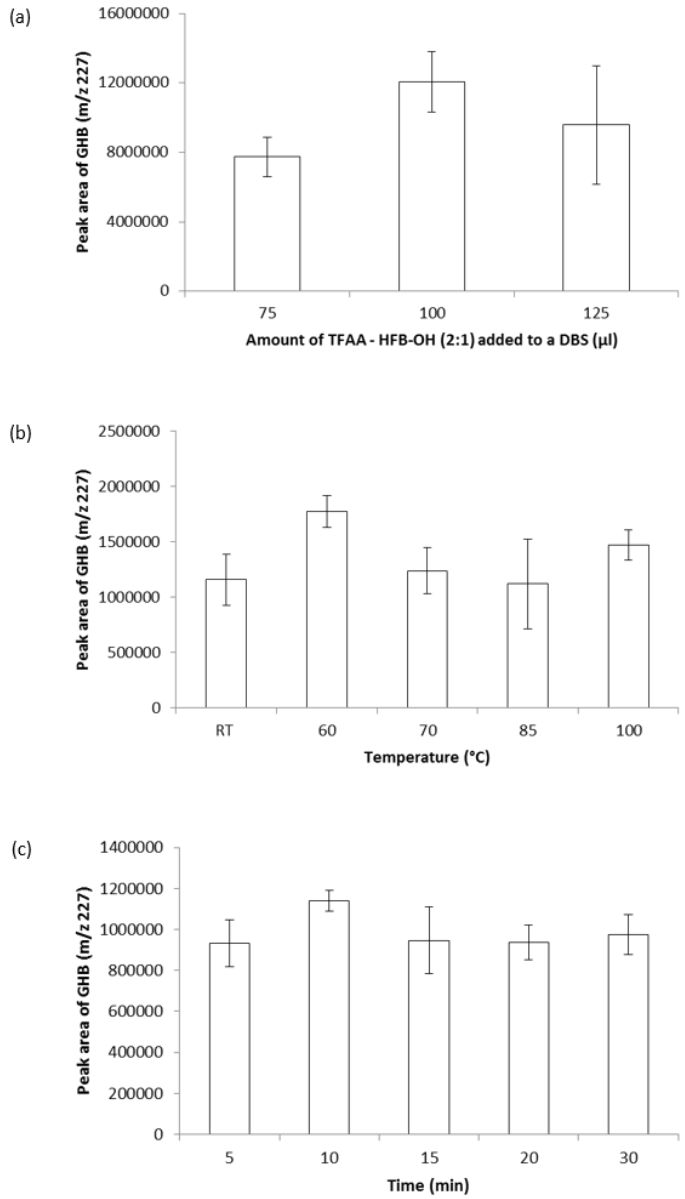


Fig. II.C.11 Optimization of the derivatization reaction using a freshly prepared mixture of TFAA/HFB-OH (2:1)

(a) Influence of amount of the reagents on the area of derivatized GHB. DBS from venous whole blood were spiked with GHB (10 µl 0.5 mg/ml) and derivatized with TFAA/HFB-OH (2:1) at 85°C for 15 min; n=5 (mean ± SD).

(b) Influence of temperature on the area of derivatized GHB. DBS from venous whole blood were spiked with GHB (10 µl 0.05 mg/ml) and derivatized with 100 µl TFAA/HFB-OH (2:1) for 15 min; n=5 (mean ± SD).

(c) Influence of derivatization time on the area of derivatized GHB. DBS from venous whole blood were spiked with GHB (10 µl 0.05 mg/ml) and derivatized with 100 µl TFAA/HFB-OH (2:1) at 60 °C; n=3 (mean ± SD).

As the polarity and the low molecular weight of GHB requires derivatization or conversion to GBL prior to GC analysis, we chose direct derivatization instead of conversion, thus avoiding extraction and resulting in an easier, less time-consuming sample preparation. Apart from a method previously published by our group, only one other published method utilizes direct derivatization of GHB [1,2]. However, both methods have as a major drawback that they require a considerably larger amount of derivatization reagent, around 1 ml for each sample, while here only 100 µl is needed, contributing to a more economic and environmentally-friendly procedure. Déglon *et al*. recently published a method for the determination of antidepressants in DBS, with simultaneous extraction using 500 µl 0.02 % triethylamine in butyl chloride in combination with direct derivatization using 100 µl derivatization reagents [45]. However, to the best of our knowledge, our procedure is the first where derivatization reagents are applied directly “on spot” without the use of any extraction solvent.

II.C.2.3.2 Analytical procedure and validation

The optimized conditions for sample pre-treatment (heating the excised DBS for 10 min at 60 °C with 100 µl of a freshly prepared mixture of TFAA and HFB-OH (2:1), followed by drying and redissolving in 200 µl ethylacetate, see section II.C.2.3.1), and analysis by GC-MS (as described in section II.C.2.2.5) were carried out for validation of the method.

II.C.2.3.2.1 Linearity, precision, accuracy and sensitivity

To assess linearity, a blank, two zero (blank whole blood + IS) and 5 calibration samples were analyzed on six different days. Representative chromatograms of the injection of a derivatized extract of a blank DBS and a DBS containing GHB in a concentration at the lower end of the calibration curve (LLOQ) are shown in Fig. II.C.12a and II.C.12b, respectively.

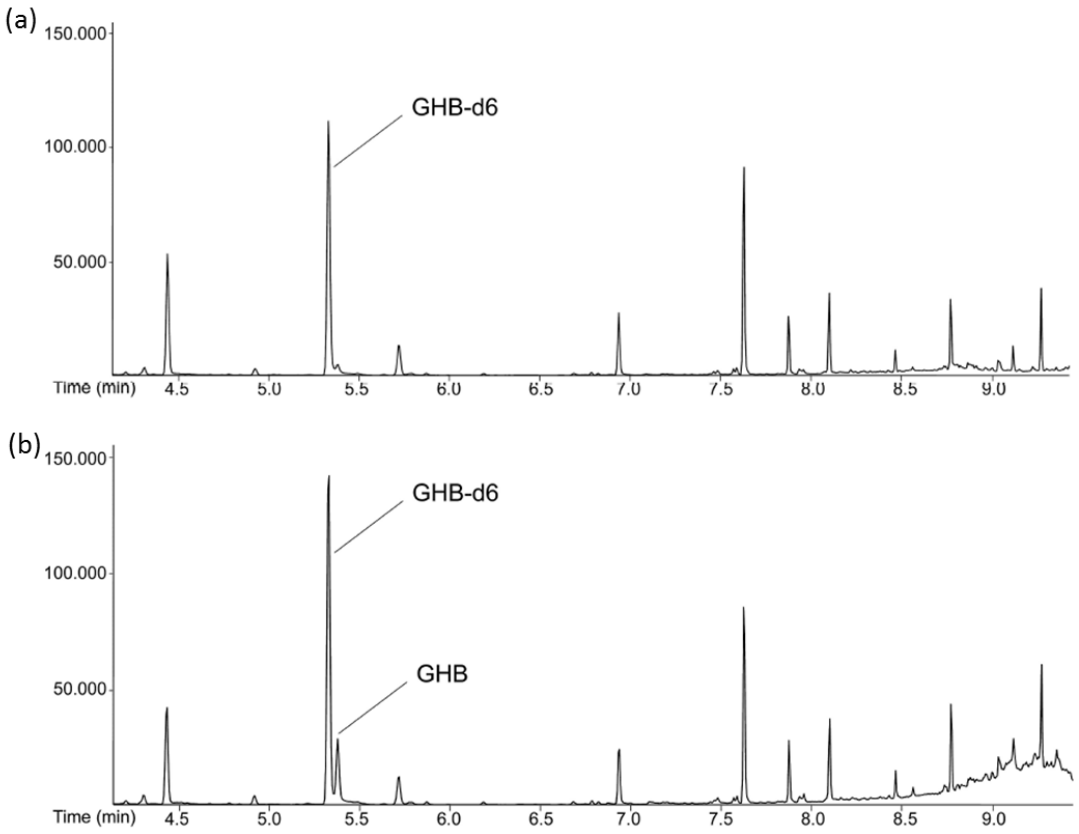


Fig. II.C.12 Representative chromatograms analyzed with GC-MS in EI-SIM mode of (**a**) a blank DBS spiked with IS at 10 µg/ml (b) a DBS spiked at LLOQ, 2 µg/ml GHB, and IS at 10 µg/ml

Table II.C.2a summarizes the calibration and sensitivity data. Ratios of GHB to GHB-d6 were calculated by dividing the peak area of GHB by the peak area of GHB-d6 and were lowered with the mean ratio of the zero samples. Results were statistically evaluated and a weighting factor $1/x^2$ was applied to the linear calibration curves. Also, the zero value was included in the 95 % confidence interval of the y-intercept, indicating absence of a constant error. As indicated in Table II.C.2b, overall intra- and inter-batch precision and accuracy were below 15 % and below 20 % at LLOQ level.

To evaluate the ability to dilute samples with a GHB concentration above the ULOQ, replicate DBS of a 200 µg/ml solution were analyzed as described in the materials and methods section (II.C.2.2).

Following a 10-fold dilution of the derivatized extract, the concentration was back-calculated using daily calibration curves, and precision and accuracy were found to be within the acceptance limits of 15 % (Table II.C.2b). No carry-over was seen following injection of a sample with GHB spiked at ULOQ level (less than 0.085 %).

Table II.C.2 Validation data for the determination of GHB in DBS using GC-MS

(a) Calibration and sensitivity data

	Slope Mean ±SD (95 % CI) (n=6)	**Intercept** Mean ±SD (95 % CI) (n=6)	**Working range** (µg/ml)	**LLOQ** (µg/ml)	**R^2** (n=6)	**Weighting factor**
50-µl DBS	0.087 ± 0.004 (0.083; 0.090)	-0.012 ± 0.03 (-0.036; 0.011)	2-100	2.0	1.000	$1/x^2$

(b) Intra- and interbatch precision (calculated as RSD %) and accuracy (calculated as % deviation from the nominal concentration) for QC low (2 µg/ml), mid (10 µg/ml) and high (100 µg/ml)

	Nominal GHB concentration (µg/ml)	**Measured GHB concentration (µg/ml)**	**Intra-batch precision (% RSD, n=5)**	**Inter-batch precision (%RSD, n=6)**	**Accuracy (Bias %)**
50-µl DBS					
QC low	2	2.14	5.1	16.1*	7.1
QC mid	10	9.49	6.0	9.1	-5.1
QC high	100	108.87	8.1	5.5	8.9
2 x ULOQ	200	198.00	8.2	9.6	-1.0

* n=5, elimination of 1 outlier based on Grubs test for outliers

II.C.2.3.2.2 Selectivity

The fact that preliminary studies indicated a small interference at the retention time of GHB by injection of a derivatized extract of a blank filter paper, combined with the endogenous presence of GHB in whole blood, rendered it impossible to prove lack of response of the blank matrix. However, as the paper signal and the signal of the used whole blood had a peak area below 10 and 20 % of that of the LLOQ, respectively, no unacceptable interferences were seen [40]. No unacceptable interferences were seen at the retention time of the IS either. When analyzing both

blank and zero samples (the latter corresponding to blank + IS), no significant difference was seen in the mean response of GHB. To further evaluate selectivity, six different sources of whole blood were spiked with a combination of structure-analogues of GHB, and certain club and date-rape drugs, with no interferences being observed.

Not unexpectedly, the presence of a high concentration of GBL in the sample resulted in an increase of the GHB signal, likely due to conversion during sample preparation. The inter-conversion between GBL and GHB is well-known and has extensively been investigated. It can be influenced by pH, temperature and time [22,46-48]. Upon spiking increasing concentrations of GBL to whole blood, followed by analyzing the resulting DBS, we saw an approximate 10 % conversion of GBL to GHB, independent from the GBL concentration. This result is consistent with that reported by Sabucedo and Furton [1], who found a 6.5 % conversion.

Because upon ingestion, GBL is metabolized to GHB by serum lactonases within minutes in humans, normally no or only minimal amounts of GBL will be present in a blood sample. However, possible saturation of serum lactonases following ingestion of larger amounts of GBL has been reported, although this still remains unclear [20]. Anyway, cases in which GBL ingestion has occurred will likely readily have high GHB blood levels and although the ± 10 % conversion of GBL to GHB may somewhat falsely elevate the quantitative result of GHB, this will likely have no relevance, neither in the forensic context, nor in the clinical setting [20,49].

II.C.2.3.2.3 Stability

The stability of GHB in DBS was thoroughly investigated as previous studies reported an increase in GHB concentration during preservation of blood samples withdrawn on certain anti-coagulants [29,36]. So, to investigate the short-term stability of GHB in DBS samples on Whatman 903 filter paper, we evaluated low and high concentration levels (n=3) at different preservation temperatures (room temperature, 4 and -20 °C). Table II.C.3 shows that GHB in DBS at both low and high concentration levels is stable when stored for at least one week at room temperature, 24 h at 4 °C and 14 days at -20 °C, as the mean percentages were within the pre-defined 15 % limits.

Based on these results, the collection of blood containing GHB on filter paper may result in a better storage manner of blood samples. Further examination is recommended to evaluate

whether DBS are indeed an alternative and reliable way for routinely storing suspicious blood samples over a longer period of time. Furthermore, although in this study the DBS were dried for minimum 4 hours at room temperature before analysis, in compliance with the manufacturer’s recommendations, a shorter drying time of 2 hours is also possible if needed.

Table II.C.3 Stability data of GHB in DBS, stored at room temperature up to 7 days, at 4 °C up to 24 h and at -20 °C up to 14 days, presented as the percentage of the results obtained when analyzed immediately after 4 h of drying, which is the recommended drying time according to the manufacturer

Room temperature			4°C			-20°C		
	Low	High		Low	High		Low	High
Time	n=3; mean ±SD		Time	n=3; mean ±SD		Time	n=3; mean ±SD	
2 h	98.2 ± 5.8	101.0 ± 12.0	24 h	97.2 ± 6.8	106.1 ± 13.8	24 h	94.1 ± 3.9	101.2 ± 9.2
8 h	95.2 ± 7.1	107.0 ± 9.2				7 days	99.1 ± 5.4	104.4 ± 3.5
12 h	96.4 ± 4.0	107.3 ± 10.8				14 days	99.8 ± 7.2	112.6 ± 5.1
24 h	95.1 ± 4.6	105.4 ± 5.2						
48 h	98.1 ± 7.7	102.8 ± 2.9						
7 days	95.5 ± 2.6	101.9 ± 6.3						

Re-injection of processed samples after a waiting period of 24 h to verify autosampler stability of the derivatized extracts proved to be no problem as nearly no differences in peak area ratio were seen (RSD < 2.1 %). Also the stability of processed samples stored for one week at -20 °C was acceptable (RSD < 2.2 %). Stock solutions were stable for minimum 14 days preserved at -20 °C and after 3 freeze-thaw cycles.

II.C.2.3.3 Application

Besides the screening and subsequent confirmation of positive findings with GC-MS and HPLC-DAD of blood and urine samples, also DBS prepared from the blood samples of two young men suspected of drug intake were analyzed with the described method. Results for the routine investigation of a blood sample of one young man led to the confirmation of the presence of alcohol (1.42 pro mille), cocaine (benzoylecgonine), cannabinoids, MDMA and ketamine. By

analyzing the DBS, a GHB concentration of less than 1 µg/ml was found (Fig. II.C.13a). In the blood of the other young man, alcohol (0.73 pro mille), cannabinoids and MDMA were present. The latter young man was also positive for GHB abuse as a 44.4 µg/ml GHB concentration was found by analyzing the DBS (Fig. II.C.13b).

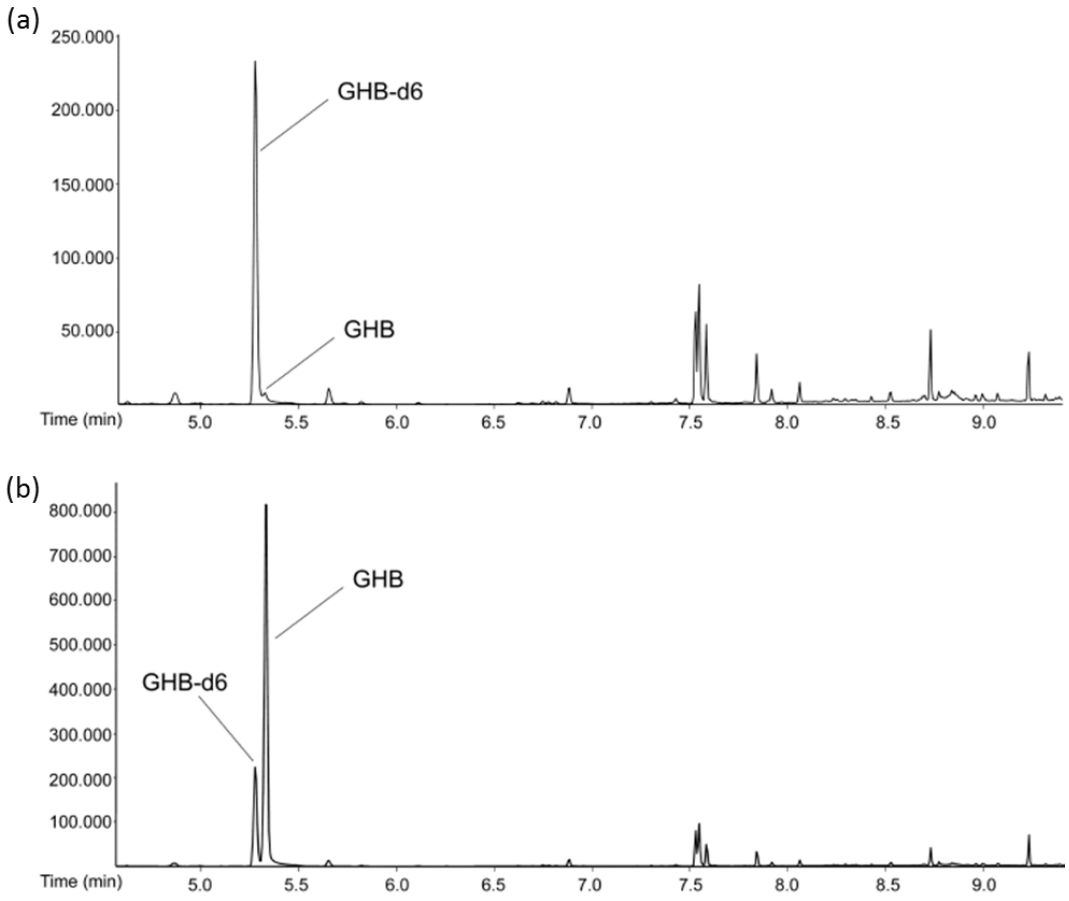


Fig. II.C.13 Chromatograms obtained when analyzing DBS from two possible GHB/GBL-users with GC-EI-MS in the SIM mode with (**a**) a GHB concentration less than 1 µg/ml in the first sample and (b) a GHB concentration of 44.4 µg/ml in the second sample

This positive and negative result were confirmed by analyzing the whole blood samples for GHB with our previously published method [2], suggesting that our newly developed method may be applicable in routine samples in a toxicological laboratory for screening purposes, as well as for the

confirmation of the presence of GHB in whole blood samples. Interesting to note in this respect is that analysis of these DBS 5 months later (storage at -20 °C) yielded similar results.

II.C.2.4 CONCLUSION

A sensitive and accurate GC-MS method was developed for the determination of GHB in whole blood samples spot on Whatman 903 filter paper. The DBS sample procedure has advantages as to the conventional blood collection, such as easy to handle and no specific storage requirements. Especially in the case of GHB, where an increase in GHB concentrations during preservation of whole blood cannot be excluded, flexibility in storage conditions is of interest. Furthermore, as the detection window is limited, it is important that samples are obtained as early as possible after ingestion. By facilitating this, DBS may consequently represent an alternative in forensic and clinical cases where there is a suspicion of illicit use of GHB, in case of DUID or when a DFSA is presumed. Our LLOQ of 2 µg/ml is well below the proposed cut-off levels of 4 and 5 µg/ml for blood samples, so this method provides enough sensitivity to distinguish between endogenous and exogenously-administered GHB, which is of major concern for the toxicological interpretation of clinical and forensic samples. Finally, our approach of direct derivatization “on spot” may also be suitable for the determination of other compounds which impose extraction problems.

II.C.3 DETERMINATION OF GHB IN 6-MM DBS PUNCHES

*(**Based on** Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. Bioanalysis. 3(20):2271-2281 (2011). Ann-Sofie M.E. Ingels, Peter De Paepe, Kurt Anseeuw, Diederik K. Van Sassenbroeck, Hugo Neels, Willy E. Lambert, and Christophe P. Stove.)*

II.C.3.1 INTRODUCTION

The short chain fatty acid gamma-hydroxybutyric acid (GHB) was synthesized in the early sixties as a structural analogue of gamma-aminobutyric acid and occurs also naturally in blood, urine and peripheral and brain tissue [19,30]. Although the function of endogenous GHB has not completely been revealed yet, evidence suggests it may act as a neuromodulator or neurotransmitter [30]. As a legal substance (sodium oxybate), GHB has a role as an anaesthetic agent, in the treatment of narcolepsy with cataplexy and of alcohol and opiate withdrawal. In addition, it has also been sold as a substance of nutritional supplements to induce sleep and increase muscle mass. Currently, illegal GHB (liquid ecstasy) as well as its precursors gamma-butyrolactone (GBL) and 1,4-butanediol are popular as club drugs and appear occasionally in drug-facilitated sexual assaults (DFSA) [7]. In those toxicological cases, the interpretation of a positive analytical result is a real challenge, because of its endogenous presence and the reported *in vitro* production [29,36]. Therefore, cut-off levels have been proposed by several authors and these are currently set at 4 or 5 μg/ml for blood (serum) samples [30]. In addition, the detection window is very limited as GHB is rapidly metabolized and eliminated after oral ingestion (plasma half-life < 1 h), so blood samples must be taken within 6 h after ingestion [14]. Consequently, a sampling delay may result in blood levels below the established cut-off level, no longer resulting in a positive case [35].

Blood sample collection may be facilitated by using dried blood spot (DBS) sampling. A DBS is capillary whole blood obtained by a finger or heel prick and collected on a filter paper card. Advantages as to a venepuncture are the easy and rapid way to collect a representative sample and the less specific sample transport and storage requirements [32]. Whereas DBS sampling has generally been used for newborn screening, more recently, this alternative sampling strategy is increasingly gaining interest in the context of therapeutic drug monitoring and (pre-) clinical studies, as well as in toxicology [50,51]. We recently reported on the development and validation of a new procedure for GHB determination in DBS, using “on spot” derivatization and gas

chromatography coupled to mass spectrometry (GC-MS) [52]. Also other drugs of forensic interest have been determined in DBS, such as MDMA, morphine, 6-acetylmorphine and cocaine [44,53,54].

To obtain a DBS on a filter paper card, a drop of blood can be spotted directly on the filter paper or with the aid of a precision capillary [32]. In our previous study, we used the second sample collection technique, and spotted a drop of blood with a fixed volume onto the filter paper card, followed by analysis of the complete DBS [52]. However, as correct sampling in this case ideally requires the presence of trained staff and in routine practice it is more convenient to collect the drop of blood directly on the filter paper cards, we modified our procedure accordingly. As we did not wish this simplification to be at the expense of sensitivity (lower limit of quantification or LLOQ of 2 µg/ml), we re-adjusted several sample pre-treatment steps. Furthermore, the analysis of DBS punches rather than of complete DBS also requires the evaluation of the impact of various blood sample properties [51]. In this study, the influence of the punch localization, of the volume spotted on the filter paper card and of the hematocrit value (Ht) was evaluated in terms of precision and accuracy of the GHB concentration measured in DBS samples [51]. Following method validation including the generally accepted parameters for bio-analytical methods, we demonstrated applicability by analyzing DBS collected from patients presenting at the emergency department with a suspected GHB-intoxication. The results obtained from capillary sampling and those obtained by conventional blood collection (venepuncture) were compared in order to evaluate the DBS sampling technique.

II.C.3.2 MATERIALS AND METHODS

II.C.3.2.1 Reagents

GHB (sodium salt, powder) and a 1-mg/ml solution in methanol of the internal standard (IS) GHB-d6 (sodium salt) were purchased from LCG standards (Molsheim, France). The derivatization reagents trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB-OH) were obtained from Sigma Aldrich (Bornem, Belgium). Methanol and ethylacetate, both of suprasolve quality suitable for GC-analysis, were delivered by Merck (Darmstadt, Germany).

Stock and working solutions to prepare calibration solutions of GHB were prepared by dissolving 10 mg of the base in 1 ml methanol, followed by appropriate dilution to obtain 1 and 0.1 mg

base/ml. To prepare quality controls (QC's), a second, independent, stock solution was used. For the IS, a 25 µg/ml methanolic solution of GHB-d6 was prepared by appropriate dilution of the commercially available stock solution of 1-mg Na-GHB-d6/ml in methanol. All solutions were stored at -20 °C.

II.C.3.2.2 DBS sampling

In the procedure to obtain DBS, the hand is first cleaned and held down or warmed for a few minutes. With the help of an automatic lancet (Becton Dickinson ref n° VAC366594, Franklin Lakes, USA), the fingertip is pricked. While the first drop of blood is wiped off with a sterile piece of cloth because of the presence of tissue fluid, the following drops are collected on a Whatman 903 filter paper card (ref n° 10334885, Dassel, Germany) with pre-printed circles. The circle (8 mm diameter) must be entirely filled with blood and although the blood is spot on just one side, both sides of the filter paper must be coloured [32].

For method development and validation, we used venous whole blood from healthy non-user volunteers with endogenous GHB concentration below the established LLOQ, collected in EDTA tubes and preserved for maximum one week at 4 °C. No significant difference (α=0.05, 95% confidence interval) was observed between the mean GHB concentration measured (nominal value 5 and 100 µg/ml, n=5) when 25 µl of blood was either directly applied with a calibrated pipette or by allowing the drops to fall from the pipette tip onto the filter paper card. So from the whole blood samples, 25-µl spots were applied with a calibrated pipette directly onto the Whatman filter paper. The resulting spots were dried for minimum 2 hours at ambient temperature and subsequently analyzed or preserved in a zip-closure plastic bag with desiccant at room temperature until analysis.

II.C.3.2.3 Sample preparation and analytical procedure

Instead of using the whole DBS, only a 6-mm (diameter) disc (corresponding to ± 10 µl) was punched out from the centre of a DBS. This influences the sample pre-treatment procedure, thus each step from our previous procedure [52] was re-evaluated (data not shown). The most important adjustments included addition of the IS (5 µl of a 25 µg/ml solution) to the punched disc, halving of the amount of derivatization reagents, TFAA and HFB-OH (2:1, by volume), and of ethylacetate to redissolve the dried derivatized sample. Fig. II.C.14 gives a detailed overview of all

adjustments. Derivatized extracts were analyzed by GC-MS as described before, using the ratio of GHB to GHB-d6 (IS) for quantification [52]. The following ions were monitored using the selected ion monitoring (SIM) mode: *m/z* 155, 183, 227 and 242 for derivatized GHB and *m/z* 161, 189, 231 and 245 for derivatized GHB-d6 (underscored ions represent the quantifier ions, the other ions were selected as qualifier ions).

Method 1: 50-µl DBS	**Method 2: 6-mm DBS punch**
Sample collection	
50 µl (capillary) whole blood is spotted onto a Whatman 903 filter paper	A drop of blood is collected directly onto a Whatman 903 filter paper
Sample pre-treatment	
The complete DBS is excised The IS is added (before excising the DBS) 10 µl of a 0.05-mg/ml methanolic solution The DBS is left to dry for 15 min 100 µl of the derivatization reagent is added	A 6-mm punch is excised The IS is added (after punching out) 5 µl of a 0.025-mg/ml methanolic solution The punch is dried for 5 min under nitrogen 50 µl of the derivatization reagent is added
TFAA /HFB-OH (2:1, by volume) freshly prepared mixture Sonication (2-5 min) Derivatization at 60 °C for 10 min The DBS is cooled down by centrifugation for 5 min at 4 °C The sample is dried under a gentle stream of nitrogen at 25 °C	
The sample is redissolved in 200 µl ethylacetate Sonication for 5 min Centrifugation 5 min at 1.6 x 1000 g (2 times)	The sample is redissolved in 100 µl ethylacetate Sonication for 2 min Centrifugation 5 min at 1.6 x 1000 g
Transfer of the supernatant to a vial	

Fig. II.C.14 Overview of the sample collection and sample pre-treatment of our previously published method (method 1 [52]) and the newly developed method (method 2) for the determination of GHB in DBS with GC-MS operating in SIM mode (with the most important changes underlined)

II.C.3.2.4 DBS method validation

As suggested by several authors, punching out a disc from a DBS has as a consequence that the impact of additional parameters needs to be evaluated, such as the punch localization (at the periphery or central in the DBS), the influence of the volume spotted on the filter paper card and of the Ht [51,55]. Furthermore, a partial validation was performed based on the FDA and EMA guidelines for the validation of bio-analytical methods [39,40]. Therefore, linearity, precision, accuracy, limits of detection and quantification, and dilution integrity were evaluated. Also long-term stability was determined [51]. Short-term stability, stability of stock solutions, and selectivity of the method were evaluated during earlier validation experiments [52].

II.C.3.2.4.1 Influence of the punch localization

Spots of 50 µl (n=5) were prepared at both low and high GHB concentration levels in whole blood with low (0.38), intermediate (0.45) and high (0.50) Ht. The difference between the mean GHB concentrations obtained when analyzing discs punched out peripherally *vs.* centrally was statistically evaluated using an independent sample T-test (α=0.05, 95% confidence interval) [56].

II.C.3.2.4.2 Influence of the blood spot volume

Venous whole blood from healthy volunteers with low (0.38), intermediate (0.45) and high (0.50) Ht was spiked at both low and high GHB concentration levels (5 and 100 µg/ml). Different volumes (20, 35 and 50 µl) were spotted (n=5 or 6) onto the filter paper card, the DBS were dried and subsequently analyzed. To calculate accuracy, the obtained GHB concentrations, when using a calibration curve prepared in whole blood with intermediate Ht (0.45), were divided by the nominal value of 5 or 100 µg/ml and multiplied by 100 %. The average % bias, which is the accuracy lowered with 100 %, needed to be within ± 15 %, while the within-volume precision needed to be < 15 % relative standard deviation (RSD), calculated by dividing the standard deviation (SD) by the mean ratio of GHB to GHB-d6 and multiplying by 100 % [55].

II.C.3.2.4.3 Influence of the hematocrit

To investigate the effect of increasing Ht on the GHB concentration, both low and high GHB concentration solutions were prepared in six whole blood samples with increasing Ht (0.34, 0.39, 0.44, 0.46, 0.51, and 0.56) and 25-µl spots were made (n=5). Therefore, we started from a whole blood sample, and after centrifugation, plasma was added or withdrawn to obtain whole blood samples with increasing Ht. The DBS were analyzed as described above, and the sample with a Ht

of 0.44 was normalized, as this is the theoretical average value of our patient population, including healthy women and men (Ht reference range of 0.37-0.47 and 0.41-0.51, respectively) [57].

II.C.3.2.4.4 Validation

To obtain the calibration data, on 4 non-consecutive days fresh calibration solutions were prepared in venous whole blood with intermediate Ht (0.45) and the resulting DBS were analyzed in duplicate. To ensure the independency of the result to the blood matrix properties, QC samples (2, 10 and 100 µg/ml) were prepared in venous whole blood samples with low (0.38), intermediate (0.45) and high (0.50) Ht values, obtained from different individuals [51]. For each day, a 6-point calibration curve was constructed by plotting the ratio (mean of the duplicates) of the area of GHB to GHB-d6 in function of the concentration (2, 5, 10, 25, 50, and 100 µg/ml). The resulting data were statistically evaluated by performing weighting if necessary. Therefore, the sum % relative error (RE) was calculated and the % RE versus concentration was plotted, where % RE is the concentration found lowered with the nominal concentration, divided by the nominal concentration and multiplied by 100 %. Linearity was assessed by performing Fisher’s test [41].

Intra- and interbatch precision were evaluated by analyzing QC solutions prepared in blood with low, intermediate and high Ht on 5 separate days in duplicate. The RSD was calculated using one-way-ANOVA as recently described by Wille *et al*., and needed to be < 15 and < 20 % at LLOQ [43]. Accuracy needed to be within 85 to 115 % of the nominal value and within 80 to 120 % at LLOQ level (expressed as % bias).

To evaluate sensitivity, the limit of detection (LOD) was estimated as the minimum GHB concentration with a signal-to-noise ratio equal to or larger than 3. Furthermore, the LLOQ was defined as the lowest GHB concentration still measured with % RSD < 20 % and accuracy between 80 and 120 %.

The possibility to dilute the final derivatized extract of samples with a GHB concentration higher than the highest point of the calibration curve (100 µg/ml), was assessed by spiking venous whole blood with low (0.38), intermediate (0.45) and high (0.50) Ht at 200 µg/ml and 25-µl spots were made (3 days, n=2). The spots were analyzed as described and 10 µl of the final derivatized extract was diluted to 100 µl with ethylacetate (as a result, also the derivatized internal standard is diluted

10-fold). The mean GHB concentration was back-calculated by using the daily calibration curve, and was corrected for by the dilution factor. Inter-batch precision (% RSD) and accuracy were evaluated as described above and needed to be < 15 % and within 85-115 %, respectively.

Finally, drying times of 30 min to 2h and of 72 h at room temperature or 4 °C in a study box were investigated (n=3 for each condition at 5 and 100 µg/ml). Long-term stability at room temperature was investigated by analyzing DBS (n=6) at both low and high GHB concentration levels (5 and 100 µg/ml) at time point zero, and after 14, 48 and 148 days of storage. The mean concentration measured must be within ± 15 % of the nominal concentration, when using a freshly prepared calibration curve.

II.C.3.2.5 Determination of GHB in DBS collected at the emergency department

In a first study (approved by the local medical ethical boards), patients transported to the emergency room of the cooperating hospitals (Ghent and Antwerp), and with moderate to severe loss of consciousness and/or with indications of a GHB-ingestion were included. A venepuncture was performed (EDTA as anti-coagulant) and within 10 minutes capillary DBS were obtained as described above, in order to compare the GHB concentration in the venous and capillary whole blood sample [55]. Within 30 minutes after collection of the venous whole blood sample, DBS were prepared (so called venous DBS) by applying 25 µl onto the filter paper card with a calibrated pipette. The collected DBS were left to dry for minimum 2 hours at room temperature and were then placed in a zip-closure plastic bag with desiccant until analysis, while the venous whole blood samples were stored at 4 °C until analysis. The venous whole blood samples were analyzed in accordance with the routine procedure of toxicological analysis, while the DBS were analyzed as described above in order to confirm a possible GHB-intoxication. If the GHB concentration was found to be above the highest calibration level, the derivatized extract was diluted as described. In addition, an aliquot of a GHB-positive venous whole blood sample was analyzed according to the procedure of Van hee *et al.* [2]. Briefly, 20 µl of the whole blood sample was directly derivatized to obtain the di-trimethylsilyl derivative of GHB, which was analyzed by GC-MS in the SIM mode.

In a second study, capillary DBS and venous whole blood were collected at the same time from patients with a suspected GHB-intoxication in the emergency department of Guy’s and St Thomas’

hospital, London, in collaboration with the Clinical Toxicology Service. First, 4 drops of capillary whole blood were collected using a single-use lancet and then, venous samples were taken, of which 4 drops of 25 µl were spot onto a DBS card. Once dry, the DBS were placed in zip-closure plastic bags and samples were sent to the laboratory in batches by regular mail (see study flow diagram: Fig II.C.15). At the laboratory, samples were treated as described above.

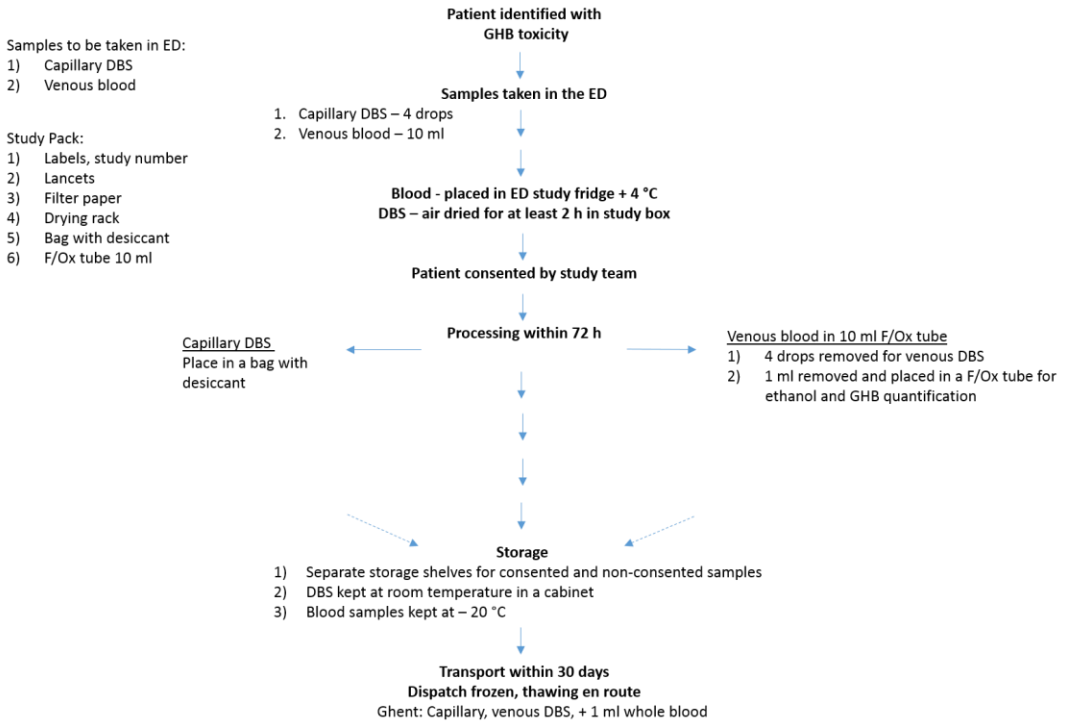


Fig. II.C.15 Comparison of capillary and venous blood analysis for GHB in patients with acute GHB-intoxication. Study flow diagram formulated by the clinical toxicology department of Guy’s and St Thomas’ hospital, London

The % difference between the various GHB measurements was calculated from the following concentration ratios: [venous DBS]/[capillary DBS] and [venous whole blood]/[venous DBS]. These respective ratios were used to evaluate whether there were consistent differences in GHB concentrations between DBS obtained from capillary *vs.* venous blood and between venous blood analyzed as such or as DBS. For method comparison, cross-validation was performed by analyzing an aliquot of the venous whole blood samples at the Laboratory of Toxicology of ZNA Stuivenberg

Hospital in Antwerp, using the method of Van hee *et al*. [2] and by analyzing venous DBS using the developed DBS method. The difference between the measured GHB concentratios in the venous DBS obtained by the newly developed DBS method and those obtained by analyzing venous whole blood with the method of van hee *et al*. [2] should be within 20 % of the mean for at least 67 % of the samples [40]. Furthermore, to evaluate if capillary GHB concentrations are a true reflection of venous GHB concentrations, the concentrations obtained from capillary and venous DBS were compared using Bland-Altman plot, Mountain plot and Passing-Bablok regression [54,58-62].

II.C.3.3 RESULTS AND DISCUSSION

Following DBS collection and drying (for a minimum of 2 hours at room temperature), a 6-mm-(diameter)-disc was punched out. After applying the IS, “on spot” derivatization was performed with a mixture of TFAA and HFB-OH (50 µl, 2:1 by volume) at 60 °C for 10 min. The derivatized sample was then centrifuged, dried under a gentle stream of nitrogen and the dried extract was redissolved in 100 µl ethylacetate. Following brief sonication and centrifugation, 1 µl of the derivatized extract was analyzed by GC-MS. Besides modification of the sample preparation, the impact of additional parameters was investigated. Finally, the procedure was validated and applicability was demonstrated using samples obtained at the emergency department of cooperating hospitals.

II.C.3.3.1 DBS method validation

II.C.3.3.1.1 Influence of the punch localization

Several publications have pointed out that the site of punching may have an effect on the measured concentration. This has been shown for both macromolecules (proteins) as for small molecules, with higher concentrations observed at the peripheral or at the central punching site, depending on the molecule under investigation. This effect, which is also influenced by the Ht, is likely owing to chromatographic effects, which are determined by interaction of the compound with both the paper and the blood [63-65]. To investigate whether the site of punching out a disc from a DBS influences the result of our analyses, discs punched out peripherally and centrally were analyzed. Irrespective of the Ht, this revealed no significant difference between the mean GHB concentrations at a confidence level of 95 %, demonstrating a homogenous GHB distribution in DBS [56].

II.C.3.3.1.2 Influence of the blood spot volume

The influence of the volume spotted on the measured analyte concentration was evaluated by replicate analysis of discs punched out in the centre of DBS with different blood volumes. Fig. II.C.15 summarizes the results and shows that the average % bias was overall within the predefined acceptance limits of ± 15 %, except for the analysis of discs punched out from 50-µl spots (5 µg/ml GHB) at the low and high Ht. The latter is probably due to an overload of the filter paper, negatively influencing the spread and the homogenous distribution of the blood drop. The within-volume precision (% RSD) was overall < 15 %. So, based upon our results, the best blood volume spotted was between 20 and 35 µl, regardless of the Ht of the blood sample. This is also the volume required for filling the pre-printed circles in the case of DBS from patients (8-mm diameter, containing ± 20 µl).

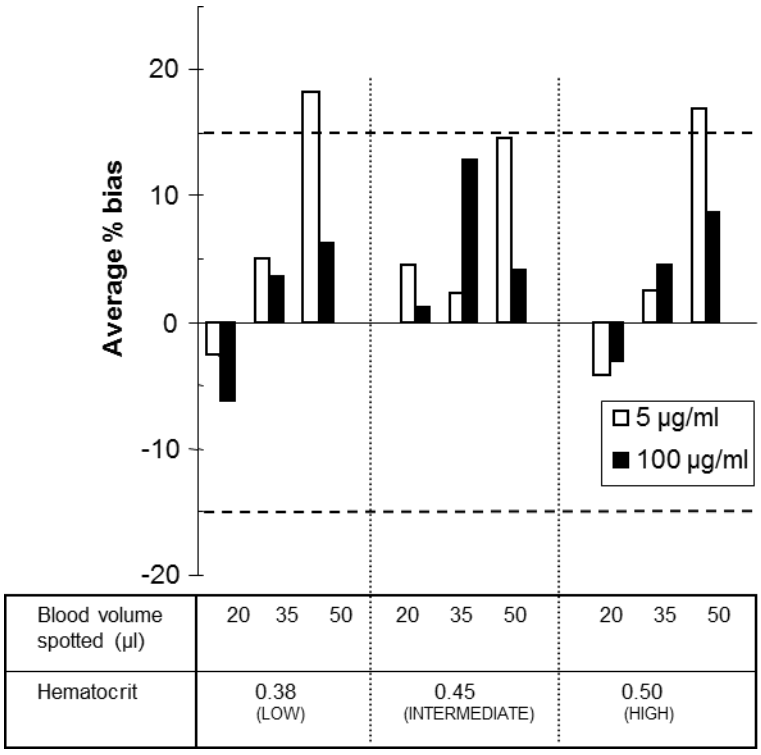


Fig. II.C.15 Average % bias vs. blood volume spotted for the determination of GHB in DBS. The DBS (n=5 or 6, at low and high nominal value) were analyzed using "on spot" derivatization and GC-MS, operating in the SIM-mode. Dotted lines indicate the ± 15 % (bias) limits.

II.C.3.3.1.3 Influence of the hematocrit

Although not unequivocally demonstrated, an equal distribution of GHB between plasma/serum and blood is assumed, which, similar to ethanol, is expected to result in a concentration ratio of blood to plasma or serum of about 0.87 (this figure being slightly lower than 1 because of the solid constituents of blood) [30,66]. As this implies an even partitioning between plasma/serum and erythrocytes, no effect of the Ht on the GHB concentration in blood *per se* is expected [67]. However, still, the influence of the Ht requires special attention, especially in the analysis of DBS, as the Ht is directly proportional to the blood viscosity, affecting flux and diffusion of the blood that is spotted on the filter paper card [68]. The Ht values in healthy women and men range from 0.37 to 0.47 and from 0.41 to 0.51, respectively [57]. Using a calibration curve obtained by analyzing DBS prepared of blood with intermediate Ht (0.45), we determined the GHB concentration (low and high nominal value) in DBS from whole blood solutions with increasing Ht. The results are summarized in Table II.C.4, presenting the % deviation from the normalized sample with average Ht [57].

Table II.C.4 Influence of the hematocrit on the GHB concentration measured in DBS samples, using GC-MS, operating in SIM mode. Values indicate the mean % deviation from the GHB concentration obtained for the sample with a hematocrit of 0.44, which was used for normalization, given the reference interval of 0.37 to 0.51 for healthy women and men.

Hematocrit	**Low GHB concentration (5 µg/ml, n=5)**	**High GHB concentration (100 µg/ml, n=5)**
0.34	-15.0	-5.17
0.39	-3.45	-2.73
0.44	Normalized	Normalized
0.46	-2.32	3.85
0.51	-0.910	1.18
0.56	11.1	10.9

Overall, we observed little or negligible influence in the Ht range of 0.39 to 0.51, covering the expected range of Ht in our patient population. Analysis of DBS prepared from whole blood with Ht deviating from the reference range may no longer result in accurate measurements. Therefore, based upon this experiment and in agreement with other reports, for quantification purposes, it is

recommended to prepare calibration and QC samples in whole blood with a Ht within the reference range and most preferably with an intermediate Ht, and this to minimize its effect on accuracy [32,51].

II.C.3.3.1.4 Validation

The obtained calibration data were statistically evaluated as described in the experimental section, and a weighting factor of $1/x^2$ was applied. The resulting calibration and sensitivity data are summarized in Table II.C.5. Fig. II.C.16 shows a representative chromatogram for the LLOQ sample (2 µg/ml). Linearity was demonstrated within the working range using Fisher's test.

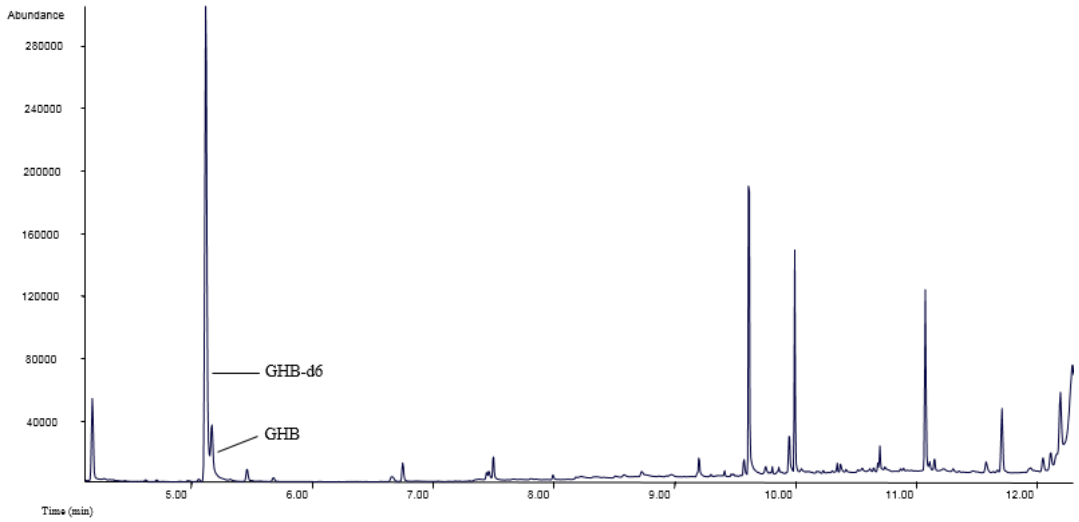


Fig. II.C.16 Representative chromatogram obtained after analysis of a 6-mm disc punched out from a DBS prepared from blood spiked with GHB at 2 µg/ml (LLOQ). Five µl of a 25 µg/ml IS solution was added to the punch before derivatization and analysis with GC-MS operating in SIM mode.

Table II.C.5 Calibration and sensitivity data for the determination of GHB in 6-mm punches using GC-MS in SIM mode

	Slope Mean ±SD (95 % CI) (n=4 x 2)	**Intercept** Mean ±SD (95 % CI) (n=4 x 2)	**Working range** (µg/ml)	**LLOQ** (µg/ml)	**R^2** (n=4 x 2)	**Weighting factor**
6-mm punch	0.044 ± 0.003 (0.040; 0.047)	0.000 ± 0.007 (-0.007; 0.007)	2-100	2.0	0.999	$1/x^2$

As shown in Table II.C.6, precision and accuracy were within the predefined acceptance limits (< 15 % RSD and bias). Results of the dilution experiment are also summarized in Table II.C.6. Precision and accuracy were acceptable, so the derivatized extract of samples with a GHB concentration of more than 100 µg/ml can be diluted 1 to 10 with ethylacetate prior to GC-MS analysis.

Table II.C.6 Intra- and interbatch precision and accuracy data for the QC’s prepared in whole blood with low, intermediate and high Ht

	Nominal GHB concentration (µg/ml)	Measured GHB concentration (µg/ml)	Intra-batch precision (% RSD, n=5 x 2)	Inter-batch precision (%RSD, n=5 x 2)	Accuracy (Bias %)
Low Ht (0.38)					
QC low	2	1.99	5.3	7.9	-0.6
QC mid	10	9.07	6.7	14.6	-9.2
QC high	100	97.17	5.2	12.5	-2.8
2 x ULOQ	200	191.30	6.0	9.5	-4.4
Intermediate Ht (0.45)					
QC low	2	2.10	11.6	11.6	5.1
QC mid	10	9.65	3.7	5.5	-3.5
QC high	100	103.02	4.9	14.7	3.0
2 x ULOQ	200	209.94	4.5	11.1	5.0
High Ht (0.50)					
QC low	2	2.12	6.0	10.3	6.0
QC mid	10	10.10	6.3	12.5	1.0
QC high	100	106.60	7.3	8.0	6.6
2 x ULOQ	200	206.50	4.1	6.8	3.3

Furthermore, 2 h drying at room temperature was required to obtain completely dry DBS suitable for punching out a 6-mm disc. Drying DBS for 72 h was possible at room temperature or 4 °C in a study box with desiccant, before placing the DBS card in a sealable plastic bag for storage at room temperature. Finally, DBS appeared to be stable when stored at room temperature in a zip-closure plastic bag with desiccant for at least 148 days, as the average calculated GHB concentration deviated less than 15 % from the nominal value (Fig. II.C.17).

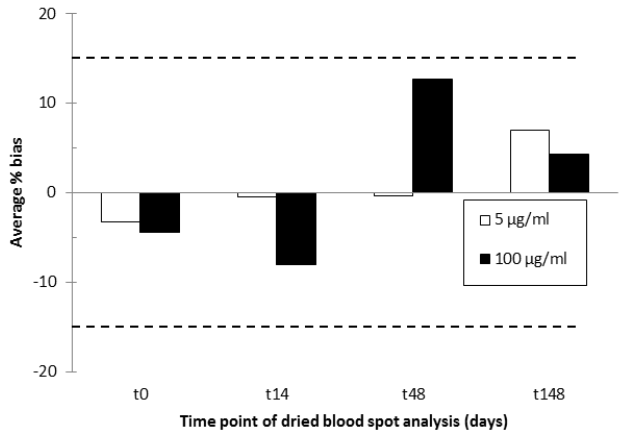


Fig. II.C.17 Long-term stability of GHB in DBS (n=6, low and high GHB concentration level) stored at room temperature in a zip-closure plastic bag with desiccant up to 148 days. The average % bias *vs.* time point of DBS analysis (days) is plotted and needed to be within the ± 15 % limits, indicated by the dotted lines. T_0 refers to time point zero (DBS analysis after 2 hours of drying).

II.C.3.3.2 Determination of GHB in DBS collected in the emergency room

Given the required drying time of minimum 2 h at room temperature, the DBS analyses will most likely have no influence on clinical patient management in an emergency department setting. This also holds true for the majority of methods used to determine GHB, given the rapid onset and disappearance of (side-) effects. Because of the availability of presumed GHB positive patients at emergency departments, we chose to collect DBS samples from these patients, to demonstrate the applicability of our procedure. So, two separate studies were conducted to evaluate the DBS sampling technique in a real-life setting, as well as to make a first comparison between the GHB concentrations measured in venous *vs.* capillary whole blood. In the first study, a total of 14 patients (between 18 and 35 years old, 13 men and 1 woman) were included. They were brought to the emergency department with unknown cause of coma and/or signs of drug intoxication. Blood sample analysis confirmed 7 GHB intoxications (Table II.C.7, patients 1-7). Consistent with other reports describing GHB abuse in the context of multi-drug use, we found GHB to be combined in all cases with other drugs such as alcohol, cocaine and cannabinoids [20]. We previously demonstrated that these do not interfere with the GC-MS determination [52]. Within the same study, also during one dance festival and one techno party, we collected samples at a

first aid post. Also here, 2 samples were GHB-positive, consistent with the clinical diagnosis (Table II.C.7, patients 8-9).

From the study in collaboration with Guy’s and St Thomas’ hospital in London, we received 15 paired samples (Table II.C.7, patients 10-24). Broad toxicological screening of corresponding plasma samples using time-of-flight mass spectrometry was performed [68]. Only 3 samples were negative for additional recreational drugs (N° 13,22 and 24). All other samples screened positive for mephedrone (4-methylmethcathinone, 4-MMC) and its metabolites. In addition, other commonly abused recreational drugs were also present (e.g. synthetic cathinone, ketamine and amphetamines). Other detected drugs were benzodiazepines, antidepressants, antibiotics, antiretroviral drugs and over-the-counter medications such as antihistamines. Furthermore, patient 13 and 19 had a blood alcohol concentration of 1.1 and 0.27 ‰, respectively; the rest of these patients (10 to 24) were negative (for alcohol ingestion).

Fig. II.C.18 shows representative chromatograms (overlay) obtained by GC-MS analysis of a derivatized extract of a capillary and venous DBS collected from the first GHB-positive patient (case N^{o}1, in Table II.C.7). The measured GHB concentrations in the collected DBS and whole blood are summarized in Table II.C.7.

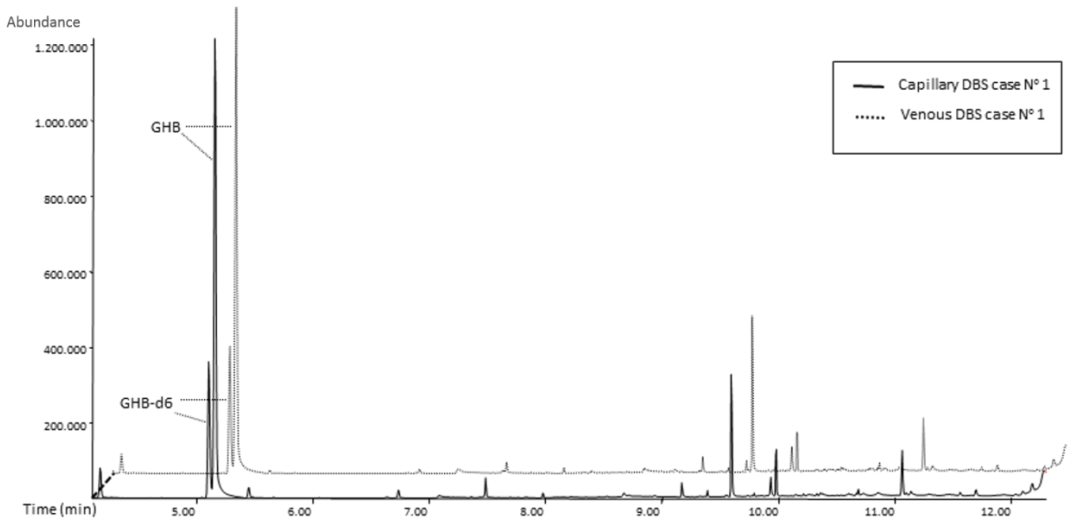


Fig. II.C.18 Overlay of representative chromatograms obtained by analyzing the derivatized extract of the capillary and venous DBS, collected from the first patient who tested positive for GHB use (GHB-positive case N^{o} 1 in Table II.C.7), by using “on spot” derivatization and GC-MS, operating in the SIM-mode

Table II.C.7 GHB level (µg/ml) in paired venous and capillary DBS, and venous whole blood samples in 24 patients presenting at emergency departments with a suspected GHB-intoxication. A DBS (single analysis) was analyzed using "on spot" derivatization and GC-MS, operating in SIM mode, while venous whole blood (in duplicate, average of duplicate measurements is shown) was analyzed according to the procedure of Van hee *et al*. [2]

GHB-positive case N°	Venous DBS (µg/ml GHB)	Capillary DBS (µg/ml GHB)	Venous whole blood (µg/ml GHB)
1	81	79	89
2	170	150	173
3	153	169	154
4	56	92*	44
5	118	142	126
6	107	116	91
7	127	132	97
8	121	137	125
9	170	163	145
10	275	269	261
11	121	135	145
12	113	91	85
13	100	102	102
14	98	89	85.0
15	624	580	526
16	131	127	128
17	130	140	121
18	142	120	118
19	101	128	109
20	121	126	96.7
21	225	218	229
22	163	142	136
23	123	112	100
24	201	170	166

* We suspect this higher value to be due to contamination of the fingertip

To ensure that no consistent change in GHB concentration occurred during drying of the DBS, an aliquot of the GHB-positive samples was also analyzed using the procedure of Van hee *et al*. [2]. Overall, as shown in Table II.C.7, analyzing a venous whole blood sample directly or after it has been spotted onto a filter paper card yielded similar analyte concentrations (mean difference of

8.5 %) with a ratio [venous whole blood]/[venous DBS] equal to 0.93. According to the general guidelines for cross-validation, for more than two-thirds of the samples the difference between a repeated measurement and the mean of the first and repeat measurement should be less than 20 % [40]. This requirement was fulfilled when calculating the % difference in GHB concentrations obtained with the 2 methods.

Comparing the measured GHB concentrations in capillary and venous DBS using Bland-Altman analysis revealed no systematic difference, with the zero value included in the 95 % CI of the mean difference in GHB concentration. Passing-Bablok regression resulted in a linear model to fit the data, with an intercept of 17.91 (95 % CI ranging from -5.94; 36.17) and a slope of 0.89 (95 % CI ranging from 0.75; 1.03), so with 1 and zero value included in the respective CI. This suggests that there are no systematic differences between GHB concentrations measured in capillary *vs*. venous DBS (Fig. C.II.19). The average ratio [venous DBS]/[capillary DBS] was 0.99, suggesting that capillary GHB concentrations can be used as an alternative for venous GHB concentrations. Since the analyses mentioned above were conducted with mere 24 paired samples, a more extensive paired sample analysis is recommended to confirm these preliminary findings.

In addition, re-analysis of a subset of capillary DBS (n=6) and venous DBS (n=4) was performed after at least 3 weeks of storage of the DBS at room temperature in a plastic bag with dessicant (incurred sample re-analysis, ISR). The requirement that for more than two-thirds of the samples the initial concentration and that obtained by reanalysis should be within 20 % of their mean for at least 67 % of the repeats [40], was fulfilled, with all the percentages below 15 %. Concerning the sampling technique, collecting the drops of blood directly on the filter paper card was generally experienced as easy and quick. Also, thorough cleaning of the fingertips before sampling appeared to be very important to exclude contamination [32]. For example, since there were only 2 min between collection of venous whole blood and capillary DBS in case N^{o} 4, with the venous whole blood being collected first, we suspect the higher concentration found in the capillary DBS in this case to be due to contamination of the fingertip. Nevertheless, the advantages of DBS sampling make this technique highly suitable for drug determination in a real-life setting, as it is less invasive than a venepuncture, the obtained DBS can be collected fast and only little sample volume is needed.

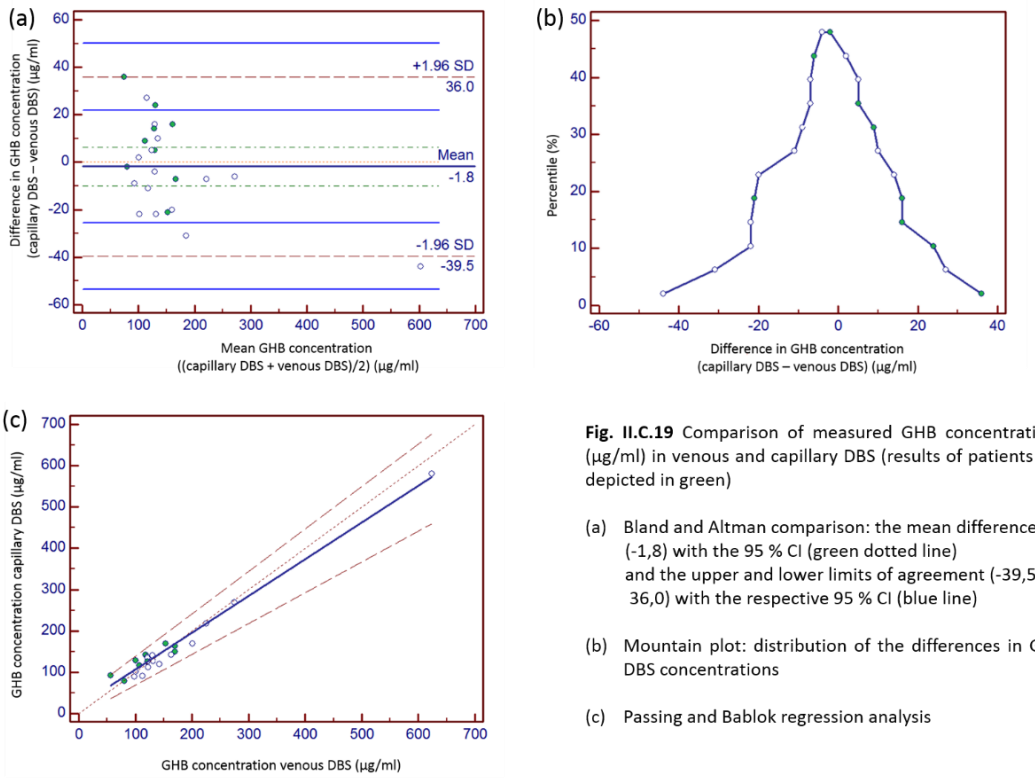


Fig. II.C.19 Comparison of measured GHB concentrations (µg/ml) in venous and capillary DBS (results of patients 1-9 depicted in green)

(a) Bland and Altman comparison: the mean difference (-1,8) with the 95 % CI (green dotted line) and the upper and lower limits of agreement (-39,5 & 36,0) with the respective 95 % CI (blue line)

(b) Mountain plot: distribution of the differences in GHB DBS concentrations

(c) Passing and Bablok regression analysis

II.C.3.4 Conclusion

A method for the determination of GHB in DBS samples, previously developed in our laboratory, was successfully adjusted in order to collect drops of blood directly on filter paper cards. Consequently, no longer a fixed volume of blood was analyzed, but only a 6-mm (diameter) disc punched out of the obtained DBS. This report includes the re-evaluation of the sample pre-treatment steps, to maintain a LLOQ of 2 µg/ml, as well as the evaluation of the impact of various blood sample properties and method validation. We applied this procedure on DBS from intoxicated patients from collaborating emergency departments. Collecting the drops of blood directly on the filter paper card can be considered as a more convenient technique than the use of a precision capillary in a real-life setting. Moreover, GHB in DBS was found to be stable for at least 148 days stored at room temperature in a zip-closure plastic bag with desiccant, so the DBS matrix overcomes the reported possibility of *in vitro* production by storage of whole blood samples at temperatures above 2 to 8 °C [30]. To conclude, the collection and analysis of DBS may be a useful tool to confirm a suspected GHB ingestion, even outside a hospital environment, because of the general advantages coupled to this sampling technique.

II.C.4 DETERMINATION OF GHB IN DBS COLLECTED BY PATIENTS WHO USE XYREM® (SODIUM OXYBATE) FOR THE TREATMENT OF NARCOLEPSY WITH CATAPLEXY

*(**Based on** Feasibility of Following up Gamma-Hydroxybutyric Acid Concentrations in Sodium Oxybate (Xyrem®)-Treated Narcoleptic Patients Using Dried Blood Spot Sampling at Home. An Exploratory Study. CNS Drugs. 27(3):233-237 (2013). Ann-Sofie M.E. Ingels, Katrien B. Hertegonne, Willy E. Lambert and Christophe P. Stove.)*

II.C.4.1 INTRODUCTION

Gamma-hydroxybutyric acid (GHB) is a short-chain fatty acid synthesized in the early 1960s as a structural analogue of gamma-aminobutyric acid. GHB is also naturally present in blood, urine and peripheral and brain tissue [19,30]. The sodium salt of GHB, which is a popular club drug, is used as an orphan drug (sodium oxybate, Xyrem®) in the treatment of narcolepsy (with cataplexy or with excessive daytime sleepiness [EDS]), a chronic neurological sleep disorder [7]. This orphan disease is characterized by EDS, cataplexy (a sudden loss of muscle tone provoked by emotional stimuli), disturbed nocturnal sleep, hypnagogic hallucinations and sleep paralysis. It has been demonstrated that GHB administration in narcoleptic patients with cataplexy increases slow-wave sleep duration, improves EDS and reduces the number of awakenings at night [69-71].

Therefore, sodium oxybate has been approved in 2002 by the US Food and Drug Administration (FDA) for the treatment of cataplexy in narcolepsy patients, and subsequently in 2005 for the treatment of EDS in narcolepsy patients. Also, in 2005, the European Medicines Agency (EMA) approved sodium oxybate for the treatment of narcolepsy with cataplexy. A maximum of 9 g can be administered each night, split in two equal doses because of the short half-life (plasma and whole blood half-life less than 1 h). The first dose should be taken at bedtime and the second 2.5–4 h later [69-71]. Intra- and interindividual variation in clinical effect has been seen with sodium oxybate; however, it is not known whether this correlates with variation in obtained GHB concentrations. Therefore, the DBS sampling technique, stated to be easy and minimally invasive, may be useful to obtain patient samples in a non-hospital-based setting.

The present study was designed to determine the GHB blood concentration obtained after the first intake of sodium oxybate by the use of the DBS sampling technique. The first objective was to evaluate the applicability of a recently developed and validated DBS-based GC-MS method [72].

Whereas DBS sampling has been used for decades in newborn screening, more recently, this alternative sampling strategy is increasingly gaining interest in the context of therapeutic drug monitoring, (pre-) clinical studies and toxicology. Having advantages such as being easy to perform and minimally invasive, DBS sampling renders blood sampling by the patient at home a feasible option, allowing better insight to be gained into GHB concentrations following sodium oxybate administration [32,50,51,73]. However, only a few studies have evaluated true home-based sampling; therefore, a second objective was to evaluate DBS collection in a real-life setting [74,75].

II.C.4.2 METHODS

Since narcolepsy with cataplexy is an orphan disease and sodium oxybate may only be prescribed in selected cases, there is very limited access to patients. In Belgium, around 80 of the approximately 200 patients diagnosed with narcolepsy are currently using sodium oxybate (Xyrem®; UCB Pharma Ltd, Brussels, Belgium), of whom 7 are treated in the Department for Respiratory Diseases of Ghent University Hospital. Those 7 patients were included in this study, approved by the local medical ethical board.

The patients, taking sodium oxybate on a daily basis, were asked to fill a maximum of four pre-printed circles (8-mm diameter) on a DBS card approximately 20 min after the first sodium oxybate intake on 7 consecutive days. They received DBS cards (Whatman 903 filter paper; reference no. WHA10334885, Dassel, Germany), single-use automatic lancets for capillary blood collection (Becton Dickinson; reference no. VAC366594, Franklin Lakes, NJ, USA), disinfection tissues, zip-closure plastic bags to store the DBS cards and a pre-paid envelope to send the material back to the laboratory. Each patient provided informed consent and was given a 30-min explanation concerning the aim of the study and the DBS collection, together with an illustrating folder. Also, a logbook (Fig. II.C.20) and a questionnaire, with questions concerning ease of sample collection and pain, as well as inconvenience experienced with this collection technique, were provided. Patients were asked to complete and return this form and questionnaire at the end of the collection period.

Datum: ../../.. Patiëntencode:....

Xyrem® inname	Andere ingenomen geneesmiddelen	Samenstelling laatst genuttigde maaltijd (indien van toepassing: ook tijdstip + hoeveelheid ingenomen alcohol)	Opmerkingen
- tijdstip - hoeveelheid (ml Xyrem® verdund in 60 ml water)			

Fig. II.C.20 Patients were asked to fill this logbook in correctly for each day they participated to the study

II.C.4.2.1 Dried blood spot (DBS) collection

To obtain a DBS, the hand was first cleaned and held down or warmed for a few minutes. With the help of an automatic lancet, the fingertip was pricked. While the first drop was wiped off with a sterile piece of cloth because of the presence of tissue fluid, the subsequent drops were collected on the DBS card [32]. After overnight drying (horizontal on a clean and empty glass), the card was placed in a zip-closure plastic bag and, finally, all the cards were sent to the laboratory by regular mail. Fig. II.C.21 gives an overview of the DBS collection (part of the illustration folder provided to the patients).

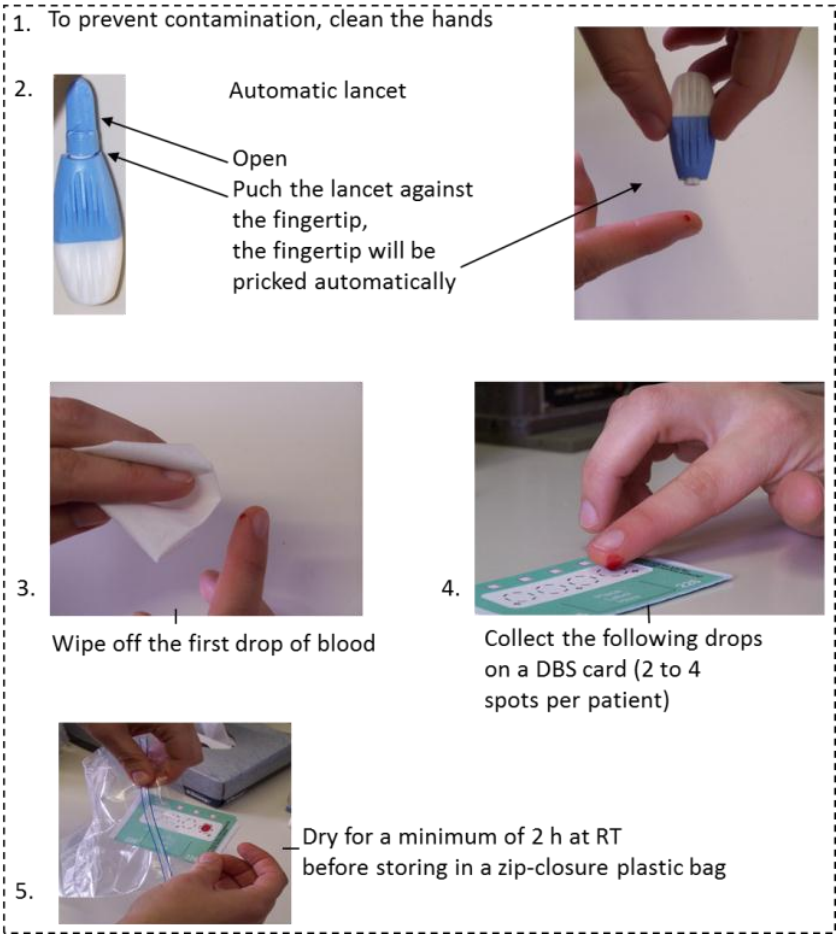


Fig. II.C.21 Schematic overview of how to collect DBS at home, information supplied to the participating patients using an illustrating folder

II.C.4.2.2 DBS analysis

Upon arrival at the laboratory, DBS were visually inspected according to Edelbroek *et al*. [32]. DBS were considered of acceptable quality when these were large enough, symmetrically spread on the filter paper and had even colouration on both sides of the filter paper [32]. Fig. II.C.22 shows examples of well and not well collected DBS. The number of well collected DBS for each day was recorded and DBS analysis was performed in duplicate, if possible, using a previously developed and fully validated GC-MS method. Briefly, a 6-mm disc was punched out from a DBS and placed in a test tube. The internal standard GHB-d6 was added directly on the punch (5 µL of a 25-µg/mL methanolic solution), and subsequently dried under nitrogen at 25°C. Derivatization took place by adding ‘on spot’ 50 µl of a freshly prepared mixture of trifluoroacetic acid anhydride and heptafluorobutanol, and by placing the test tube in a heating block at 60°C for 10 min. The sample was cooled down during centrifugation, dried under nitrogen and then redissolved in 100 µl of ethylacetate. Finally, after centrifugation, the supernatant was transferred to a vial. One µL of the derivatized extract was injected into the GC-MS, operating in selected ion monitoring (SIM) mode for quantification [72].

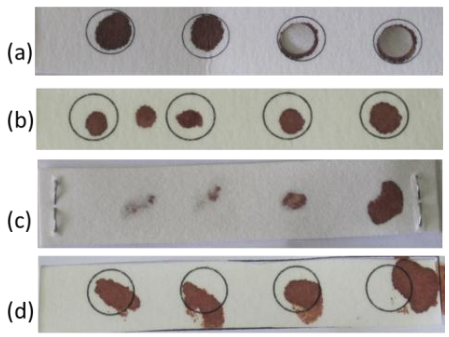


Fig. II.C.22 Overview of well (a) and not well (b, c, d) collected DBS. Sometimes, DBS are too small (b), both sides of the filter paper are not evenly coloured (c) or the DBS are not spread symmetrically on the filter paper (d).

The method used has a lower and upper limit of quantification of 2 and 100 µg/ml GHB, respectively. Of those DBS with a GHB concentration above 100 µg/ml, the final ethylacetate extracts were diluted ten-fold according to the previously validated dilution technique. Furthermore, in our previous work we demonstrated that similar GHB concentrations are found in

capillary DBS, in DBS prepared from venous blood and in venous blood, collected simultaneously from GHB-intoxicated patients. Based on that study, it can be concluded that DBS from capillary blood can be used as a suitable alternative for venous blood [72].

II.C.4.3 RESULTS AND DISCUSSION

In total, five series of DBS were collected by three different patients. One patient sent back a single series, the two other patients two series. An overview of the GHB concentrations (µg/ml) in DBS collected by the three patients is depicted in Fig. II.C.23. Appendix 2 gives a detailed overview of the sodium oxybate dose, which was taken at bedtime, the measured GHB concentrations, the time between sodium oxybate intake and DBS collection, the number of usable DBS and remarks.

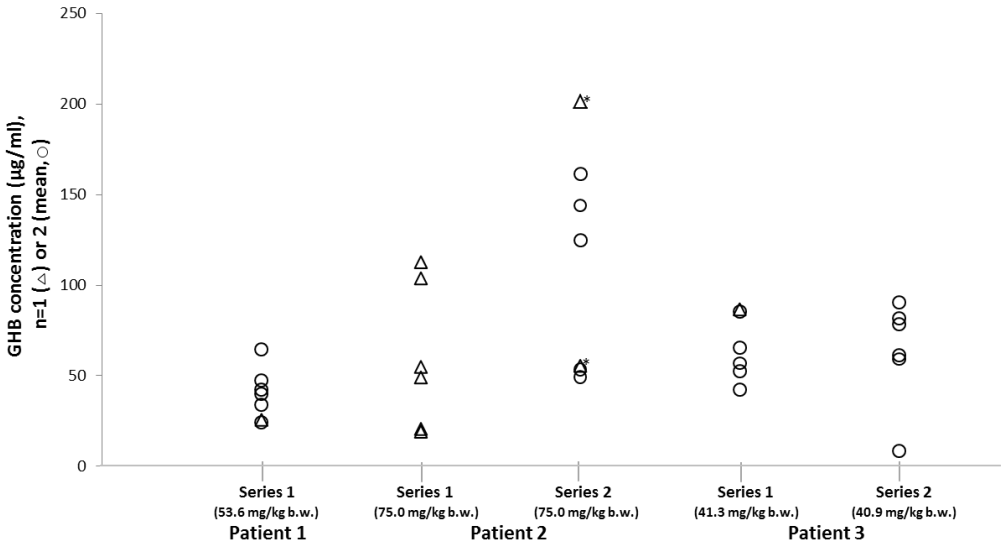


Fig. II.C.23 Overview of the measured GHB concentrations (µg/ml, analysis of 1 spot (△) or mean of analysis of 2 spots (○) on one card) in DBS collected by three narcoleptic patients approximately 20 min after the intake of their sodium oxybate dose during a maximum period of 7 days. Patients 2 and 3 collected two series with an interval of 5 and 9 months between series, respectively. DBS analysis was performed using a GC-MS method with ‘on spot’ derivatization. *b.w.* bodyweight, * DBS collected after the second dose of Xyrem®

Table II.C.8 gives an overview of the within-card precision, which was calculated as the percentage relative standard deviation (% RSD, standard deviation/mean × 100) of two DBS, obtained at the same time points. Upon evaluation of the day-to-day GHB concentrations found in the collected DBS (Fig. II.C.23), it becomes clear that patient 2 had more intra-individual variation than patients 1 and 3. When this patient performed a second sampling 5 months later, day-to-day variation was again observed. On the other hand, analysis of DBS of the second series of patient 3 resulted in similar GHB concentrations as compared with series 1, with the exception of one day. To rule out variation in analytical performance as a cause for this exception, the two remaining DBS were also analyzed, resulting in similar GHB concentrations (mean: 8.8 µg/ml, % RSD: 5.9 %, $n = 2 \times 2$).

Table II.C.8 Within-DBS card precision (% RSD), calculated for each duplicate measurement; a indicates two usable DBS, but only one was analyzed, together with one DBS collected in the morning

Day	Patient 1	Patient 2		Patient 3	
		Series 1	Series 2	Series 1	Series 2
1	5.4	ND	11.0	ND	7.7
2	1.7	ND	2.0	4.5	11.4
3	3.2	ND	ND	2.2	0.1
4	24.6	ND	8.5	2.2	1.0
5	a	ND	7.9	-	7.6
6	7.5	ND	10.1	5.6	6.5
7	10.4	ND	ND	1.4	-

ND: no duplicate measurements possible, - indicates no DBS collected

Concerning the DBS sampling technique, the three patients reported that DBS were easy or quite easy to obtain. According to the first and third volunteer, no pain or inconvenience was experienced; patient 2 reported inconvenience of the finger prick itself. Despite the positive evaluation, visual inspection at the laboratory (using guidelines from Edelbroek *et al*. [32] revealed that not all DBS fulfilled the requirements (Fig. II.C.24). Since a maximum of four DBS was requested, DBS cards with at least two suitable DBS were considered to have acceptable quality

[76]. For the first collection period, this criterion was fulfilled by 100 % of total cards collected by patient 1, none of the cards by patient 2 (analysis of single spots was however possible on 6 out of 7 days) and 83 % of the cards by patient 3. When repeating the study (series 2), the number of well collected DBS increased, to 71 % by patient 2 and to 100 % by patient 3 (Fig. II.C.24).

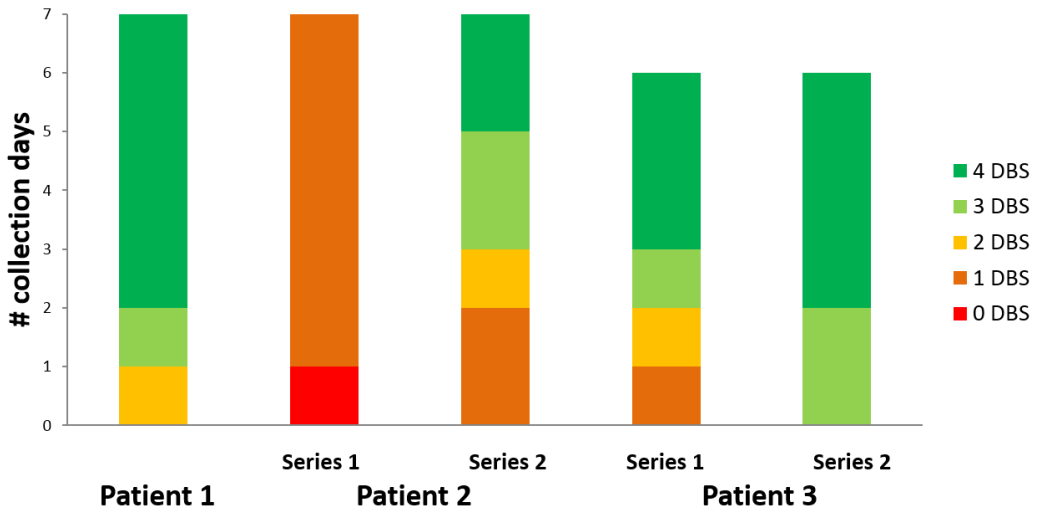


Fig. II.C.24 Overview of the number of well collected dried blood spots (DBS) per day per patient

Of the 7 patients, one patient decided to cease participation after 3 days because of nausea, one was lost in follow-up and two stated falling asleep almost immediately after the intake of sodium oxybate. The GHB concentrations found in the three patients that completed the study are in line with those previously reported in pharmacokinetic studies using plasma samples of narcoleptic patients ingesting twice-nightly a 3-g dose [77]. In addition, no interference is to be expected from sodium oxybate intake on the previous day, since in all cases there were more than 21 hours between the intake of two first doses on subsequent days. Scharf *et al*. [77] using a dosing scheme similar to the one in our report (two doses some 4 h apart), reported a decrease to endogenous levels in less than 11 hours, consistent with the reported rapid metabolism of GHB in - and elimination from - the human body [14].

With a single exception, the variation in GHB concentrations in DBS collected at the same time (within-DBS card precision) was acceptable for all duplicate measurements (< 11.5 % RSD, Table II.C.8). Besides confirming the precision of the analytical procedure, this also demonstrates the suitability of the collection technique. Patients were able to send back representative and independent samples collected in an ambulant setting, leading to valid measurements.

The intra-individual variation found in the narcoleptic patients of this study is in line with reports describing variation of serum levels following ingestion of low GHB doses in healthy subjects [78,79]. Also, variation in clinical effects has been reported [78,80]; however, it is not known whether this relates to differently obtained GHB concentrations. Although not within the scope of this exploratory study, our results demonstrate that in future studies, it may be possible to explore the relationship between GHB blood levels and sleep quality in a real-life setting using DBS sampling. In addition, although here, we opted for the most challenging scenario, in which sampling was performed shortly after the intake of the first dose (which may pose a problem for some patients), the sensitivity of our method [72] also renders sampling before or several hours after intake of the second dose a feasible option. Introduction of DBS-based sampling at home may not only allow to gain better insight to be gained into the intra-individual variations in GHB concentration from day-to-day, but may also give relevant information about the concentrations attained at a certain time point. Indeed, in addition to sampling shortly after the first sodium oxybate dose - as was done here - it may be relevant to know the GHB concentration at the time of awakening at night (just before the second dose) or in the morning (e.g. when the patient wakes up or leaves to work). Interesting to mention in this respect is the fact that hitherto it is not known whether the spontaneous awakening at night of sodium oxybate-treated patients is associated with the drop of GHB levels below a certain threshold (and - if so - whether this threshold is similar in different patients). The most representative insights into this matter may be obtained in a home setting, rather than in a hospital environment. Also, from a legal perspective, it is relevant to know whether at the time a sodium oxybate-treated patient gets into a car to drive, the GHB concentrations have dropped below 4–5 µg/ml, the cut-off value used in forensic toxicology.

Overall, the sampling technique was positively evaluated by the patients, and when patients 2 and 3 repeated the collection, improvement in DBS quality was seen. This improvement supports the idea that if in future studies adequate training is provided, the DBS collection technique may be used as an alternative to venepuncture in an ambulant setting [74-76]. No guidelines are available

on the percentage of spots that should fulfill a certain quality standard. As we asked the patients in this study to generate 4 DBS and as we wished to perform duplicate analysis (i.e., analysis on two different spots on every time point), we put forward an acceptance criterion of 50 % (i.e., there should be at least two DBS with acceptable quality: large enough, symmetrically spread on the filter paper, both sides of the filter paper being evenly coloured). In fact, for any given DBS-based method, we would recommend to strive for at least 2 suitable DBS, in order to allow sample re-analysis, when deemed necessary.

II.C.4.4 CONCLUSION

This study shows that the DBS sampling technique may be easily adapted in a real-life setting, since DBS cards with acceptable quality are obtained by non-medically trained patients without any supervision or aid of a trained person. In addition, our results demonstrate the acceptable precision associated with execution of the complete procedure, from patient self-sampling to analysis in the laboratory. Given the nature of the medication (requiring intake just before going to bed and having a very short half-life), monitoring of GHB concentrations was hitherto only possible in a hospital setting. Our study is the first to demonstrate that unsupervised sampling by sodium oxybate-treated patients at home is not only feasible, but also leads to samples with acceptable quality. Therefore, in future studies, this minimally invasive sampling technique, in which samples can be obtained in an easy and convenient way for the patient, may be used to acquire additional information on the relationship between GHB blood concentrations, the corresponding effects and adverse effects and sleep quality.

REFERENCES

1. Sabucedo AJ, Furton KG. Extractionless GC/MS analysis of gamma-hydroxybutyrate and gamma-butyrolactone with trifluoroacetic anhydride and heptafluoro-1-butanol from aqueous samples. *J Sep Sci*. 27(9):703-709 (2004).
2. Van hee P, Neels H, De Doncker M, Vrydags N, Schatteman K, Uyttenbroeck W, Hamers N, Himpe D, Lambert WE. Analysis of gamma-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. *Clin Chem Lab Med.* 42(11):1341-1345 (2004).
3. Grob K. Split and splitless injection in capillary GC. Hüthig Buch Verlag, Heidelberg, Germany, third edition (1993).
4. Wang FS, Shanfield H, Zlatklis A. Injection temperature effects using column and split sampling in capillary gas chromatography. *J High Resolution Chrom & Chrom Commun*. 6(9):471-479 (1983).
5. Rood D. A practical guide to the care, maintenance, and troubleshooting of capillary gas chromatographic systems. Wiley-VCH, Weinheim, Germany (1999).
6. Wille S. Quantitative analysis of new generation antidepressants using gas chromatography-mass spectrometry. Applications in clinical and forensic toxicology. Chapter 5 (2008).
7. Carter LP, Pardi D, Gorsline J, Griffiths RR. Illicit gamma-hydroxybutyrate (ghb) and pharmaceutical sodium oxybate (xyrem): Differences in characteristics and misuse. *Drug Alcohol Depend*. 104(1-2):1-10 (2009).
8. ElSohly MA, Salamone SJ. Prevalence of drugs used in cases of alleged sexual assault. *J Anal Toxicol*. 23(3):141-146 (1999).
9. Jones AW, Kugelberg FC, Holmgren A, Ahlner J. Occurrence of ethanol and other drugs in blood and urine specimens from female victims of alleged sexual assault. *Forensic Sci Int*. 181(1-3):40-46 (2008).
10. Koesters SC, Rogers PD, Rajasingham CR. MDMA ('ecstasy') and other 'club drugs'. The new epidemic. *Pediatr Clin North Am*. 49(2):415-433 (2002).
11. Sumnall HR, Woolfall K, Edwards S, Cole JC, Beynon CM. Use, function, and subjective experiences of gamma-hydroxybutyrate (ghb). *Drug Alcohol Depend*. 92(1-3):286-290 (2008).
12. Miotto K, Darakjian J, Basch J, Murray S, Zogg J, Rawson R. Gamma-hydroxybutyric acid: Patterns of use, effects and withdrawal. *Am J Addict*. 10(3):232-241 (2001).
13. Snead OC, 3rd, Gibson KM. Gamma-hydroxybutyric acid. *N Engl J Med*. 352(26):2721-2732 (2005).
14. Abanades S, Farre M, Segura M, Pichini S, Barral D, Pacifici R, Pellegrini M, Fonseca F, Langohr K, De La Torre R. Gamma-hydroxybutyrate (GHB) in humans: Pharmacodynamics and pharmacokinetics. *Ann N Y Acad Sci*. 1074:559-576 (2006).
15. Chin RL, Sporer KA, Cullison B, Dyer JE, Wu TD. Clinical course of gamma-hydroxybutyrate overdose. *Ann Emerg Med*. 31(6):716-722 (1998).

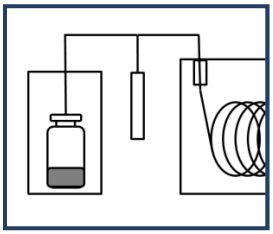
16. Ingels M, Rangan C, Bellezzo J, Clark RF. Coma and respiratory depression following the ingestion of GHB and its precursors: Three cases. *J Emerg Med*. 19(1):47-50 (2000).
17. Bosman IJ, Lusthof KJ. Forensic cases involving the use of GHB in The Netherlands. *Forensic Sci Int.* 133(1-2):17-21 (2003).
18. Kintz P, Villain M, Pelissier AL, Cirimele V, Leonetti G. Unusually high concentrations in a fatal GHB case. *J Anal Toxicol*. 29(6):582-585 (2005).
19. Andresen H, Stimpfl T, Sprys N, Schnitgerhans T, Muller A. Liquid ecstasy - a significant drug problem. *Dtsch Arztebl Int*. 105(36):599-603 (2008).
20. Elliott SP. Nonfatal instances of intoxication with gamma-hydroxybutyrate in the United Kingdom. *Ther Drug Monit*. 26(4):432-440 (2004).
21. Morris-Kukoski CL. Gamma-hydroxybutyrate: Bridging the clinical-analytical gap. *Toxicol Rev*. 23(1):33-43 (2004).
22. Wood M, Laloup M, Samyn N, Morris MR, de Bruijn EA, Maes RA, Young MS, Maes V, De Boeck G. Simultaneous analysis of gamma-hydroxybutyric acid and its precursors in urine using liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 1056(1-2):83-90 (2004).
23. Zacharis CK, Raikos N, Giouvalakis N, Tsoukali-Papadopoulou H, Theodoridis GA. A new method for the hplc determination of gamma-hydroxybutyric acid (ghb) following derivatization with a coumarin analogue and fluorescence detection: Application in the analysis of biological fluids. *Talanta*. 75(2):356-361 (2008).
24. Gong XY, Kuban P, Scholer A, Hauser PC. Determination of gamma-hydroxybutyric acid in clinical samples using capillary electrophoresis with contactless conductivity detection. *J Chromatogr A*. 1213(1):100-104 (2008).
25. Brown SD, Rhodes DJ, Pritchard BJ A validated SPME-GC-MS method for simultaneous quantification of club drugs in human urine. *Forensic Sci Int*. 171(2-3):142-150 (2007).
26. Lenz D, Kroner L, Rothschild MA. Determination of gamma-hydroxybutyric acid in serum and urine by headspace solid-phase dynamic extraction combined with gas chromatography-positive chemical ionization mass spectrometry. *J Chromatogr A*. 1216(18):4090-4096 (2009).
27. LeBeau MA, Christenson RH, Levine B, Darwin WD, Huestis MA. Intra- and interindividual variations in urinary concentrations of endogenous gamma-hydroxybutyrate. *J Anal Toxicol*. 26(6):340-346 (2002).
28. Elian AA. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Sci Int*. 128(3):120-122 (2002).
29. Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H. Endogenous level and in vitro production of GHB in blood from healthy humans, and the interpretation of GHB levels detected in antemortem blood samples. *J Health Sci*. 51(2):147-154 (2005).

30. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S. Gamma-hydroxybutyrate in urine and serum: Additional data supporting current cut-off recommendations. *Forensic Sci Int*. 200(1-3):93-99 (2010).
31. McDade TW, Williams S, Snodgrass JJ. What a drop can do: Dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography*. 44(4):899-925 (2007).
32. Edelbroek PM, van der Heijden J, Stolk LM. Dried blood spot methods in therapeutic drug monitoring: Methods, assays, and pitfalls. *Ther Drug Monit*. 31(3):327-336 (2009).
33. Lejeune D, Souletie I, Houze S, Le bricon T, Le bras J, Gourmel B, Houze P. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. *J Pharm Biomed Anal*. 43(3):1106-1115 (2007).
34. Meesters RJ, van Kampen JJ, Reedijk ML, Scheuer RD, Dekker LJ, Burger DM, Hartwig NG, Osterhaus AD, Luider TM, Gruters RA. Ultrafast and high-throughput mass spectrometric assay for therapeutic drug monitoring of antiretroviral drugs in pediatric hiv-1 infection applying dried blood spots. *Anal Bioanal Chem*. 398(1):319-328 (2010).
35. Jones AW, Holmgren A, Kugelberg FC. Driving under the influence of gamma-hydroxybutyrate (GHB). *Forensic Sci Med Pathol*. 4(4):205-211 (2008).
36. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci Int*. 161(2-3):158-(2006).
37. Forni S, Pearl PL, Gibson KM, Yu Y, Sweetman L. Quantitation of gamma-hydroxybutyric acid in dried blood spots: feasibility assessment for newborn screening of succinic semialdehyde dehydrogenase (SSADH) deficiency. Mol Genet Metab. 109(3):255-259 (2013).
38. Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Sci Int*. 165(2-3):216-224 (2007).
39. Guidance for industry, bioanalytical method validation (2001) U.S. Department of health and human services, food and drug administration. Rockville, USA. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf. Accessed 14 July 2010.
40. EMA Guideline on bioanalytical method validation, Committee for Medicinal Products for Human Use (CHMP), 21 July 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf
41. Almeida AM, Castel-Branco MM, Falcao AC. Linear regression for calibration lines revisited: Weighting schemes for bioanalytical methods. *J Chromatogr B Analyt Technol Biomed Life Sci*. 774(2):215-222 (2002).

42. Rivier L. Criteria for the identification of compounds by liquid chromatography-mass spectrometry and liquid chromatography-multiple mass spectrometry in forensic toxicology and doping analysis. *Analytica Chimica Acta*. 492(1-2):69-82 (2003).
43. Wille SMR, Peters FT, Di Fazio V and Samyn N. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. *Accred Qual Assur*. 16(6):279-292 (2011).
44. Garcia Boy R, Henseler J, Mattern R, Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. *Ther Drug Monit*. 30(6):733-739 (2008).
45. Déglon J, Lauer E, Thomas A, Mangin P, Staub C. Use of the dried blood spot sampling process coupled with fast gas chromatography and negative-ion chemical ionization tandem mass spectrometry: Application to fluoxetine, norfluoxetine, reboxetine, and paroxetine analysis. *Anal Bioanal Chem*. 396(7):2523-2532 (2010).
46. Chappell JS, Meyn AW, Ngim KK. The extraction and infrared identification of gamma-hydroxybutyric acid (ghb) from aqueous solutions. *J Forensic Sci*. 49(1):52-59 (2004).
47. Hennessy SA, Moane SM, McDermott SD. The reactivity of gamma-hydroxybutyric acid (GHB) and gamma-butyrolactone (GBL) in alcoholic solutions. *J Forensic Sci*. 49(6):1220-1229 (2004).
48. Ciolino LA, Mesmer MZ, Satzger RD, Machal AC, McCauley HA, Mohrhaus AS. The chemical interconversion of GHB and GBL: Forensic issues and implications. *J Forensic Sci*. 46(6):1315-1323 (2001).
49. Lenz D, Rothschild MA, Kroner L. Intoxications due to ingestion of gamma-butyrolactone: Organ distribution of gamma-hydroxybutyric acid and gamma-butyrolactone. *Ther Drug Monit*. 30(6):755-761 (2008).
50. de Boer T, Wieling J, Meulman E, Reuvers M, Renkema G, den Daas I, van Iersel T, Wemer J, Chen L. Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. *Biomed. Chromatogr.* 25:1112-1123 (2011).
51. Li W, Tse F. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed. Chromatogr.* 24(1), 49-65 (2010).
52. Ingels ASME, Lambert W and Stove C. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal. Bioanal. Chem.* 398(5), 2173-2182 (2010).
53. Mercolini L, Mandrioli R, Gerra G and Raggi MA. Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection A novel test for cocaine and alcohol intake. *J Chromatogr A.* 1217(46):7242-7248 (2010).
54. Jantos R, Veldstra JL, Mattern R, Brookhuis KA and Skopp G. Analysis of 3,4-Methylenedioxymetamphetamine: Whole Blood Versus Dried Blood Spots. *J Anal Tox.* 35(5):269-273 (2011).

55. Spooner N, Lad R and Barfield M. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: Considerations for the validation of a quantitative bioanalytical method. *Anal Chem.* 81(4):1557-1563 (2009).
56. Kolocouri F, Dotsikas Y and Loukas YL. Dried plasma spots as an alternative sample collection technique for the quantitative LC-MS/MS determination of gabapentin. *Anal Bioanal Chem.* 398(3):1339-1347 (2010).
57. Wilhelm AJ, Den Burger JCG, Vos RM, Chahbouni A and Sinjewel A. Analysis of Cyclosporin a in Dried Blood Spots using Liquid Chromatography Tandem Mass Spectrometry. *J Chromatogr B*. 877:1595-1598 (2009).
58. Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Methods Med Res*. 8:135-160 (1999).
59. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clincal chemistry, Part I. *J Clin Chem Clin Biochem*. 21:709-720 (1983).
60. Capiau S, Stove VV, Lambert WE, Stove CP. Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer. *Anal Chem*. 85(1):404-410 (2013).
61. Wilhelm AJ, Klijn A, den Burger JC, Visser OJ, Veldkamp AI, Janssen JJ, Swart EL. Clinical validation of dried blood spot sampling in therapeutic drug monitoring of ciclosporin A in allogeneic stem cell transplant recipients: direct comparison between capillary and venous sampling. *Ther Drug Monit*. 35(1):92-95 (2013).
62. Schutt BS, Weber K, Elmlinger MW and Ranke MB. Measuring IGF-I, IGFBP-2 and IGFBP-3 from dried blood spots on filter paper is not only practical but also reliable. *Growth Horm IGF Res.* 13(2-3):75-80 (2003).
63. Holub M, Tuschl K, Ratschmann R, Strnadová KA, Mühl A, Heinze G, Sperl W, Bodamer OA. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. *Clin Chim Acta.* 373(1-2): 27-31 (2006).
64. Vu DH, Koster RA, Alffenaar JW, Brouwers JR and Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. *J Chromatogr B Analyt Technol Biomed Life Sci.* 879(15-16):1063-1070 (2011).
65. Rainey PM. Relation between serum and whole-blood ethanol concentrations. *Clin Chem.* 39(11):2288-2292 (1993).
66. Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis*. 2(11):1791-1796 (2010).
67. De Kesel PM, Sadones N, Capiau S, Lambert WE, Stove CP. Hemato-critical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis*. 5(16):2023-2041 (2013).

68. Wood M, Dargan PI, Wood DM, Mistry N, Goshawk J, Archer JRH. Broad toxicological screening results from suspected GHB/GBL overdose cases. Proceedings of the 51th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). Funchal, Madeira, Portugal (2013).
69. Fuller DE, Hornfeldt CS, Kelloway JS, et al. The Xyrem® risk management program. *Drug Saf*. 27(5):293-306 (2004).
70. Pardi D, Black J. γ-Hydroxybutyrate/sodium oxybate neurobiology, and impact on sleep and wakefulness. *CNS Drug.* 20(12):993-1018 (2006).
71. Robinson DM, Keating GM. Sodium oxybate: a review of its use in the management of narcolepsy. *CNS Drugs.* 21(4):337-354 (2007).
72. Ingels ASME, De Paepe P, Anseeuw K, et al. Dried blood spot punches for the confirmation of suspected γ-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis*. 3(20):2271-2281 (2011).
73. Stove CP, Ingels ASME, De Kesel PMM, Lambert WE. Dried blood spots in toxicology: from the cradle to the grave? *Crit Rev Toxicol*. 42(3):230-243 (2012).
74. Leichtle AB, Ceglarek U, Witzigmann H, Gäbel G, Thiery J, Fiedler GM. Potential of dried blood self-sampling for cyclosporine C_2 monitoring in transplant outpatients. *J Transplant.* 2010: 201918 (2010).
75. Fokkema MR, Bakker AJ, de Boer F, Kooistra J, de Vries S, Wolthuis A. HbA1c measurements from dried blood spots: validation and patient satisfaction. *Clin Chem Lab Med*. 47(10):1259-1264 (2009).
76. Williams SR, McDade TW. The use of dried blood spot sampling in the national social life, health and aging project. *J Gerontol B Psycol Sci Soc Sci.* 64 (Suppl. 1):i131-136 (2009).
77. Scharf MB, Lai AA, Branigan B, Stover R, Berkowitz DB. Pharmacokinetics of gamma-hydroxybutyrate (GHB) in narcoleptic patients. *Sleep*. 21(5):507-514 (1998).
78. Zvosec DL, Smith SW, Hall BJ. Three deaths associated with the use of Xyrem®. *Sleep Med*. 10(4):490-493 (2009).
79. Brenneisen R, Elsohly MA, Murphy TP, Passarelli J, Russmann S, Salamone SJ, Watson DE. Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. *J Anal Toxicol*. 28(8):625-630 (2004).
80. Dyer JE. Gamma-Hydroxybutyrate: a health-food product producing coma and seizure-like activity. *Am J Emerg Med*. 9(4):321-324 (1999).



PART III
DETERMINATION OF GHB IN BIOFLUIDS USING A ONE-STEP PROCEDURE WITH "IN-VIAL" DERIVATIZATION AND HS-TRAP GC-MS

The aim of this part was to evaluate if a relatively new commercially available technique, headspace-trap (HS-trap), combined with GC-MS may be a valuable alternative for existing methods to determine GHB in biofluids. First, in ***Chapter III.A****, a brief overview of headspace techniques is given, with the focus on headspace-trap and its published applications.*

Secondly, ***Chapter III.B*** *describes the optimization of the derivatization reaction, as well as of the headspace-trap GC-MS method to determine GHB in urine, plasma, serum and whole blood. Using a (HS-) trap for sample introduction should decrease sample treatment and increase method sensitivity. Indeed, sample treatment only consists of the addition of salt and derivatization reagents to an aliquot of the sample, followed by proper sealing of the vial which is subsequently placed in the HS oven. Different parameters such as sample volume, amount of salt added, the derivatization procedure, the HS oven temperature, time to reach equilibrium, trap*

settings and GC-MS parameters were optimized to determine low levels of GHB in only 100 µl of various biofluids. Since GHB requires derivatization, we opted for an "in-vial" derivatization reaction, which, when combined with HS-trap, extends the application range of this technique to non-volatile analytes, with a negligible increase in workload. Finally, validation has been performed and the applicability of the method was demonstrated by the analysis of samples collected from suspected GHB-intoxicated patients.

CHAPTER III.A

HEADSPACE INJECTION TECHNIQUES: FOCUS ON HEADSPACE-TRAP

III.A.1 INTRODUCTION

Extraction of various body fluids or tissue samples to separate analytes of interest (e.g. toxic compounds or their metabolites in forensic and clinical toxicology) from the complex biological matrix is an important step of almost every analysis. Hereby, one of the aims is to minimize the disturbing response of interferences caused by matrix constituents during subsequent chromatographic-spectrometric analysis [1]. If the analytes of interest are volatile enough, headspace (HS) techniques may be of interest as they combine extraction and injection in a single step. When applying static headspace, volatile analytes are extracted from a solid or liquid sample and injected onto a gas chromatographic (GC)-column as a gaseous phase. Only those analytes that are volatile enough will reach the GC-column, and can thus be analyzed without the interference of the non-volatile matrix [2]. Modifications of the classical procedure may involve adsorption of the analytes of interest on or in surfaces of fibers, inside an injection needle or inside a capillary, followed by desorption by evaporation at high injector temperatures (GC) or by dissolution in the mobile phase (liquid chromatography, LC) [1] .

III.A.2 STATIC AND DYNAMIC HEADSPACE

When analyzing samples with static or classical headspace, a liquid or solid sample is placed in a HS vial, the vial is sealed properly and subsequently placed in the HS oven (Fig. III.A.1). Volatile components will partition between the gas phase and the sample, until equilibrium has been reached. Next, an aliquot of the headspace is brought onto the GC column [2].

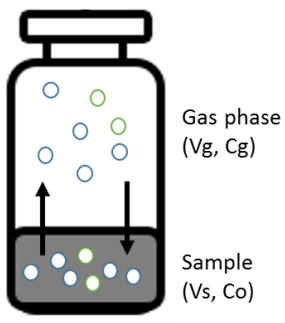


Fig. III.A.1 Principle of static headspace: by heating the vial, volatile components will partition between the gas phase and the sample until equilibrium has been reached.

The peak area is proportional to the concentration of the analyte in the gas phase:

$$A \sim Cg = Co / K + \beta$$

Where A = peak area

Cg = concentration in the gas phase (mg/l)

Co = original concentration in the sample (mg/l)

K = partition coefficient

$\beta = Vg/Vs$ = phase ratio

To improve the sensitivity of a HS method for a given sample with concentration Co of the analyte of interest, K and β can be optimized. The partition coefficient or K can be influenced by changing the HS oven temperature (equilibration temperature), while by changing the sample volume (Vs) the phase ratio or β can be optimized. The influence of K and β is analyte-specific and should be evaluated separately in each case. The HS oven temperature is limited by the sample matrix (e.g. for aqueous samples, the oven temperature should not exceed 100 °C) and by the developed pressure when heating the sample vial. Also the sample volume (Vs) is limited: the needle may not enter the sample phase and there should be enough gas phase that can be transferred to the analytical column [2].

Furthermore:

$$K \sim 1 / p_i \times \gamma_i$$

With p_i = pressure

γ_i = activation coefficient

Ideally, the analyte(s) of interest should resolve better in the gaseous phase than in the matrix. For aqueous samples with polar compounds, sensitivity may be improved substantially by adjusting the matrix, so by influencing K by adjusting γ_i. This can be accomplished by the addition of electrolytes or by salting out, thereby decreasing the solubility of the analytes in the matrix and

increasing the concentration in the gaseous phase. In addition, β is changed *via* an additional volume-effect. Finally, besides matrix adjustments, the chemical properties of the analytes of interest can also be modified by derivatization, resulting in less polar and more volatile analytes more suitable for headspace analysis [2].

Furthermore, dissolving inorganic salts may improve sample-to-sample reproducibility. For this reason, the liquid sample can also be diluted with a solvent reducing the influence of the sample matrix on the analyte distribution or the sample can be evaporated completely no longer requiring partitioning between the 2 phases (total or full vaporization technique) [2]. Also the addition of an internal standard can eliminate the effect of small variations in the matrix.

A second headspace technique is dynamic headspace or purge and trap, based on a continuous gas extraction by a continuous flow of new carrier gas in or through the sample, to remove all volatile analytes from the sample matrix. The analytes in the gas phase are first collected onto a trap, where they are concentrated. At the end of the extraction, the trap is heated to release the analytes of interest (desorption), which are then brought onto the analytical column for analysis [2]. This technique mostly has a better sensitivity than when using static headspace, but may suffer from carry-over and may have difficulties to eliminate water before injection [3].

A modification allowing miniaturization, sample enrichment and sample transfer is solid-phase micro-extraction (SPME). Here, the analytes of interest are extracted from a gas or liquid sample by adsorption in or on coated surfaces of fibers, inside needles or inside capillaries. In the case of HS extraction, a fiber or needle is placed in the gas phase, allowing analytes in the gas phase to adsorb and to concentrate before desorption. Besides the general advantage of SPME, being a solvent-free and fully automatable technique, headspace SPME has as an additional advantage over classical HS that it provides cleaner extracts, since it allows injection without excess of air. Furthermore, when compared to SPME where the fiber or needle is placed in the liquid sample, headspace SPME also minimizes fiber damage by aggressive or irreversibly adsorbed matrix components [1].

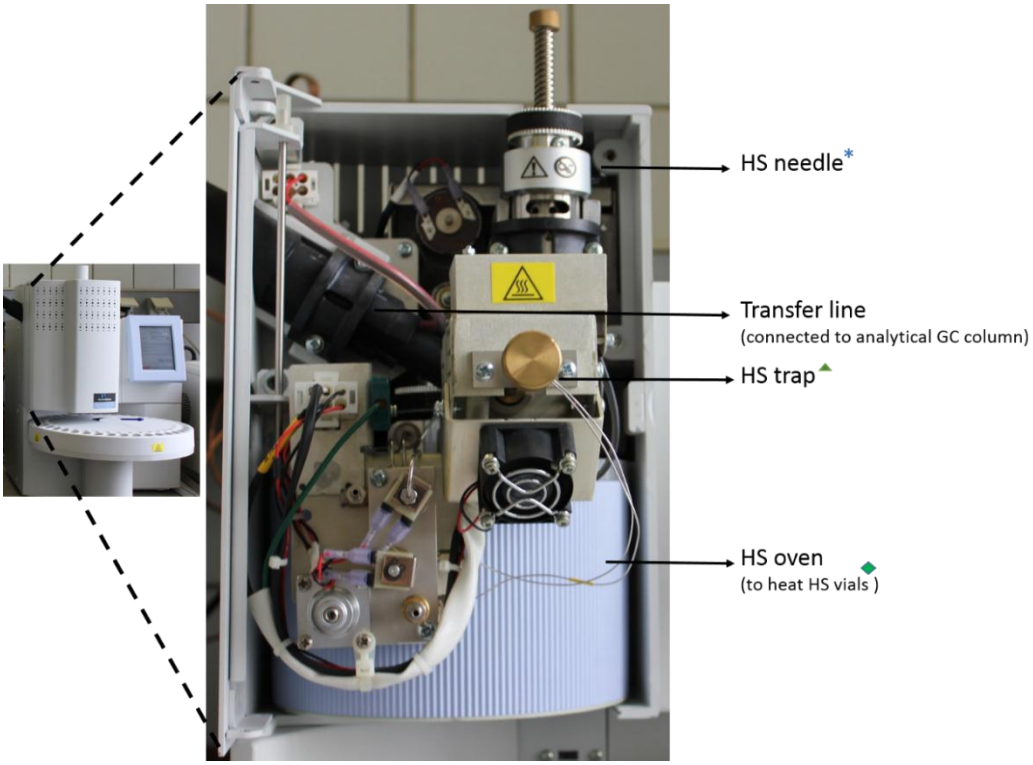
III.A.3 HS-TRAP

A relatively new commercialized technique, HS-trap, encompasses an enhanced static HS system covering both static and dynamic HS properties (Fig. III.A.2). The principle of the HS-trap method has been illustrated by Barani *et al*. [3] and Schulz *et al*. [4]. A brief description of the procedure is given below (variables are given in italics) and shown in Fig. III.A.2.

A vial is heated *(thermostat time and oven temperature)* and when the analytes reach equilibrium between headspace and sample matrix, an aliquot of the headspace is withdrawn. To this end, the vial is pressurized *(vial pressure and pressurization time)*, followed by trap loading until the pressure in the HS vial reaches atmospheric pressure *(decay time)*. The trap is maintained at lower temperature *(trap low temperature)*, just above ambient, leading to condensation of the gas phase, while water can be removed by purging helium through the trap *(dry purge time and pressure)*. Then, the trap is heated *(trap high temperature)* and backflushed, leading to desorption of the analytes that enter the chromatographic system *(desorption time and pressure)* [5].

As mentioned above, an advantage of this technique is the possibility to eliminate water before injection (dry purge) by purging helium through the trap. This is important since injection of large amounts of water may disturb flame ionization detection (FID) or mass spectrometric (MS) signal stability, resulting in peak broadening and in higher background. Furthermore, similar to dynamic HS, a larger fraction of the HS can be analyzed compared to static HS, since the analytes are first concentrated on the trap before desorption, enhancing sensitivity. To further increase sensitivity, without excessively increasing analysis time, loading of the trap can be repeated up to 4 times (trap load) [3,4].

(a)



(b)

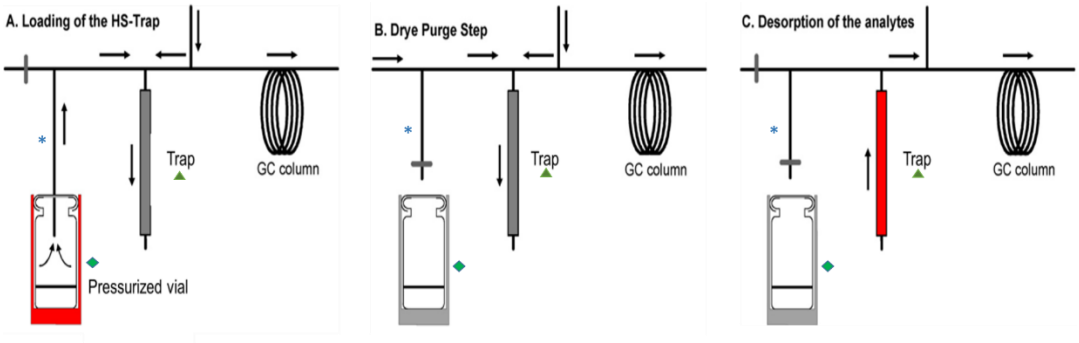


Fig. III.A.2 A headspace (HS)-trap system from PerkinElmer (a), together with a schematic overview of the principle of sample preparation using HS-trap (b, figure modified from Schulz *et al.* [4])

A. Trap loading is accomplished by pressurizing the sample vial and allowing the pressure to decay through the cooled adsorbent trap
B. A drying step removes moisture from the trap
C. After thermal desorption, the analytes are transported by the carrier gas into the GC column for separation

The HS-trap technique has already been successfully applied to determine various volatile compounds in water [3,5], spirits [4], soil [6], apple juices [7] and plant matrices [8] (Table III.A.1) in combination with GC-FID or GC-MS analysis.

Table III.A.1 Overview of other published methods using headspace-trap

Reference	Analytes of interest	Matrix	Analytical column (run time)	Equilibration time and temperature	Trap	Mode/ Type of injection
Barani et al.; 2006 [3]	Volatile organic compounds (VOCs)	Water	6 % cyanopropylphenyl - 94 % dimethyl-polysiloxane 60 m x 0.32 mm; 1.8 µm (GC-FID) 60 m x 0.25 mm; 1.4 µm (GC-MS) (12 min)	20 min at 70 °C Shaker ON	Air Toxic®	Constant pressure mode 33 psi/ NS
Schulz et al.; 2007 [4]	Volatile constituents	Spirits	6 % cyanopropylphenyl - 94 % dimethyl-polysiloxane 60 m x 0.53 mm; 1.5 µm (45 min)	20 min at 80 °C	Air Toxic®	Constant pressure mode 13 psi/ Splitless injection
Røen et al.; 2010a and b [5,6]	Sulphur mustard and related compounds	Water Soil	5 % diphenyl - 95 % dimethyl-polysiloxane 30 m x 0.25 mm; 0.25 µm (20 min)	15 min at 80 °C Shaker ON	Tenax™	Constant pressure mode 15 psi/ Direct connection
Nikfardjam and Maier; 2011 [7]	Aldehydes Ketones Aroma's Alcohols	Apple juices	6 % cyanopropylphenyl - 94 % dimethyl-polysiloxane 30 m x 0.25 mm; 1.4 µm (45 min)	20 min at 80 °C	Air Toxic®	Constant pressure mode 40 psi/ NS
Aberl and Coelhan; 2012 [8]	Volatile constituents in hops	Plant matrices	5 % diphenyl - 95 % dimethyl-polysiloxane 60 m x 0.25 mm; 0.25 µm (38.5 min)	45 min at 85 °C	Air Toxic®	Constant pressure mode 22 psi/ NS

NS: not specified

There are currently 2 traps (fused silica capillaries filled with adsorbent material) commercially available by PerkinElmer, Air Toxic© and Tenax™. According to the manufacturer, Air Toxic© contains graphitized carbon, a relatively weak adsorbent for very volatile analytes, and a molecular sieve with strong adsorbing material. This trap is typically used for the determination of volatile organic components (VOC) such as aromatics and volatile alkanes. On the other hand, the turbomatrix thermal desorber trap packed with Tenax™ consists of a porous polymer of 2,6-diphenyl oxide and shows better results for volatile analytes, with little or no retention of the very volatile components. The highest recommended working temperatures for Air Toxic© and Tenax™ are 375 and 280 °C, respectively [4,5].

REFERENCES

1. Pragst F. Application of solid-phase microextraction in analytical toxicology. *Anal Bioanal Chem*. 388:1393-1414 (2007).
2. Kolb B, Ettre LS, Static headspace – gas chromatography: theory and practice, John Wiley and sons inc., Hoboken, New Jersey, 2nd edition (2006).
3. Barani F, Dell'Amico N, Griffone L, Santoro M, Tarabella C. Determination of volatile organic compounds by headspace trap. *J Chromatogr Sci*. 44(10):625-630 (2006).
4. Schulz K, Dreβler J, Sohnius EM, Lachenmeier DW. Determination of volatile constituents in spirits using headspace trap technology. *J Chrom A*. 1145(1-2):204-209 (2007).
5. Røen BT, Unneberg E, Tørnes JAa, Lundanes E. Trace determination of sulphur mustard and related compounds in water by headspace-trap gas chromatography–mass spectrometry. *J Chrom A*. 1217(5):761-767 (2010).
6. Røen BT, Unneberg E, Tørnes JAa, Lundanes E. Headspace-trap gas chromatography–mass spectrometry for determination of sulphur mustard and related compounds in soil. . *J Chrom A*. 1217(14):2171-2178 (2010).
7. Nikfardjam MP, Maier D. Development of a headspace trap HRGC/MS method for the assessment of the relevance of certain aroma compounds on the sensorial characteristics of commercial apple juice. *Food Chem*. 126(4):1926-1933 (2011).
8. Aberl A, Coelhan M. Determination of volatile compounds in different hop varieties by headspace-trap GC/MS in comparison with conventional hop essential oil analysis. *J Agric Food Chem*. 60(11):2785-2792 (2012).

CHAPTER III.B
DETERMINATION OF GHB IN BIOFLUIDS USING "IN-VIAL" DERIVATIZATION AND HEADSPACE-TRAP GC-MS

Based on

Determination of gamma-hydroxybutyric acid in biofluids using a one-step procedure with "in-vial" derivatization and headspace-trap gas chromatography-mass spectrometry. J Chrom A, 1296:84-92 (2013). Ann-Sofie ME Ingels, Hugo Neels, Willy E Lambert, Christophe P Stove.

III.B.1 INTRODUCTION

In numerous analytical methods to detect and quantify drugs in biological matrices, sample preparation is regarded as the most laborious and time-consuming step [1,2]. Hence, a tendency towards less demanding procedures has been noted, such as automated solid phase extraction (SPE), dilution or filtration of the samples prior to injection, thereby reducing manual operations, often with a simultaneous reduction of the initial sample size [1,2]. Moreover, if the analytes of interest are volatile enough, headspace (HS) extraction techniques can be used as these combine extraction and injection in a single step. Only relatively recently, a new HS technique has been commercialized: HS-trap, encompassing an enhanced static headspace system covering both static and dynamic HS properties. This technique, already successfully applied to determine various volatile compounds in water [3], spirits [4], soil [5], apple juices [6] and plant matrices [7], was evaluated for its possibility to determine gamma-hydroxybutyric acid (GHB), a low molecular weight compound and drug of abuse, in biofluids such as urine, plasma, serum and whole blood. As mentioned earlier, detection and quantification of GHB in biological matrices is of interest in toxicological cases, since recreational use with limited awareness of the possibility to suddenly fall into a coma has resulted in an increase in GHB intoxications at raves or other dance parties [8,9].

Reported procedures, mostly using gas chromatography (GC) -although liquid chromatography (LC) and capillary zone electrophoresis have been applied as well- often include laborious sample preparation steps. While in a UHPLC-MS/MS method mere filtration and dilution prior to injection was shown to suffice as sample preparation for urine samples, extraction with SPE was required for whole blood samples [10]. For GC analysis, either derivatization to a more volatile derivative or conversion to the more volatile lactone-form gamma-butyrolactone (GBL) is required [11].

Several options have been described that allow reducing the work-load associated with GC-MS-based GHB determination in biological fluids. These include the addition of excess derivatization reagents directly to biological samples, thus omitting the extraction step [12-15], the use of HS injection after conversion of GHB to gamma-butyrolactone (GBL) [16-18] or “in-vial” derivatization with for example hexylchloroformate prior to solid-phase microextraction (SPME) [19] or HS injection [20]. The available HS-based methods, either ‘classical’ static HS [16], SPME [17,20], or solid-phase dynamic extraction (SPDE) [18], all start from 0.5-1 ml of biofluid (water, urine, plasma, serum or whole blood), providing sufficient sensitivity (LLOQ from 0.1 to 5.0 µg/ml). In this study,

we evaluated whether the combination of two advantageous options, “in-vial” derivatization/conversion and headspace injection, may represent a valuable alternative for existing methods. A third advantage is that smaller sample volumes may be analyzed, while still providing enough sensitivity. This is because, when compared to SPME or SPDE, the trap described here allows sampling of a larger headspace fraction, since its adsorptive capacity (having a solid sorbent volume of 160 mm^3) is much larger than that of the small fibers or coated capillaries (having volumes of 0.94-5.99 mm^3) used in SPME or SPDE [4]. Furthermore, by combining “in-vial” derivatization and HS-trap, the application range can be extended to non-volatile analytes, with a negligible increase in workload [21].

The aim of this study was to develop a HS-trap GC-MS procedure to determine GHB in a small sample volume (100 µl) of biofluids with minimal sample handling: sample handling is limited to the addition of a sample and reagents to a single vial, followed by closing the vial. Method validation was performed and applicability was demonstrated by analysis of samples from suspected GHB-intoxicated patients.

III.B.2 MATERIALS AND METHODS

III.B.2.1 CHEMICALS AND REAGENTS

Na-GHB and its internal standard (IS) Na-GHB-d6 were supplied by LGC standards (Molsheim, France). Dimethyl sulphate (DMS) and tetrabutylammonium-hydrogensulphate (TBA-HSO_4) were delivered by Sigma-Aldrich (Steinheim, Germany), while anhydrous sodium sulphate (Na_2SO_4) and methanol (MeOH) of analytical grade were supplied by Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was supplied by VWR (Stockholm, Sweden). Ultrapure water from a Synergy ultrapure water purification system (Millipore, Brussels, Belgium) was used in the experiments.

III.B.2.2 PREPARATION OF STOCK AND WORKING SOLUTIONS

A 10 mg/ml stock solution of GHB was prepared by dissolving 12.2 mg Na-GHB powder in 1 ml MeOH. Working solutions of GHB of 1 and 0.1 mg/ml were prepared by appropriate dilution of the stock solution in MeOH. From a second, separately prepared 10 mg/ml stock solution of GHB, 1 and 0.1 mg/ml working solutions were prepared for quality control (QC) samples.

Similar to the preparation of the GHB stock solutions, a 10 mg/ml stock solution of GHB-d6 was prepared in MeOH. By appropriate dilution of this stock solution in water, a 0.30 mg/ml GHB-d6 working solution was freshly prepared each week or as needed, and stored at -20 °C.

III.B.2.3 HEADSPACE-TRAP SETTINGS

A PerkinElmer Turbomatrix HS-40 trap automatic headspace sampler with trap enrichment (Shelton, CT, USA) was used. There are currently 2 traps commercially available by PerkinElmer, Air Toxic© and Tenax™. Both packing materials were tested and based on preliminary results Tenax™ was used in further experiments, providing a signal for methylated GHB in the chromatogram. HS vials of 22 ml with polytetrafluoroethylene (PTFE)/silicone septa (delivered by PerkinElmer), were used once, as recommended by the manufacturer; the derivatization reagent required caution and proper sample handling and waste removal [22]. The HS oven can thermostat several vials at the same time, the period from injection to injection was automatically calculated by the HS system and was set at 30 min.

HS-trap conditions were optimized to determine GHB in urine, plasma and whole blood. To optimize trap parameters and vial pressure and pressurization time, water samples spiked with GHB were analyzed. Other parameters were optimized using spiked biofluids, as described below and more into detail in the results and discussion section. Table III.B.1 gives an overview of all the parameters that need to be optimized.

Table III.B.1 Overview of HS and trap variables

Headspace conditions		
Oven temperature Needle temperature	Thermostat time Vial pressurization time Decay time = Trap load time	Column pressure Vial pressure
Trap settings		
Trap low temperature Trap high temperature	Dry purge time Desorption time Trap hold time	Desorption pressure (same pressure during dry purge: Dry purge pressure)

III.B.2.3.1 Effect of salting out

The effect of adding anhydrous Na_2SO_4 to the sample matrix was evaluated by analyzing samples with increasing amounts of salt. Therefore, to 100 µl of biofluid spiked with 10 (urine) or 25 (plasma and whole blood) µg/ml GHB, no salt, 50, 100, 120 or 150 mg Na_2SO_4 (n=3) was added. Fifteen µl of a 5-M NaOH solution and 30 µl DMS were added to derivatize GHB to its corresponding di-methyl derivative. The vials were closed immediately and analyzed within 24 h. Resulting peak areas of GHB were compared (n=3 for each condition) and significance of differences was checked at 0.05 significance level using one-way ANOVA with post hoc Bonferroni correction.

III.B.2.3.2 Equilibration time and temperature

To determine the equilibration time, 100 µl of biofluid was spiked with 25 µg/ml GHB; 100 or 50 mg (whole blood) Na_2SO_4, 15 µl NaOH 5 M and 30 µl DMS were added and the HS vial was closed. Analysis was performed in triplicate and the time to reach equilibrium was varied from 10 to 60 min. The resulting peak areas of GHB were plotted in function of time; when the signal reaches a plateau, this indicates that equilibrium is reached [23].

III.B.2.4 GC-MS ANALYSIS

The HS-trap was connected to a 6890 Agilent gas chromatographic system and a 5973 mass spectrometer (Agilent, Avondale, PA, USA). The GC-MS procedure was optimized to selectively determine GHB in biofluids using HS-trap as injection technique. During preliminary experiments, better results were obtained when using a capillary GC column with a stationary phase consisting of 94 % dimethyl 6 % cyanopropylphenyl polysiloxane (DB-624; Agilent, Avondale, PA, USA; Fig. III.B.1) instead of the commonly used 95 % methyl 5 % phenyl polysiloxane (30 m x 0.25 mm i.d., 0.25 µm film thickness). The column used had a length of 30 m, a 0.25 mm internal diameter with a 1.4 µm film thickness. Helium was used as carrier gas, at constant column inlet pressure of 15 psi. To separate derivatized GHB from interfering components, various temperature programs were evaluated, and finally, the following temperature program was chosen: The GC temperature program started at 40 °C, which was maintained for 2 min, then ramped at 10 °C/min to 170 °C, resulting in a total run time of 15 min. Solvent delay was set at 9 min, and derivatized GHB-d6 and GHB eluted at 11.82 and 11.88 min, respectively. The HS transfer line was directly coupled to the analytical column using a butt connector, and was maintained at 150 °C, as recommended by the manufacturer, to avoid condensation on possible cold spots [3].

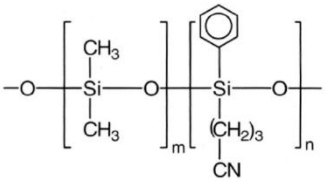


Fig. III.B.1 Structure of poly(dimethylcyanopropylphenyl)siloxane

The mass spectrometer operated in the electron impact (EI) mode with ionization energy of 70 eV. The transfer line temperature was set at 210 °C, the ion source at 230 °C, and the quadrupole temperature at 150 °C. Following the initial acquisition of the mass spectra in SCAN mode, selective *m/z* fragments were selected of derivatized GHB and its IS (Fig. III.B.2). For GHB quantification in various biological matrices, the mass spectrometer operated in the selected ion monitoring (SIM) mode. The following *m/z* ions were chosen for GHB: 117, 101, 74 and 59 (Fig. III.B.3); and for GHB-d6: 123, 107, 77 and 63. Underscored ions represent quantifier ions, which are not the most abundant but the most selective for GHB and GHB-d6. As GHB is a low molecular weight compound, and methylation does not result in the formation of high mass fragments, quantifier and qualifier ions were not chosen based on relative intensities, but on being selective for GHB and GHB-d6 [13]. Fragment *m/z* 117 is formed by the loss of the neutral group CH_3, and the other proposed fragments are presented in Fig. III.B.3. Data acquisition and integration were carried out with Chemstation software MSDChem (Agilent, Avondale, PA, USA).

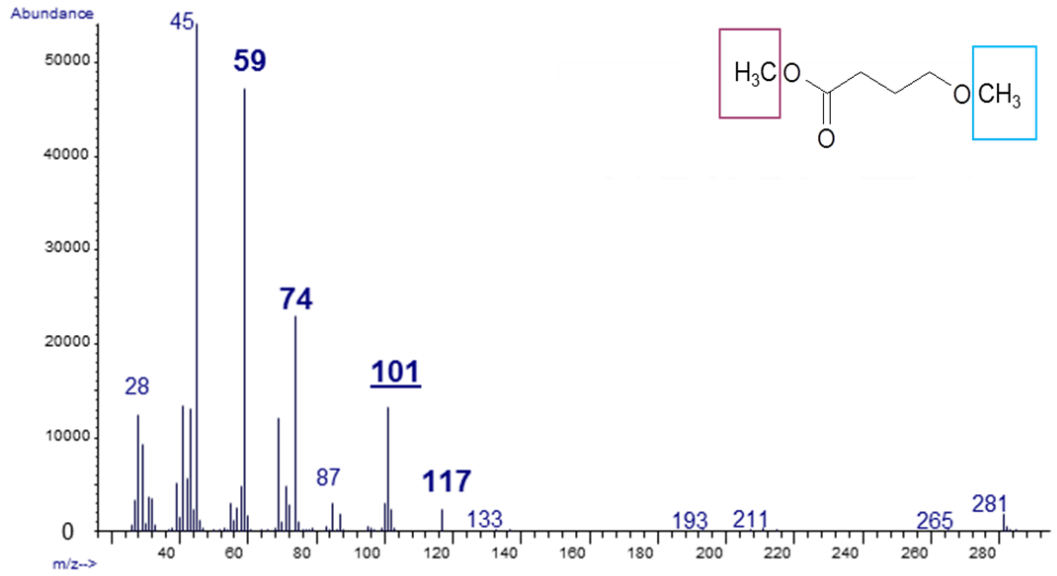


Fig. III.B.2 Mass spectrum of GHB derivatized with dimethylsulphate (bold ions were chosen to be monitored in SIM, underscored ion was used for quantification)

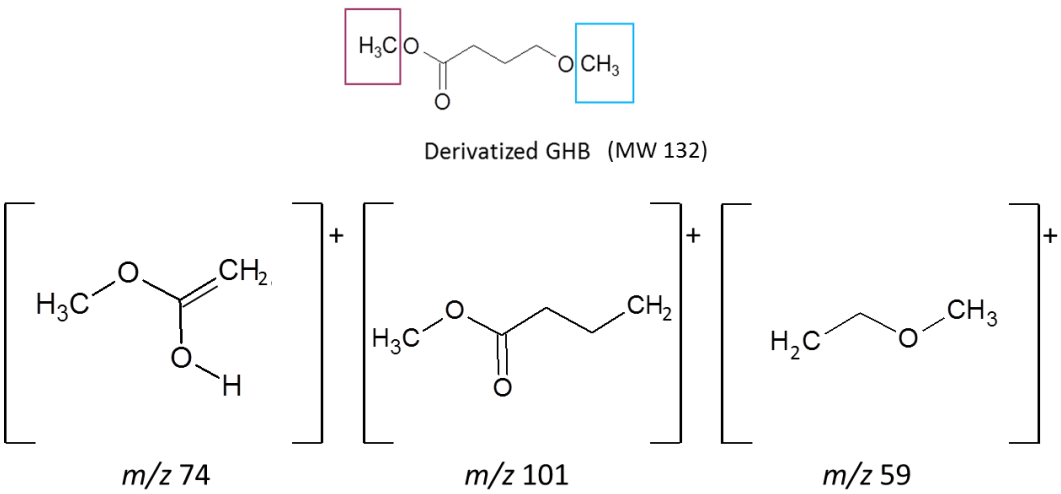


Fig. III.B.3 Proposed fragments of GHB derivatized with dimethylsulphate

III.B.2.5 “IN-VIAL” DERIVATIZATION

The “in-vial” derivatization is a modification of the procedure recently published by Rasanen *et al*. [22]. Briefly, different volumes of the methylation reagent DMS, 5 M NaOH and TBA-HSO_4 (0.1M), were added to 0.1 ml of sample. Fig. III.B.4 shows the reaction scheme for GHB. TBA-HSO_4 is an ion pairing agent, which may activate the analytes during derivatization, thereby increasing esterification yields, thus improving the sensitivity of a derivatization procedure. Preliminary experiments in water and biofluids showed no improvement of derivatization and extraction yield of GHB using the ion-pairing agent (data not shown), so TBA-HSO_4 was no longer added to a HS vial during further experiments. Furthermore, during further optimization (see III.B.3.1 “In-vial” derivatization), we evaluated various volumes of NaOH (15, 30 and 60 µl). To this end, we spiked 100 µl of urine, plasma and whole blood with 25 µg/ml GHB and compared the resulting peak areas of GHB (mean ± st dev, n=3).

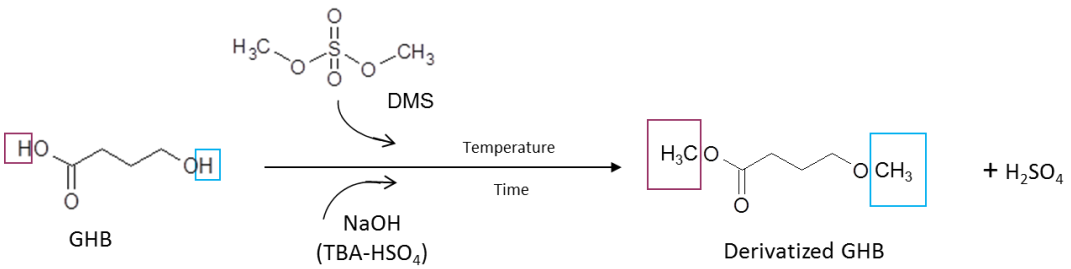


Fig. III.B.4 Reaction scheme of the applied “in-vial” derivatization reaction of GHB with dimethylsulphate in biofluids

III.B.2.6 SAMPLE PREPARATION

One hundred µl sample was added to a 22 ml HS vial. Ten µl of a 0.30 mg/ml solution in water of the IS GHB-d6 was added, followed by addition of 100 mg anhydrous Na_2SO_4, 30 µl 5 M NaOH and 30 µl DMS. The vial was properly sealed and in the case of whole blood and plasma, samples were vortexed for 10 sec before placing the vial in the HS autosampler. To conduct method development and validation, healthy drug-free volunteers donated EDTA-anticoagulated whole blood and urine.

III.B.2.7 ANALYTICAL PERFORMANCE AND APPLICATION

The analytical performance of the optimized method was evaluated based on guidelines of the European Medicines Agency (EMA) for validation of bioanalytical methods [24]. To determine inter- and intra-batch precision, QC’s (LLOQ, low, medium, high, 2 x ULOQ) were analyzed in duplicate on 4 different days, and precision was calculated using one-way ANOVA [25]. Accuracy (% bias) was determined by dividing the difference between the measured mean and the nominal value by the nominal value and multiplying it with 100 %. Bias and imprecision should be < 15 % for all QC’s, except at LLOQ, where it should be < 20 % [24].

To evaluate if plasma calibrators can be used for quantification of GHB in serum samples, QC samples prepared in serum were also measured together with calibration samples prepared in plasma. Similarly, to evaluate if calibrators prepared in whole blood are suitable for quantification of GHB in lyzed blood samples such as postmortem blood samples, QC samples were prepared in fresh whole blood (stored for a maximum of 7 days at 4 °C after collection) and lyzed blood (submitted to 2 freeze-thaw cycles before spiking). Also linearity, sensitivity, stability of processed samples, selectivity and carry-over were evaluated, as described more into detail below (III.B.3.5 Analytical performance). It was defined that no carry-over was seen if in a blank sample injected after a 200 µg/ml GHB water sample, the area at the retention time of GHB was < 20 % of the area of the LLOQ [24].

The applicability of the HS-trap injection technique was demonstrated using patient samples. To this end, we analyzed urine and serum samples (anonymous leftovers) from suspected GHB-intoxicated patients, kindly provided by the Laboratory of Toxicology of Ghent University Hospital and by the AZ St-Jan Hospital in Bruges, and a whole blood sample received in our own laboratory. To evaluate assay reproducibility, incurred sample reanalysis (ISR) was performed after a minimum of 7 days of storage at -20 °C. The initial concentration and that obtained by reanalysis should be within 20 % of their mean for at least 67 % of the repeats [24]. Furthermore, for method comparison, we performed a cross-validation by analyzing an aliquot of the samples at the Laboratory of Toxicology of ZNA Stuivenberg Hospital in Antwerp, using the method of Van hee *et al*. [12]. The difference between the result obtained by the newly developed HS-trap method and that obtained by the previously published method for the determination of GHB should be within 20 % of the mean for at least 67 % of the samples [24].

III.B.3 RESULTS AND DISCUSSION

III.B.3.1 “IN-VIAL” DERIVATIZATION

Using HS injection techniques, only volatile compounds will partition between the sample matrix and the headspace. Consequently, an aliquot of the sample can be brought directly into the HS vial, requiring a minimum of sample preparation [23]. “In-vial” or “in-situ” derivatization techniques are compatible with this general advantage The HS vial is then used as reaction vessel to perform derivatization reactions of the analytes of interest. However, one should bear in mind that derivatization reagents added directly to the HS vial, if they are volatile and added in excess, that they can also enter the chromatographic system and may interfere in the chromatogram. Furthermore, non-volatile impurities from the sample matrix may also be derivatized and generate peaks in the chromatogram, with additional increase of the vial pressure. Finally, most derivatization reagents require water-free conditions for the derivatization reaction to occur, restricting their use to derivatize samples in real practice. Reagents suitable for “in-situ” or “in-vial” derivatization are therefore preferentially combined with headspace techniques. Examples of those reagents are alkylchloroformates and methylation reagents such as dimethylsulphate [23].

In literature, “in-situ” hexylchloroformation of GHB has been reported in urine samples, followed by SPME [19, 20]. However, when applying this derivatization reagent to water samples spiked with GHB, we had difficulties to desorb hexyl-GHB from the trap (preliminary results, data not shown). Furthermore, only two fragments were formed using the MS in EI mode, requiring background substraction to properly identify the presence of hexyl-GHB. Therefore, methylation was considered, as also used by Rasanen *et al*. [22] for “in-vial” derivatization of hydroxylic analytes, including GHB, in urine and extracted blood samples. Safety measures should be taken into account, and excess reagent should be avoided, as it can enter the gaseous phase and interfere with the chromatographic detection of di-methyl GHB [26]. We evaluated this “in-vial” methylation reaction on 100 µl of urine, plasma and whole blood, spiked at 25 µg/ml GHB. Comparison of the resulting peak areas of derivatized GHB led us to choose a 1:1 ratio of NaOH (5M) and DMS (30 µl for both) in the final procedure (Fig. III.B.5).

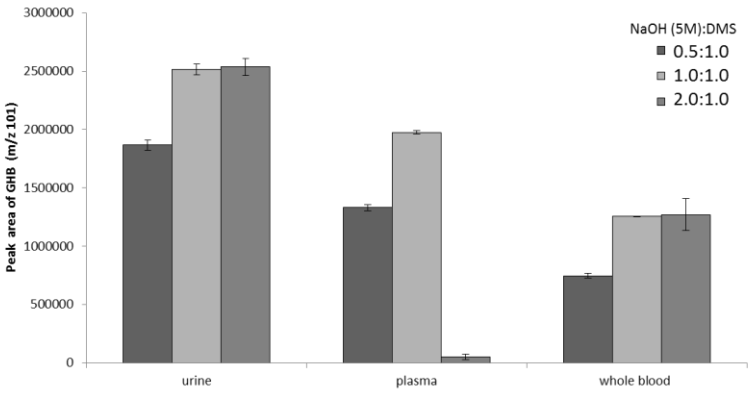


Fig. III.B.5 Optimization of the “in-vial” derivatization:
NaOH 5M-solution and dimethylsulphate were added to 100 µl urine, plasma and whole blood spiked with 25 µg/ml GHB in three different ratio’s: 0.5:1.0, 1.0:1.0, 2.0:1.0 (v:v). Samples were analyzed using a HS-trap GC-MS method and resulting peak areas of GHB for each ratio (mean ± SD; n=3) were plotted for each matrix.

III.B.3.2 EFFECT OF SALTING OUT

Adding salt to a HS vial may decrease analyte solubility in a water-based matrix and result in an additional volume-effect, facilitating the transfer of the analyte from the sample matrix to the headspace [23]. As shown in Fig. III.B.6, the addition of salt indeed increased the amount of derivatized GHB in the vapour phase, the difference between no salt and salt added being significant in all matrices, with no significant difference between the different salt concentrations added (one-way ANOVA with Bonferroni correction, α=0.05, n=3). Based upon these results, we opted to add 100 mg (± 5 mg) of anhydrous Na_2SO_4 to each HS vial in the final procedure.

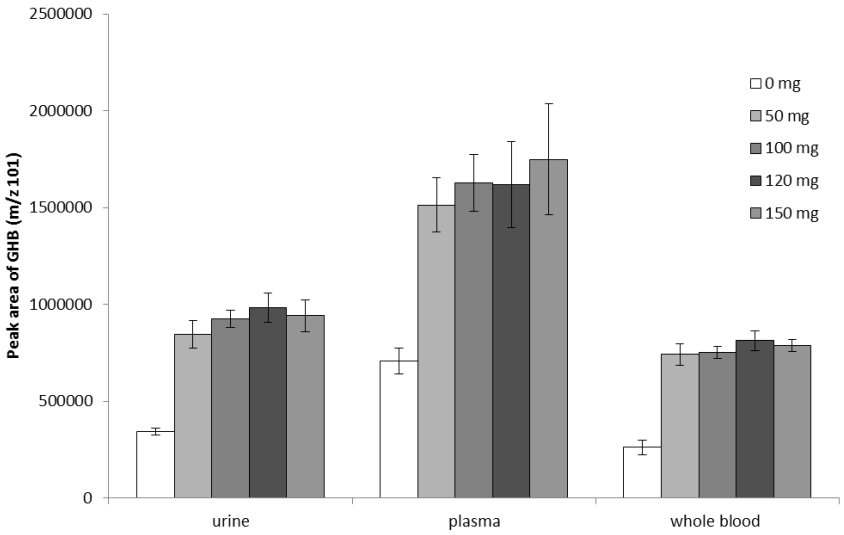


Fig. III.B.6 Effect of salting out:

To 100 µl urine, plasma and whole blood, spiked with 10 (urine) or 25 (plasma and whole blood) µg/ml GHB, varying amounts of anhydrous Na_2SO_4 were added. Samples were analyzed using a HS-trap-GC-MS method. The resulting peak areas of GHB (mean ± SD; n=3) for the various amounts of salt added were plotted for each matrix.

III.B.3.3 TRAP SETTINGS

Trap parameters (overview Table III.B.2) such as trap temperature during trap load and trap desorption were optimized to retain the analytes of interest and to maximize sample transfer to the GC-MS system [4]. In addition, when dealing with aqueous matrices, water removal prior to GC-MS analysis needs special consideration. The latter was achieved by dry purging the trap between its loading and desorption [3]. Conditions of the dry purging step were selected to obtain a maximum of water loss accompanied by a minimum of analyte loss from the trap [4]. For 100 µl water samples thermostatted for 50 min at 90 °C, a dry purge time of 10 min at a trap low temperature of 50 °C was required to efficiently remove water from the trap. To evaluate possible losses during this drying step, we compared the extraction yields obtained under these conditions with those obtained by a 5 min dry purge at 40 °C, the latter being associated with incomplete drying of the trap (as measured by following *m/z* 18, corresponding to H_2O). Comparing the means of the resulting peak areas using a t-test revealed no significant difference at the 0.05 significance level (n=3; relative standard deviations or RSDs < 3 %) [3].

Table III.B.2 Evaluated HS and trap settings for the determination of GHB in 100 µl urine, serum, plasma and whole blood samples (22 ml vial) using "in-vial" derivatization and GC-MS. Underlined values indicate the initially tested settings; bold values indicate the optimal setting chosen for the final method. * Decay time= Trap load time; $ Desorption pressure= Dry purge pressure

Headspace conditions					
Oven temperature:		**Thermostat time:**		**Column pressure:**	
70-<u>90</u> °C	**90 °C**	10-<u>20</u>-25-30-45-60 min	**30 min**	<u>15</u> psi	**15 psi**
Needle temperature:		**Vial pressurization time:**		**Vial pressure:**	
<u>95</u>°C	**95 °C**	1-<u>2</u>-3 min	**1 min**	<u>20</u>-25-30-35 psi	**30 psi**
		Decay time*:			
		0.8-<u>1.2</u>-1.4 min	**1.2 min**		
Trap settings					
Trap low temperature:		**Dry purge time:**		**Desorption pressure$:**	
<u>40</u>-50-55 °C	**50 °C**	<u>5</u>-7.5-10 min	**10 min**	<u>20</u>-25-30-35 psi	**30 psi**
Trap high temperature:		**Desorption time:**			
245-<u>250</u>-265-275°C	**265 °C**	<u>0.5</u>-2-10 min	**2 min**		
		Trap hold time:			
		<u>5</u>-8-10 min	**10 min**		

A well-known point of attention with any trapping technique is the potential of carry-over between samples. To prevent this, complete desorption of analytes from the trap is required [3]. We could minimize carry-over by setting a trap high temperature of 265 °C, desorption time of 2 min at a pressure of 30 psi, and a trap hold time of 10 min. This was evaluated by injecting a 200 µg/ml GHB spiked water sample, followed by injection of 3 blank water samples. Carry-over was no longer seen after injection of one blank sample following injection of the high concentrated GHB sample. Furthermore, analysis of blank water samples following injection of the highest calibrator prepared in matrix (urine, plasma and whole blood) also demonstrated lack of carry-over in the 2nd blank sample. Higher trap temperatures and higher desorption pressures are not recommended for routine practice, respectively to extend trap life-time and to efficiently remove water during analysis (the same pressure is also used during dry purge, negatively influencing water removal with higher pressures) [3]. Therefore, since carry-over could not be excluded completely using these mild trap settings, blank samples were analyzed between higher concentrated samples. Also, the trap can be re-used for at least 500 injections and if upon progressive use, carry-over would be seen between samples, it can be re-conditioned by heating it at 280 °C for 30 to 60 min.

III.B.3.4 HEADSPACE CONDITIONS

III.B.3.4.1 Equilibration time and temperature

Preferably, an aliquot of the vapour phase is sampled when the analytes of interest have reached equilibrium between the sample matrix and the vapour phase. The time needed to reach equilibrium depends on the sample volume, the properties of the analyte of interest and the oven temperature [23]. Preliminary experiments showed that oven temperatures of 70 °C or lower resulted in a low GHB signal (data not shown). Since the matrices of interest are water-based, the HS oven temperature may not exceed 100 °C. Therefore, 90 °C was selected for further experiments. Plotting the resulting peak areas of GHB in function of equilibration time at 90 °C, as presented in Fig. III.B.7, shows that the di-methyl derivative of GHB reaches equilibrium after 20-25 minutes in 100 µl urine, plasma and whole blood. For maximum sample throughput, the period from injection to injection should be as short as possible. The latter is calculated by the instrument, based on the optimized HS parameters. For the determination of GHB in biofluids, this calculation resulted in a minimum interval of 30 min between injections. Therefore, an equilibration time of 30 min was set, being slightly longer than the minimum required equilibration times of 20 or 25 min.

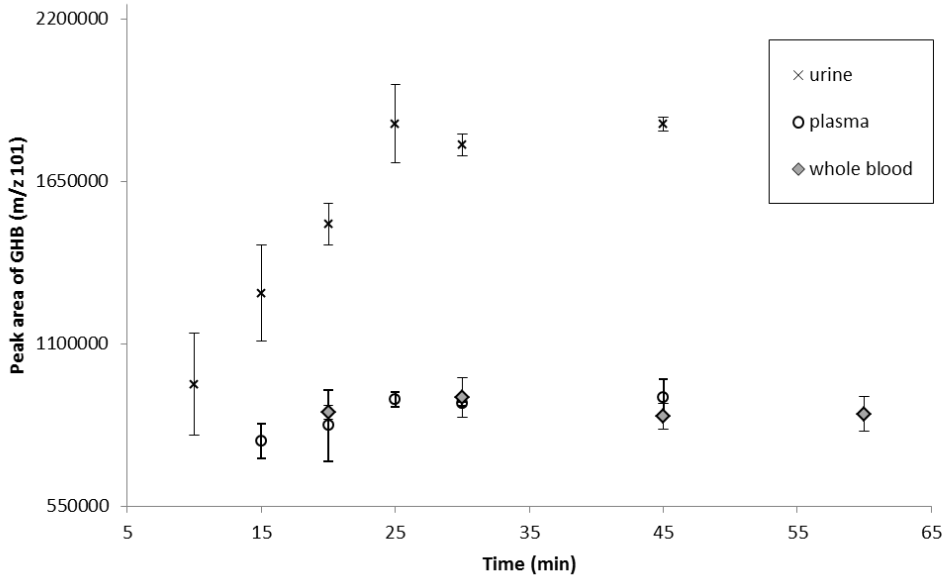


Fig. III.B.7 Time to reach equilibrium for GHB in 100 µl urine, plasma and whole blood, spiked at 25 µg/ml GHB, at 90 °C without shaking: the peak areas of GHB (mean ± SD; n=3) are plotted in function of equilibration time for each matrix

III.B.3.4.2 Sample shaking

Shaking of the sample vial during thermostatting may reduce the time needed to reach equilibrium [23]. However, for derivatized GHB no reduction in equilibration time was seen, with shaking rather introducing more variation (Fig. III.B.8). Consequently, samples were not shaken during further experiments.

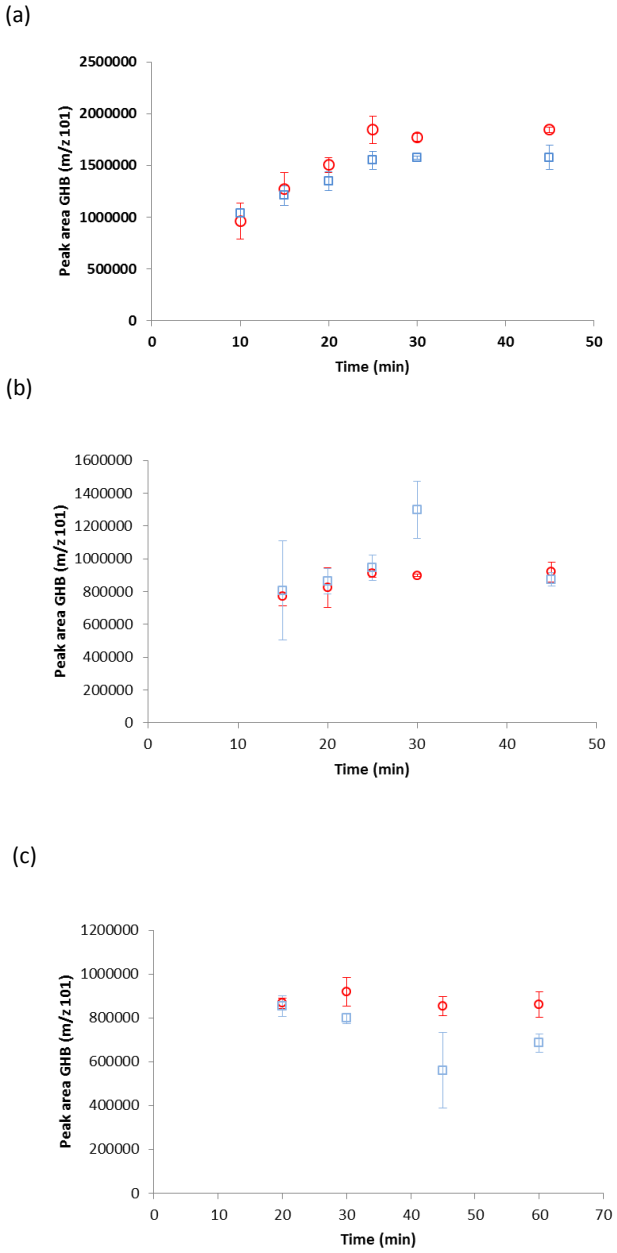


Fig. III.B.8 Equilibration time required for GHB (spiked at 25 µg/ml) in 100 µl urine (a) plasma (b) and WB (c) samples in 22 ml HS vials at 90 °C with (□) and without (O) shaking during thermostatting (n=3, mean ± SD)

III.B.3.4.3 Vial pressure, vial pressurization time and decay time

The vial pressure was optimized to give the highest sample transfer onto the trap, without risk of vial leakage at the septum or septum puncture [3]. Vial pressure was varied from 20 to 35 psi. An increase in vial pressure from 20 to 30 psi resulted in an approximate 14 % increase in GHB peak area (n=3). A pressure of 35 psi gave lower peak areas (approximate 50 % decrease in peak area, n=3). Subsequently, vial pressurization time was varied from 1 to 3 min for a vial pressure of 20 and 30 psi and it was seen that vial pressurization for 1 or 2 min at 30 psi gave highest peak areas with the lowest RSDs (< 1 %, n=3). Therefore, 1 min vial pressurization with 30 psi was chosen. Decay time or the time needed to decrease the vial pressure to atmospheric pressure after vial pressurization, was calibrated using a blank sample and was set to 1.2 min [3].

III.B.3.4.4 Repeated vial extraction

Vial pressurization followed by trap load can be repeated up to 4 times, to almost completely extract the vapour phase of the HS vial (pulse extraction). On the other hand, with each successive extraction, a larger amount of water vapour is introduced on the trap, possibly requiring adjustment of the dry purge step and prolonging analysis time. Furthermore, higher variation in measurement may be seen if equilibrium is no longer reached [3]. For 100 µl urine and plasma samples spiked at 10 and 5 µg/ml GHB, respectively, the mean GHB peak area (n=3) increased with approximately 75 % using a second extraction, with acceptable RSDs (< 5 %). A second extraction of 100 µl whole blood samples spiked at 5 µg/ml GHB, resulted in a 34 % mean increase of the peak area of GHB, as compared to a single vial extraction (Fig. III.B.9). Despite the increase in GHB peak area observed with a second vial extraction, we opted for a single vial extraction since that already resulted in sufficient sensitivity. However, it should be noted that two cycles can be used if lower detection limits would be desired.

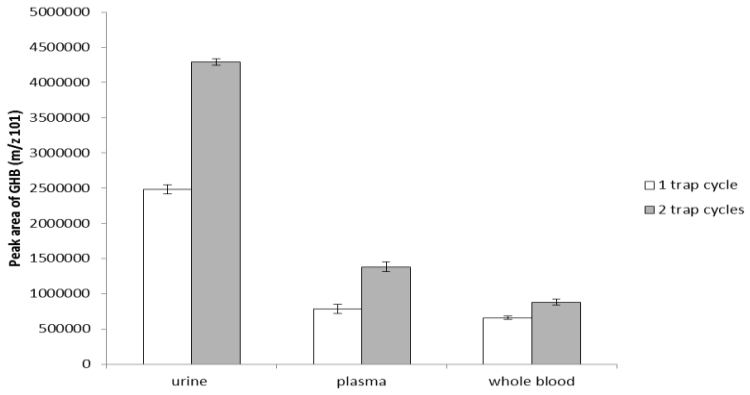


Fig. III.B.9 Repeated vial extraction: The peak areas of GHB (mean ± SD; n=3) after one and two trap load cycles are plotted for 100 µl urine (spiked at 10 µg/ml GHB), plasma (spiked at 5 µg/ml GHB) and whole blood (spiked at 5 µg/ml GHB).

III.B.3.5 ANALYTICAL PERFORMANCE

The resulting optimized HS-trap GC-MS method was validated based on EMA guidelines [24] to determine GHB in biofluids: to 100 µl sample, 10 µl 0.30 mg/ml GHB-d6, 100 mg anhydrous Na_2SO_4, 30 µl 5M NaOH and 30 µl DMS were added. Subsequently, the vial was properly sealed and placed in the HS autosampler. The sample was equilibrated for 30 min at 90 °C, before transferring an aliquot of the HS to the Tenax trap (50 °C) after vial pressurization (30 psi for 1 min). Water was removed during dry purge (30 psi for 10 min), followed by desorption (30 psi for 2 min) of di-methylated GHB by heating the trap (265 °C). The GC-MS operated in SIM mode for GHB quantification.

III.B.3.5.1 Selectivity

To evaluate selectivity, 6 different sources were analyzed for each matrix (urine, plasma, serum and whole blood). Blank (GHB-free) matrices are unavailable since GHB is naturally present in biofluids, and small elevations of *m/z* ions 59, 74, 101 and 117 at the retention time of GHB were sometimes seen when analyzing these non-spiked samples. To evaluate the interference of matrix compounds and structural analogues such as beta-hydroxybutyric acid, alpha-hydroxybutyric acid, gamma-aminobutyric acid, 1,4-butanediol, ethyleneglycol, diethyleneglycol, glycolic acid, lactic acid, urea (urine samples), succinic semi-aldehyde and hydroxy-isovaleric acid, the latter compounds were spiked at 100 µg/ml, together with GHB at 10 µg/ml (urine) or 5 µg/ml (plasma,

serum and whole blood) and GHB-d6, and analysis was performed as described above (n=1 for each matrix). Since at all instances the measured GHB concentration was within ± 15 % of the nominal value, we concluded that no interference with the GHB determination was seen.

Also the possible interference by common drugs of abuse, such as ketamine, flunitrazepam, amphetamine, 3,4-methylenedioxy-N-methylamphetamine (MDMA), cocaine, benzoylecgonine, tetrahydrocannabinol (THC), 11-nor-9-carboxy-THC, and ethanol was evaluated. Therefore, 100 µl spiked urine (10 µg/ml GHB), plasma (5 µg/ml GHB) and whole blood (5 µg/ml GHB) samples containing these interferences in concentrations above therapeutic or toxic range were analyzed (n=1 for each matrix). Also here, GHB measurements were within ± 15 % of the nominal value in all cases, meaning that there was no interference with the GHB determination.

Since derivatization occurs in an alkaline environment, reported to favour hydrolysis of the lactone GBL to GHB in aqueous matrices [27], samples were spiked at 100 µg/ml GBL (n=6) to evaluate GBL-GHB conversion. In the urine samples 109 ± 3.3 µg/ml GHB was measured and 111 ± 7.0 µg/ml GHB in the plasma samples, 103 ± 9.0 µg/ml GHB in the serum samples, 113 ± 4.9 µg/ml GHB in the whole blood samples, and finally 98 ± 12.0 µg/ml GHB in the lyzed blood samples, meaning that GBL had been completely converted to GHB during analysis. Therefore, this method determines total GHB (GHB+GBL) in biofluids. Since GBL is converted to GHB within minutes after oral ingestion, GHB is the analyte of choice to search for in samples collected from suspected GBL/GHB-intoxicated patients [17,28,29].

III.B.3.5.2 Linearity, precision, accuracy, dilution integrity and sensitivity

To evaluate the calibration model for determining GHB in 100 µl of biofluid using HS-trap, in total 8 curves were constructed in the different biofluids, by preparing and analyzing on 4 different days, a blank (non-spiked), a zero (blank + IS) and 2 x 9 calibrators (2, 5, 10, 25, 50, 100, 150, 200 and 250 µg/ml GHB in urine and plasma; 2, 3.5, 5, 10, 25, 50, 100, 150 and 200 µg/ml GHB in whole blood). Sensitivity and calibration data are summarized in Table III.B.3.

Table III.B.3 Sensitivity and calibration data: HS-trap GC-MS analysis of 100 µl of urine, plasma or whole blood samples using "in-vial" derivatization

	Slope Mean ±SD (95 % CI) (n=4 x 2)	**Intercept** Mean ±SD (95 % CI) (n=4 x 2)	**Working range** (µg/ml)	**LLOQ** (µg/ml)	**R^2** (n=4 x2)	**Weighting factor**
Urine	0.0505 ± 0.005 (0.0470; 0.0539)	0.0178 ± 0.0306 (-0.003; 0.0389)	5.0-150	5.0	0.999	$1/x^2$
Plasma	0.0477 ± 0.002 (0.0461; 0.0493)	0.0035 ± 0.0107 (-0.004; 0.0110)	2.0-150	2.0	0.996	$1/x^2$
Whole blood	0.0420 ± 0.003 (0.0398; 0.0442)	0.0063 ± 0.0066 (0.002; 0.0109)	3.5-200	3.5	1.000	$1/y^2$

Only for urine samples, calibrator ratios were lowered with the blank signal (ratio GHB/GHB-d6) and the calibration curve was found to be linear (using Fisher's test) from 2 to 150 µg/ml GHB. A working range of 5 to 150 µg/ml was selected for accuracy and precision experiments. The latter range includes the proposed cut-off level for GHB in urine (6 or 10 µg/ml) [30-33] and can be extended to 1500 µg/ml using a 10-fold dilution technique. Fig. III.B.10 shows representative chromatograms of a blank and zero urine sample, as well as of a urine sample spiked at LLOQ and a patient sample positive for GHB. For plasma samples, the calibration curve was also found to be linear from 2 to 150 µg/ml, and this range was also selected as working range (using the following 6 calibrators: 2, 5, 25, 50, 100 and 150 µg/ml). Furthermore, the calibration curve was linear from 2 to 200 µg/ml GHB for whole blood samples, and a working range of 3.5 to 200 µg/ml was chosen (using the following 6 calibrators: 3.5, 10, 50, 100, 150 and 200 µg/ml). The lower limit of this range is below the proposed cut-off level for GHB in blood, 4 or 5 µg/ml, used to distinguish between endo- and exogenous GHB [32,33]. Upon evaluation of the resulting data, heteroscedasticity was observed in all matrices and $1/x^2$ was chosen as weighting factor for urine and plasma, while for whole blood $1/y^2$ was chosen.

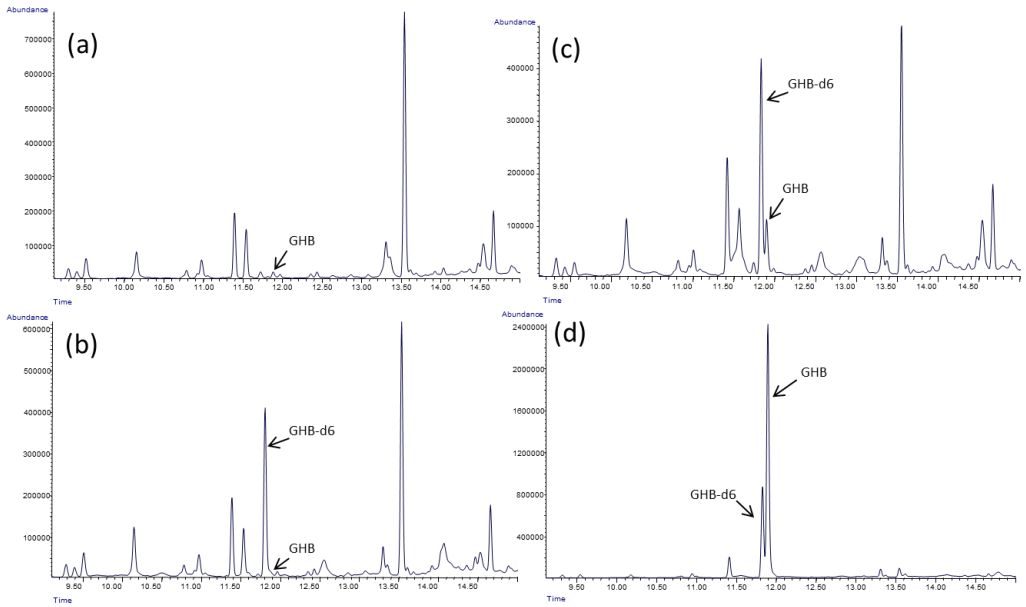


Fig. III.B.10 Representative chromatograms of a blank (a), a zero (b) and a 5 µg/ml-GHB-spiked (LLOQ) (c) urine sample, as well as a positive urine sample (1/10 dilution with water) containing 596 µg/ml GHB (sample U5, see Table III.B.6)

As shown in Table III.B.4, where precision and accuracy data are summarized, requirements were fulfilled for all QC samples, prepared in the different biofluids and analyzed using the method described above. Importantly, the results for the QC’s prepared in serum and lyzed blood demonstrate that calibration curves prepared in respectively plasma and whole blood can be used for quantification in these matrices. As to method sensitivity, as mentioned above, no blank (GHB-free) matrices are available. Therefore, the lower limit of quantification was arbitrarily set at the lowest point of the calibration curve (5 µg/ml for urine, 2 µg/ml for plasma and serum, and 3.5 µg/ml for whole blood). Samples with a GHB concentration above the upper limit of quantification can be diluted 10-fold with ultrapure water and in the case of plasma, serum and whole blood, also with non-spiked matrix, and can be analyzed as described above with acceptable precision and accuracy (see Table III.B.4, QC 2 x ULOQ).

Table III.B.4 Precision and accuracy data of the quality control samples (100 µl urine, plasma, serum, whole and lyzed blood) analyzed with HS-trap GC-MS in combination with "in-vial" derivatization (n=4 x 2).

	Nominal GHB concentration (µg/ml)	Measured GHB concentration (µg/ml)	Intra-batch precision (% RSD)	Inter-batch precision (%RSD)	Accuracy (Bias %)
Urine					
LLOQ	5	4.7	1.3	9.6	-6.7
QC low	10	9.9	3.1	4.6	-1.4
QC mid	75	71.9	1.9	6.1	-4.1
QC high	125	125.4	8.5	12.6	0.3
2 x ULOQ	300	296.1	1.5	4.6	-1.3
Plasma					
LLOQ	2	1.8	4.3	9.9	-10.4
QC low	5	5.0	3.5	8.9	0.1
QC mid	75	67.5	2.1	4.3	-10.0
QC high	125	117.0	0.5	6.4	-6.4
2 x ULOQ	300	273.1	2.9	5.4	-9.0
2 x ULOQ (matrix)[a]	300	310.8	5.6	11.0	3.6
Serum					
LLOQ	2	1.8	4.4	10.6	-9.2
QC low	5	4.9	2.3	9.0	-1.1
QC mid	75	69.4	2.3	8.5	-7.5
QC high	125	114.1	3.6	5.2	-8.7
2 x ULOQ	300	266.6	3.3	4.7	-11.2
2 x ULOQ (matrix)[a]	300	313.6	4.6	8.4	4.5
Whole blood					
LLOQ	3.5	3.4	4.3	11.8	-1.7
QC low	7.5	7.8	3.9	4.7	4.3
QC mid	75	75.8	4.9	7.8	1.0
QC high	160	158.3	5.0	7.6	-1.1
2 x ULOQ	400	360.0	3.3	9.5	-10.0
2 x ULOQ (matrix)[a]	400	388.3	4.5	11.1	-2.9
Lyzed blood					
LLOQ	3.5	3.9[b]	6.4[b]	7.7[b]	10.4[b]
QC low	7.5	7.8	4.5	6.6	4.2
QC mid	75	71.6	6.7	8.2	-4.6
QC high	160	159.8	6.4	8.6	-0.1
2 x ULOQ	400	375.0	6.1	10.3	-6.3
2 x ULOQ (matrix)[a]	400	379.3	2.8	8.2	-5.2

[a] 10-fold dilution with blank matrix, [b] 1 outlier (Grubbs test for outliers)

III.B.3.5.3 Processed sample stability

Low and high QC samples prepared in the different matrices, with all reagents added in a closed vial, were stored for at least 24 h at RT and for one week at 4 °C (n=3). The measured GHB concentration was within 15 % deviation from the original concentration in all cases. Thus, processed samples are stable under these storage conditions (Table III.B.5), which further contributes to the convenience of the developed procedure.

Table III.B.5 Processed sample stability: The average % deviation from the original concentration (T_0) is given for low and high processed QC samples (n=3) stored for at least 24h at room temperature and for at least 7 days at 4 °C.

	Nominal GHB concentration (µg/ml)	**Room temperature 24 h** % deviation from T_0 concentration	**4 °C 7 days** % deviation from T_0 concentration
QC low			
Urine	10	2.2	0.6
Plasma	5	1.7	-12.4
Serum	5	13.9	-14.8
Whole blood	7.5	7.2	-9.1
Lyzed blood	7.5	7.0	-5.1
QC high			
Urine	125	-0.5	-4.1
Plasma	125	-8.2	-6.3
Serum	125	1.8	-0.7
Whole blood	160	13.7	-12.2
Lyzed blood	160	4.6	-10.8

III.B.3.6 APPLICATION

The applicability of the validated method using HS-trap as injection technique in combination with GC-MS for the determination of GHB in patient samples was demonstrated by analyzing 5 urine, 5 serum, and 1 whole blood sample collected from suspected GHB-intoxicated patients. An aliquot of these samples was also analyzed using the method of Van hee *et al*. [12]. Results are

summarized in Table III.B.6. Both the initial concentration and the concentration obtained by reanalysis using our described method varied from -4 to 4 % from their mean, well in line with the above mentioned requirement for ISR. Furthermore, analyzing the study samples by the use of HS-trap and by the use of the method by Van hee *et al*. [12] led to similar GHB concentrations for the same sample. The difference between the two obtained results was within -9.4 to 16.7 % from the mean, calculated by dividing the difference between the two results (separately obtained by the two above-mentioned methods for the same sample), by the mean of those two results and multiplying this quotient with 100.

Table III.B.6 Measured GHB concentrations (µg/ml) in real samples using the HS-trap GC-MS method (HS-trap) and the method of Van hee *et al*. [12]. Urine samples (U) were frozen at -20 °C before reanalysis after 14 days; serum samples (S) were frozen at -20 °C before reanalysis after 30 days; whole blood sample (WB) was frozen at -20°C before reanalysis after 7 days.

	GHB concentration (µg/ml)		
Sample	**HS-trap**	**HS-trap (ISR)**	**Van hee et al.**
U1	546	555	600
U2	991	1024	1052
U3	385	393	387
U4	58	59	57
U5	596	587	531
S1	13	12	11
S2	70	68	69
S3	128	124	134
S4	259	247	224
S5	220	231	220
WB1	183	176	186

ISR: incurred sample re-analysis

III.B.4 CONCLUSION

In the study presented here, “in-vial” derivatization and HS-trap injection are combined into an application with minimal hands-on time. This combination has resulted in a simple and accurate GC-MS method for determination of total GHB (GHB+GBL) in urine, plasma, serum, whole blood and lyzed blood. In contrast to other published methods, no extra sample pretreatment step is required for quantitative determination of GHB in e.g. blood: the same procedure can be applied to all biofluids, which can simply be added to the HS vial together with the reagents, followed by closure of the vial. Moreover, the fact that these samples can be stored for at least 24 h at RT or 7 days at 4 °C further adds to the convenience of the procedure.

Besides the simplicity of the sample preparation, requiring a minimum of technical time, an important reduction of sample volume was accomplished in comparison to other HS-based methods, as a result of the trap and its associated gain in sensitivity. A sample volume of only 100 µl is used, which is markedly lower than previously reported HS-based methods for GHB determination, which require 0.5 to 1 ml sample volume. In conclusion, the use of HS-trap as injection technique results in a quick, simple and universal sample preparation protocol, only including the addition of salt and derivatization reagents directly to a given biological matrix.

The method was shown to be selective and sensitive enough to quantify GHB in samples collected from suspected GHB-intoxicated patients with LLOQ’s below the proposed cut-off levels. In addition, incurred sample reanalysis demonstrated good assay reproducibility, while cross-validation with another method demonstrated comparable results. Furthermore, according to preliminary experiments, this method shows great potential to determine other compounds of interest in emergency toxicology or post-mortem cases, such as GBL itself, as well as 1,4-BD, beta-hydroxybutyric acid, diethylene glycol, glycolic acid and ethylene glycol, derivatized to their corresponding (di)-methyl derivatives.

REFERENCES

1. Smith RM. Before the injection. Modern methods of sample preparation for separation techniques. *J Chrom A*. 1000(1-2):3-27 (2003).
2. Wille SM, Lambert WE. Recent developments in extraction procedures relevant to analytical toxicology. *Anal Bioanal Chem*. 388:1381-1391 (2007).
3. Røen BT, Unneberg E, Tørnes JAa, Lundanes E. Trace determination of sulphur mustard and related compounds in water by headspace-trap gas chromatography–mass spectrometry. *J Chrom A*. 1217(5):761-767 (2010).
4. Schulz K, Dreβler J, Sohnius EM, Lachenmeier DW. Determination of volatile constituents in spirits using headspace trap technology. *J Chrom A*. 1145(1-2):204-209 (2007).
5. Røen BT, Unneberg E, Tørnes JAa, Lundanes E. Headspace-trap gas chromatography–mass spectrometry for determination of sulphur mustard and related compounds in soil. . *J Chrom A*. 1217(14):2171-2178 (2010).
6. Nikfardjam MP, Maier D. Development of a headspace trap HRGC/MS method for the assessment of the relevance of certain aroma compounds on the sensorial characteristics of commercial apple juice. *Food Chem*. 126(4):1926-1933 (2011).
7. Aberl A, Coelhan M. Determination of volatile compounds in different hop varieties by headspace-trap GC/MS in comparison with conventional hop essential oil analysis. *J Agric Food Chem*. 60(11):2785-2792 (2012).
8. Zvosec DL, Smith SW, Porrata T, Strobl AQ, Dyer JE. Case series of 226 γ-hydroxybutyrate–associated deaths: lethal toxicity and trauma. *Am J Emerg Med*, 29(3):319-332 (2011).
9. Van Amsterdam JGC, Brunt TM, McMaster MTB, Niesink RJM. Possible long-term effects of γ-hydroxybutyric acid (GHB) due to neurotoxicity and overdose. *Neurosci Biobehav Rev.* 36(4):1217-1227 (2012).
10. Dahl SR, Olsen KM, Strand DH. Determination of gamma-hydroxybutyrate (GHB), beta-hydroxybutyrate (BHB), pregabalin, 1,4-butanediol (1,4BD) and gamma-butyrolactone (GBL) in whole blood and urine samples by UPLC–MSMS. *J Chrom B*. 885-886:37-42 (2012).
11. Morris-Kukoski CL. γ-Hydroxybutyrate. Bridging the clinical-analytical gap. *Toxicol Rev*. 23(1):33-43 (2004).
12. Van hee P, Neels H, De Doncker M, Vrydags N, Schatteman K, Uyttenbroeck W, Hamers N, Himpe D, Lambert WE. Analysis of gamma-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. *Clin Chem Lab Med.* 42(11):1341-1345 (2004).
13. Meyer MR, Weber AA, Maurer HH. A validated GC-MS procedure for fast, simple, and cost-effective quantification of glycols and GHB in human plasma and their identification in urine and plasma developed for emergency toxicology. *Anal Bioanal Chem*. 400(2):411-414 (2011).

14. Ingels AS, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct “on spot” derivatization. *Anal Bioanal Chem.* 398(5):2173-2182 (2010).
15. Ingels ASME, De Paepe P, Anseeuw K, et al. Dried blood spot punches for the confirmation of suspected γ-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. *Bioanalysis*. 3(20):2271-2281 (2011).
16. LeBeau MA, Montgomery MA, Miller ML, Burmeister SG. Analysis of biofluids for gamma-hydroxybutyrate (GHB) and gamma-butyrolactone (GBL) by headspace GC-FID and GC-MS. *J Anal Tox*. 24(6): 421-428 (2000).
17. Frison G, Tedeschi L, Maietti S, Ferrara SD. Determination of γ-hydroxybutyric acid (GHB) in plasma and urine by headspace solid-phase microextraction and gas chromatography/positive ion chemical ionization mass spectrometry. *Rapid Com Mass Spectr*. 14(24): 2401-2407 (2000).
18. Lenz D, Kröner L, Rothschild MA. Determination of gamma-hydroxybutyric acid in serum and urine by headspace solid-phase dynamic extraction combined with gas chromatography-positive chemical ionization mass spectrometry. *J Chromatogr A*. 1216(18):4090-4096 (2009).
19. Blair S, Song M, Hall B, Brodbelt J. Determination of gamma-hydroxybutyrate in water and human urine by solid phase microextraction-gas chromatography/quadrupole ion trap spectrometry. *J Forensic Sci.* 46(3):688-693 (2001).
20. Brown SD, Rhodes DJ, Pritchard BJ. A validated SPME-GC–MS method for simultaneous quantification of club drugs in human urine. *Forensic Sci I.* 171(2-3):142-150 (2007).
21. Harvey SD, Wahl JH. On-matrix derivatization for dynamic headspace sampling of nonvolatile surface residues. *J Chrom A*. 1256:58-66 (2012).
22. Rasanen I, Viinamäki J, Vuori E, Ojanperä I. Headspace in-tube extraction gas chromatography-mass spectrometry for the analysis of hydroxylic methyl-derivatized and volatile organic compounds in blood and urine. *J Anal Tox*. 34(3):113-121 (2010).
23. Kolb B, Ettre LS, Static headspace – gas chromatography: theory and practice, John Wiley and sons inc., Hoboken, New Jersey, 2nd edition (2006).
24. EMA Guideline on bioanalytical method validation, Committee for Medicinal Products for Human Use (CHMP), 21 July 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf
25. Wille SMR, Peters FT, Di Fazio V and Samyn N. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. *Accred Qual Assur*. 16(6):279-292 (2011).

26. Cardador MJ, Gallego M. Optimisation and comparison of several microextraction/methylation methods for determining haloacetic acids in water using gas chromatography. *Anal Bioanal Chem*. 396(3):1331-1343 (2010).
27. Ciolini LA, Mesmer MZ, Satzger RD, Machal AC, McCauley HA, Mohrhaus AS. The chemical interconversion of GHB and GBL: forensic issues and implications. *J Forensic Sci.* 46:1315-1323 (2001).
28. Roberts DM, Smith MW, Gopalakrishnan M, Whittaker G, Day RO. Extreme γ-butyrolactone overdose with severe metabolic acidosis requiring hemodialysis. *Ann Emerg Med*. 58(1):83-85 (2011).
29. Elliott SP. Nonfatal instances of intoxication with gamma-hydroxybutyrate in the United Kingdom. *Ther Drug Monit*. 26(4):432-440 (2004).
30. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S. Gamma-hydroxybutyrate in urine and serum: Additional data supporting current cut-off recommendations. *Forensic Sci Int.* 200(1-3):93-99 (2010).
31. LeBeau MA, Montgomery MA. Comments on “gamma-hydroxybutyrate in urine and serum: Additional data supporting current cut-off recommendations”. *Forensic Sci Int*. 207(1):e61 (2011).
32. Elian AA. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Sci Int.* 128(30):120-122 (2002).
33. Elliott SP. Gamma-hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. *Forensic Sci Int*. 133(1-2):9-16 (2003).

PART IV

CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS AND FUTURE PERSPECTIVES

In this work, several new methods to determine gamma-hydroxybutyric acid (GHB) in microvolumes (≤ 100 µl) of biofluids have been successfully developed, validated and applied. The polar nature of GHB renders derivatization necessary prior to analysis by gas chromatography-mass spectrometry (GC-MS). This is generally seen as laborious, so we opted to use direct derivatization approaches. Applying derivatization reagents directly to biological matrices, thereby combining derivatization and extraction, resulted in efficient and minimal sample treatment procedures.

First, GC-MS-based methods to determine GHB in dried blood spots (DBS) were successfully developed and validated. This included a method for the analysis of GHB in 50-µl DBS, where a fixed volume is applied on a filter paper, as well as a procedure to analyze 6-mm discs punched out of complete DBS. The latter offers the possibility to directly apply the drops of blood on the filter paper, which is useful and practical in real-life settings. Method sensitivity allowed for the discrimination between endogenous GHB concentrations and concentrations arising from exogenous intake, with a lower limit of quantification below the current cut-off value of 5 µg/ml GHB in blood samples. Moreover, the second method has been used to determine GHB in DBS collected from suspected GHB-intoxicated patients brought to the emergency room. Results demonstrated that combining “on spot” derivatization, followed by analysis with GC-MS, led to a reliable procedure applicable in routine toxicology. Also, a first comparison could be made between GHB concentrations measured in the collected capillary DBS, in the DBS prepared from the venous whole blood sample, and in the venous blood, collected simultaneously from the same patient (not self-sampling by the patients). In the 24 samples (each consisting of venous and capillary DBS and venous blood), we found similar GHB concentrations. Although more patients samples should be collected, these findings suggest that for GHB determination in blood, DBS from capillary blood could be a suitable alternative for venous blood.

Furthermore, in a second study that was set-up to evaluate the feasibility of the DBS sampling technique at home, acceptable precision of the complete procedure - from sampling at home to quantitative analysis in the laboratory - has been demonstrated. Given the intra- and inter-individual variability in clinical effects seen with GHB/sodium oxybate, the possibility of DBS sampling *via* a fingerprick may allow a better follow-up of GHB concentrations in future studies.

These can be set-up in e.g. patients discontinuing chronic use of GHB (to explore the correlation between GHB concentration and withdrawal symptoms) or in patients using sodium oxybate in real-life settings, e.g. to measure GHB concentrations after awakening in the morning.

Secondly, another one-step procedure has been successfully developed and validated to determine GHB in various biofluids using a relatively recently commercialized HS technique, HS-trap. Here, "in-vial" methylation was chosen to directly derivatize GHB, allowing for a straightforward approach. The developed procedure only included the addition of salt and derivatization reagents to a 100-µl sample in a HS-vial, and could be used for all tested biofluids (urine, plasma, serum, whole blood or lyzed blood). The implementation of the trap and its associated gain in sensitivity led to a reduction of the required sample volume compared to previously reported HS-based methods for GHB determination (which require 0.5 to 1 ml sample volume). For all biofluids, the method was shown to be selective and sensitive enough to discriminate between exo- and endogenous GHB concentrations with LLOQ's below proposed cut-off values.

Comparable results were obtained when analyzing samples (collected from suspected GHB-intoxicated patients) using the novel method and using another GC-MS method, implying that our method is a valuable alternative for GHB determination in toxicological samples. Moreover, re-analysis of these samples led to similar results, showing assay reproducibility. Finally, according to preliminary experiments, this method shows great potential to determine other compounds of interest in emergency toxicology or post-mortem cases, such as GBL itself, as well as 1,4-BD, beta-hydroxybutyric acid, diethylene glycol, glycolic acid and ethylene glycol, derivatized to their corresponding (di)-methyl derivatives.

APPENDIX

Appendix 1 Overview of the drugs of abuse determined in DBS, as discussed in Table II.A.1. *Update until July 2013*.

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Amphetamine	Jantos & Skopp, 2011	LC-MS/MS	3.0 ng/ml 5-50 ng/ml	C: 100 µl (18 mm ∅ punch covers complete spot) PAPER: Whatman 903 EXTR: 1 ml 0.01M NaOH ⇒ LLE (ethylacetate)	Intra- and interassay precision Linearity No carry-over	Yes (venous DBS) (DRUID study) Cross-comparison with whole blood	NS
	Langel et al., 2011	GC-MS	20-2000 ng/ml	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 89.2%	Intra- and interassay precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	±40 ng/ml	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Saussereau et al., 2012	LC-MS/MS	5-200 ng/ml	P: 3 mm ∅ of 30 µl DBS PAPER: Whatman 903 EXTR: 150 µl H_2O RECOV: 97.4%	Intra- and interassay precision Accuracy Linearity No ion suppression	Yes (venous DBS) (DUID) Cross-comparison with whole blood	6 months at -20°C 6 months at 4°C: ± 40% ↓
	Ambach et al., 2013	LC-MS/MS	NS Screening technique LOD 5.0 ng/ ml	C: 10 mm ∅ punch, 10 µl spotted PAPER: Bioanalysis cards 226 EXTR: 500 µl MeOH RECOV: 95.2%	For screening purposes: selectivity, specificity, matrix effect, LOD, extraction efficiency, stability	Yes (venous DBS)	2 w RT and 4°C
MDMA, MDA	Skopp et al., 2007	LC-MS/MS	NS	C: 100 µl PAPER: Whatman 903 EXTR: 0.1M NaOH ⇒ LLE	Precision Linearity No ion suppression	Yes (venous)	NS
	Jantos & Skopp, 2011 Jantos et al., 2011a	LC-MS/MS	5-40 & 50-400 ng/ml 5.7 ng/ml (MDMA) 0.25-3 & 2.5-30 ng/ml 0.40 ng/ml (MDA)	C: 100µl (18 mm ∅ punch covers complete spot) PAPER: Whatman 903 EXTR: 1 ml 0.01M NaOH ⇒ LLE (ethylacetate) RECOV: >85% (MDMA) >95% (MDA)	Intra- and interassay precision Accuracy Linearity No ion suppression No carry-over	Yes (venous DBS) (DRUID study) Cross-comparison with whole blood	NS

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
MDMA, MDA ***continued***	Langel et al., 2011	GC-MS	20-2000 ng/ml	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 90.9% (MDMA) 98.6% (MDA)	Intra- and interassay precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 2 ng/ml (MDMA) ± 20 ng/ml (MDA)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Saussereau et al., 2012	LC-MS/MS	5-200 ng/ml	P: 3 mm ∅ of 30 µl DBS PAPER: Whatman 903 EXTR: 150 µl H_2O RECOV: 101.6% (MDMA) 121.6% (MDA)	Intra- and interassay precision Accuracy Linearity No ion suppression	No	6 months at -20°C 6 months at 4°C: ±25-30% ↓
	Thomas et al., 2012	LC-HRMS	(0.5-20 ng/ml) (qualitative)	C: 20 µl PAPER: Sartorius TFN EXTR: 100 µl MeOH + 400 µl TBME 300 µl acetone RECOV: 62% (MDMA) 78% (MDA)	Precision Linearity	No	7d at 4°C
	Ambach et al., 2013	LC-MS/MS	NS Screening technique LOD 2.5 ng/ ml	C: 10 mm ∅ punch, 10 µl spotted PAPER: Bioanalysis cards 226 EXTR: 500 µl MeOH RECOV: 87.6% (MDA) 96.4 % (MDMA)	For screening purposes: selectivity, specificity, matrix effect, LOD, extraction efficiency, stability	Yes (venous DBS)	2 w RT and 4°C
MDEA	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 2 ng/ml (MDEA)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Saussereau et al., 2012		5-200 ng/ml	P: 3 mm ∅ of 30 µl DBS PAPER: Whatman 903 EXTR: 150 µl H_2O RECOV: NS	Intra- and interassay precision Accuracy Linearity No ion suppression	No	NS
	Ambach et al., 2013	LC-MS/MS	NS Screening technique LOD 1.0 ng/ ml	C: 10 mm ∅ punch, 10 µl spotted PAPER: Bioanalysis cards 226 EXTR: 500 µl MeOH	For screening purposes: selectivity, specificity, matrix effect,	Yes (venous DBS)	2 w RT and 4°C

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
MDEA ***continued***	Ambach et al., 2013 *continued*				LOD, extraction efficiency, stability		
Methamphetamine	Langel et al., 2011	GC-MS	20-2000 ng/ml	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 87.3%	Intra- and interassay precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 50 ng/ml	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Saussereau et al., 2012	LC-MS/MS	5 ng/ml 5-200 ng/ml	P: 3 mm ∅ of 30 µl DBS PAPER: Whatman 903 EXTR: 150 µl H_2O RECOV: 88.6%	Intra- and interassay precision Accuracy Linearity No ion suppression	No	6 months at -20°C 6 months at 4°C: ±40-50% ↓
	Ambach et al., 2013	LC-MS/MS	NS Screening technique LOD 1.0 ng/ ml	C: 10 mm ∅ punch, 10 µl spotted PAPER: Bioanalysis cards 226 EXTR: 500 µl MeOH RECOV: 98.3%	For screening purposes: selectivity, specificity, matrix effect, LOD, extraction efficiency, stability	Yes (venous DBS)	2 w RT and 4°C
Novel psychoactive substances (64) **e.g. amphetamine and tryptamine derivatives, phenethylamines such as cathinones, piperazine derivatives**	Ambach et al., 2013	LC-MS/MS	NS Screening technique LOD's included for each compound	C: 10 mm ∅ punch, 10 µl spotted PAPER: Bioanalysis cards 226 EXTR: 500 µl MeOH	For screening purposes: selectivity, specificity, matrix effect, LOD, extraction efficiency, stability	Yes (venous blood)	2 w RT and 4°C
Cocaine, benzoylecgonine (BE), cocaethylene (CE) & ecgonine methylester (EME)	Henderson et al., 1993	RIA	10-600 ng/ml (BE)	P: 6.4 mm ∅ PAPER: Whatman 903 EXTR: 18h in 200µl PBS:Tween20 RECOV: >95% (BE)	Intra- and interassay precision	Yes (autopsy samples & newborn DBS)	>1024h at 25, 45, 55°C (BE) 108h at 45°C (cocaine)
	Sosnoff et al., 1996	LC-MS/MS	± 2 -166 ng/ml (BE)	P: 6.4 mm ∅ (reconstituted blood) PAPER: NS EXTR: 200 µl 2mM CH_3COONH_4	Linearity	Yes (fingerprick from drug abusers) (DBS from new-borns & mothers)	NS

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Cocaine, benzoylecgonine (BE), cocaethylene (CE) & ecgonine methylester (EME) ***continued***	Henderson et al., 1997	RIA, LC-MS	/ (BE)	P: 6.4 mm ∅ Procedures of Henderson et al., 1993 & Sosnoff et al., 1996	/	Yes (newborn DBS)	/
	Alfazil & Anderson, 2008	LC-MS/MS	24.6 ng/ml 50-2000 ng/ml (cocaine)	C: 100 µl of reconstituted blood PAPER: Whatman 903 EXTR: phosphate buffer (3.5 ml, pH6) RECOV: 90-97%	Intra- and interassay precision Accuracy Linearity	No	1 month at -20°C & 4°C 1 month at RT: 19.9% ↓ (DBS from spiked blood)
	Mercolini et al., 2010	LC-FLUO	20-1000 ng/ml (cocaine & BE) 12-1000 ng/ml (cocaethylene)	C: 10 µl PAPER: Whatman 903 EXTR: 500 µl methanol RECOV: 92-93%	Intra- and interassay precision Linearity Selectivity	Yes (fingerprick) (drug abusers)	NS
	Langel et al., 2011	GC-MS	50-1000 ng/ml (cocaine)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 83.2%	Intra- and interassay precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 1 ng/ml (cocaine & BE)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Saussereau et al., 2012	LC-MS/MS	5-200 ng/ml (cocaine, BE, CE, EME)	P: 3 mm ∅ of 30 µl DBS PAPER: Whatman 903 EXTR: 150 µl H_2O RECOV: 100% (cocaine), 99.4% (BE), 85.9% (EME)	Intra- and interassay precision Accuracy Linearity No ion suppression	Yes (venous) (BE) (DUID) Cross-comparison with whole blood	6 months at: -20°C (cocaine, BE, EME) 4°C (BE) 4°C: ±55% ↓ (cocaine) 4°C: ±90% ↓ (EME)
	Thomas et al., 2012	LC-HRMS	0.25 ng/ml (cocaine)	C: 20 µl PAPER: Sartorius TFN EXTR: 100 µl MeOH + 400 µl TBME 300 µl acetone RECOV: 67%	Precision Linearity	No	7d at 4°C (cocaine)
Benzodiazepines	Alfazil & Anderson, 2008	LC-MS/MS	9.9 ng/ml (diazepam) 15.8 ng/ml (flunitrazepam) 11 ng/ml (lorazepam) 18 ng/ml (nitrazepam) 20.6 ng/ml (oxazepam) 10.8 ng/ml (temazepam)	C: 100 µl of reconstituted blood PAPER: Whatman 903 EXTR: phosphate buffer (3.5 ml, pH6) RECOV: 83-99% (diazepam) 89-103% (flunitrazepam) 81-88% (lorazepam) 90-100% (nitrazepam)	Intra- and interassay precision Accuracy Linearity	No	(DBS from spiked blood) 1 month at -20°C 1 month at 4°C 1 month at RT: Diazepam: 12.3% ↓ Flunitrazepam: 15% ↓ Lorazepam: 11% ↓;

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Benzodiazepines ***continued***	Alfazil & Anderson, 2008 *continued*		50-2000 ng/ml	95-106% (oxazepam) 89-94% (temazepam)			Nitrazepam: 15.5% ↓ Oxazepam: 12% ↓ Temazepam: 15% ↓
	Havard et al., 2010	LC-MS/MS	100 pg/ml (midazolam)	P: 4 mm ∅ of 20 µl DBS	Interassay precision	No	1d at RT
	Thomas et al, 2010		NS (diazepam, nordiaze-pam, oxazepam, oxa-zepam glucuronide)	C: 5 µl (10 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: on-line extraction with ACN : 15 mM CH_3COONH_4 (97:3)	No ion suppression	No	NS
	Jantos & Skopp, 2011		0.7 ng/ml 2.5-50 ng/ml (alprazolam)	C: 100µl (18 mm ∅ punch covers complete spot) PAPER: Whatman 903 EXTR: 1ml borate buffer (pH 8.5) ⇒ LLE	Intra- and interassay precision Linearity No carry-over	Yes (venous DBS) (DUID study) Cross-comparison with whole blood	NS
	Langel et al., 2011	GC-MS	5-250 ng/ml (lorazepam) 5-500 ng/ml (alprazolam & clonazepam) 10-1000 ng/ml (midazolam & nitrazepam) 20-1000 ng/ml (phenazepam) 20-2000 ng/ml (nordiazepam) 50-1250 ng/ml (oxazepam) 50-2500 ng/ml (diazepam) 50-5000 ng/ml (temazepam)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 96.1% (alprazolam) 92.3% (clonazepam) 95.1% (diazepam) 94.4% (lorazepam) 92.0% (midazolam) 88.2% (nitrazepam) 96.6% (nordiazepam) 94.3% (oxazepam) 87.4% (phenazepam) 96.4% (temazepam)	Intra- and interassay Precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a		± 0.1 ng/ml (prazepam) ± 0.5 ng/ml (clobazam, flurazepam, midazolam, triazolam); ± 1 ng/ml (alprazolam, 7-aminoflunitraze-pam, nitrazepam, nordiazepam, temazepam), ± 2 ng/ml (hydroxy-midazolam) ± 5 ng/ml (flunitrazepam, lorazepam) ± 10 ng/ml (clonazepam, diazepam, oxazepam)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Benzodiazepines ***continued***	Déglon et al., 2012b	LC-MS/MS	1ng/ml 0.5-100 ng/ml (desmethylflunitraze-pam, flurazepam, lormetazepam, triazolam) 4 ng/ml 2-500 ng/ml (alprazolam, hydroxy-alprazolam, clonaze-pam, 7-aminoclonazepam, desalkylflurazepam, flunitrazepam, 7-aminofluitrazepam, lorazepam, midazo-lam, hydroxy-midazo-lam) 40 ng/ml 20-5000 ng/ml (clobazam, diazepam, nitrazepam, nordiaze-pam, oxazepam, pra-zepam, temazepam)	C: 5 µl (6 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: 100 µl MeOH (*in-vial extraction*) RECOV: 79.6-85.3% (alprazolam) 68.7-76.2% (hydroxyalprazolam) 70.1-90.2% (clobazam) 79.1-83.7% (clonazepam) 58.6-61.9% (7-aminoclonazepam) 67.7-89.3% (diazepam) 72.3-86.5% (flunitrazepam) 56.3-68.8% (7-aminoflunitrazepam) 82.2-91.4% (desmethylflunitrazepam) 51.9-70.2% (flurazepam) 71.9-81.0% (desalkylflurazepam) 69.2-78.0% (lorazepam) 78.6-78.7% (lormetazepam) 71.2-85.7% (midazolam) 68.7-76.2% (hydroxymidazolam) 72.2-93.4% (nitrazepam) 76.6-88.4% (nordiazepam) 72.2-93.4% (oxazepam) 63.0-98.0% (prazepam) 74.8-95.7% (temazepam) 73.3-73.9% (triazolam)	Intra- and interassay precision Accuracy Linearity Ion suppression overall < 15% No carry-over	Yes (venous DBS) DUID	30d at -20°C & RT
Zolpidem	Hudson et al., 2011	LC-MS/MS	(0.1-500 ng/ml)	P: 3 mm ∅ of 15 µl DBS PAPER: Bond Elut DMS EXTR: 300µl of 0.1% HCOOH in 80% MeOH RECOV: 102-110%	Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 1 ng/ml (zolpidem)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Déglon et al., 2012b	LC-MS/MS	4 ng/ml 2-500 ng/ml (zolpidem)	C: 5 µl (6 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: 100 µl MeOH (*in-vial extraction*) RECOV: 66.4-81.6%	Intra- and interassay precision Accuracy Linearity No carry-over	Yes (venous DBS) DUID	30d at -20°C & RT

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Zopiclone and ACP (2-amino-5-chloropyridine)	Langel et al., 2011	GC-MS	10-1000 ng/ml (zopiclone)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 79.3%	Intra- and interassay precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 2 ng/ml (zopiclone)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Jantos and Skopp, 2011 Jantos et al., 2012	LC-MS/MS	6.4 ng/ml (zopiclone) 0.14 ng/ml (ACP)	C: 100 µl PAPER: Whatman 903 EXTR: 1 ml borate buffer (pH 8.5) ⇒ LLE RECOV: 67.1-79.7% (zopiclone) 80.4-83.8% (ACP)	Intra- and interassay precision Accuracy Linearity No ion suppression No carry-over	Yes (venous DBS) (DRUID study) Cross-comparison with whole blood	Authentic & spiked DBS 30d at -20°C 22d at 4°C 8d-30d at 20°C 3d at 40°C
	Déglon et al., 2012b	LC-MS/MS	4 ng/ml	C: 5 µl (6 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: 100 µl MeOH (*in-vial extraction*) RECOV: 61.6-67.2%	Intra- and interassay precision Accuracy Linearity No carry-over	No	30d at -20°C & RT
Zaleplon	Déglon et al., 2012b	LC-MS/MS	4 ng/ml	C: 5 µl (6 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: 100 µl MeOH (*in-vial extraction*) RECOV: 77.4-89.8%	Intra- and interassay precision Accuracy Linearity No carry-over	No	30d at -20°C & RT
Ketamine and norketamine	Moll et al., 2009	LC-MS/MS	5-2500 ng/ml 5 ng/ml (ketamine & norketamine)	P: 3.2 mm ∅ from 19 µl spot PAPER: NS EXTR: MeOH/0.2M $ZnSO_4$ (7:3) ⇒ dilute with H_2O	Precision & Accuracy No matrix interferences No carry-over	No	NS
	Ambach et al., 2013 (ketamine)	LC-MS/MS	NS Screening technique LOD 1.0 ng/ ml	C: 10 mm ∅ punch, 10 µl spotted PAPER: Bioanalysis cards 226 EXTR: 500 µl MeOH RECOV: 96.3%	For screening purposes: selectivity, specificity, matrix effect, LOD, extraction efficiency, stability	Yes (venous blood)	2 w RT and 4°C

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Gamma-hydroxybutyric acid (GHB)	Forni et al., 2013	LC-MS/MS	1-128 µg/ml	P: 3 x4.6 mm ∅ PAPER: Whatman 903 EXTR: 200 µl MeOH RECOV: > 70 %	Linearity Recovery Matrix effect Intra- and interday precision	Yes (newborn DBS)	NS
	Ingels et al., 2010	GC-MS	2-100 µg/ml	C: 50 µl PAPER: Whatman 903 EXTR: *on-spot derivatization* with 100 µl TFAA & HFB-OH (2:1) RECOV: /	Intra- and interassay precision Accuracy Linearity No carry-over	Yes (venous DBS) (drug abuser)	> 7d at RT > 14d at -20°C
	Ingels et al., 2011	GC-MS	2-100 µg/ml	P: 6 mm ∅ PAPER: Whatman 903 EXTR: *on-spot derivatization* with 50 µl TFAA & HFB-OH (2:1) RECOV: /	Intra- and interassay precision Accuracy Linearity Effect of hematocrit, volume spotted, site of punching	Yes (fingerprick) Cross-comparison with venous blood & DBS from venous blood	> 148d at RT
Opiates & metabolites: morphine, codeine, 6-monoacetylmor-phine (6MAM), morphine 3-glucuronide (M3G), morphine 6-glucuronide (M6G), oxycodone, noroxycodone, hydromorphone	Skopp et al., 2007	LC-MS/MS	NS	C: 100µl PAPER: Whatman 903 EXTR: 0.5% NH_4OH ⇒ LLE	NS	No	6d at 4°C, RT & 40°C
	Garcia Boy et al., 2008	LC-MS/MS	14 ng/ml (morphine) 27 ng/ml (6MAM) 50-500 ng/ml (morphine & 6MAM)	C: 100 µl PAPER: Whatman BFC 180 EXTR: 1ml 0.1M borate buffer (pH 8.5) RECOV: 23-37% (morphine) 76-86% (6MAM)	Intra- and interassay precision Linearity No ion suppression No carry-over	Yes (venous DBS) (drug abusers) Cross-comparison with whole blood	10% ↓ in both morphine & 6MAM upon drying Morphine: 7d at 4°C, -20°C; 5d@ 40°C 6MAM: 5d at 40°C: ±50% ↓
	Thomas et al., 2010	LC-MS/MS	NS Morphine, codeine and their glucuronides	C: 5 µl (10 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: on-line extraction with ACN : 15 mM CH_3COONH_4 (97:3)	No ion suppression	No	NS

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Opiates & metabolites ***continued***	Marin et al., 2010	LC-MS/MS	100 pg/ml (oxycodone)	RECOV: ±85%	NS	No	NS
	Clavijo et al., 2011a	LC-MS/MS	1-1000 ng/ml (morphine & M3G) 2.5-1000 ng/ml (M6G)	P: 6.4 mm ∅ from 50 µl spot PAPER: Whatman 903 EXTR: 100 µl HPLC-water RECOV: 99.6-108.3% (morphine) 95.6-102% (M3G) 99.7-103.3% (M6G)	Intra- and interassay precision Accuracy Linearity No ion suppression No carry-over	Yes (Method used in pharmacokinetic studies)	≥ 3 freeze-thaw cycles; ≥ 3d at RT 7d at 4°C, -20°C, -80°C
	Jantos et al., 2011b	LC-MS/MS	NS (morphine, hydro-morphone, oxycodo-ne, noroxycodone)	C: 100 µl PAPER: Whatman 903	NS	Yes (venous DBS) Cross-comparison with whole blood	NS
	Langel et al., 2011		10-1000 ng/ml (morphine & codeine)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 50% (morphine) >75 % (codeine)	Intra- and interassay precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	±20 ng/ml (morphine) ± 5 ng/ml (codeine) ± 1 ng/ml (6MAM, hydrocodone)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Saussereau et al., 2012	LC-MS/MS	5-200 ng/ml (morphine, codeine) 10-200 ng/ml (M3G & M6G)	P: 3 mm ∅ of 30 µl DBS PAPER: Whatman 903 EXTR: 150 µl H_2O RECOV: 90% (morphine) 93.2% (codeine)	Intra- and interassay precision Accuracy Linearity No ion suppression	Yes (venous DBS) Cross-comparison with whole blood (morphine)	6 months at -20°C
	Mommers et al., 2013	LC-MS/MS	4-1000 ppb morphine	P: 3 mm ∅ PAPER: Whatman 903 EXTR: 100 µl MeOH-H_2O (1:9)	Linearity Precision Accuracy	No	NS
Buprenorphine and metabolites	Thomas et al., 2010	LC-MS/MS	NS Buprenorphine, norbuprenorphine and their glucuronides	C: 5 µl (10 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: on-line extraction with ACN : 15 mM CH_3COONH_4 (97:3)	No ion suppression	Yes (animal study)	NS
	Marin et al., 2010	LC-MS/MS	NS (>25 pg/ml)	RECOV: ±45%	NS	No	NS
	Langel et al., 2011	GC-MS	5-100 ng/ml (buprenorphine)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and	Inter- and intraday precision	No	NS

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Buprenorphine and metabolites *continued*	Langel et al., 2011 *continued*			2 ml Butylacetate RECOV: 49.8%	Accuracy Linearity		
	Lauer et al., 2011	LC-MS/MS	NS	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
Methadone and metabolites	Clavijo et al., 2010	LC-MS/MS	0.1-100 ng/ml (methadone, EDDP, EMDP)	P: 6.4 mm ∅ from X µl spot PAPER: Whatman 903 EXTR: 100 µl HPLC water ⇒ PP RECOV: NS	Precision & Accuracy Linearity No ion suppression No carry-over	No	NS
	Langel et al., 2011	GC-MS	10-1000 ng/ml (methadone)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 78.5%	Inter- and intraday precision Accuracy Linearity	No	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 1 ng/ml (methadone) ± 5 ng/ml (EDDP)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	NS	NS
	Addolorata Saracino et al., 2012	LC-Coulometry	4-500 ng/ml (on-column concentration)	C: Theoretical volume calculated from ∅ PAPER: Whatman FTA® classic EXTR: 250 µl phosphate buffer: CH_3CN (20:80) & 250 µl phosphate buffer RECOV: 90.1-94.8%	Inter- and intraday Prec Linearity	Yes (fingerprick) (methadone maintenance patients) Cross-comparison with plasma	1 month at RT (methadone) (patient samples)
Fentanyl and metabolites	Clavijo et al., 2011b	LC-MS/MS	0.1 - 100 ng/ml (fentanyl) 0.25-100 ng/ml (norfentanyl & despropionylfentanyl)	P: 6.4 mm ∅ from 50 µl spot PAPER: Whatman 903 EXTR: 100 µl HPLC-water RECOV: 78, 75, 77% for resp. fentanyl, norfentanyl and despropionylfentanyl	Intra- and interassay precision Accuracy Linearity No ion suppression No carry-over	Yes (pharmacokinetic study in neonates & children)	DBS from spiked blood 6d at -20°C & -80°C 3d at RT (all 3 analytes) 5d at RT (fentanyl)
	Jantos et al., 2011b	LC-MS/MS	NS (fentanyl & norfentanyl)	C: 100 µl PAPER: Whatman 903	NS	Yes (venous DBS) Cross-comparison with whole blood	NS
	Lauer et al., 2011 Déglon et al., 2012a	LC-MS/MS	± 0.2 ng/ml (fentanyl)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	NS	NS
Tramadol	Langel et al., 2011	GC-MS	50-5000 ng/ml	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate	Inter- and intraday precision Accuracy	No	NS

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Tramadol ***continued***	Langel et al., 2011 *continued*			RECOV: 78%	Linearity		
	Déglon et al., 2012a	LC-MS/MS	± 0.5 ng/ml (Tramadol)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	NS	NS
Tetrahydrocannabinol and metabolites	Thomas et al., 2010	LC-MS/MS	NS THC, carboxy-THC and its glucuronide, 11-OH-THC	C: 5 µl (10 mm ∅ punch covers complete spot) PAPER:Whatman 903 EXTR: on-line extraction with ACN : 15 mM CH_3COONH_4 (97:3)	No ion suppression	No	NS
	Langel et al., 2011	GC-MS	5-100 ng/ml (THC) 5-500 ng/ml (THC-COOH)	C: 100 µl PAPER: NS EXTR: saturated borate buffer (pH 10) and 2 ml Butylacetate RECOV: 41.2% (THC) 32.2% (THC-COOH)	Inter- and intraday precision Accuracy Linearity	No	NS
	Déglon et al., 2012a	LC-MS/MS	± 50 ng/ml (THC)	C: 5 µl PAPER: NS EXTR: 100% MeOH (on-line extraction)	NS	No	NS
	Thomas et al., 2012	LC-HRMS	1 ng/ml (THC & THC-COOH)	C: 20 µl PAPER: Sartorius TFN EXTR: 100 µl MeOH + 400 µl TBME 300 µl acetone RECOV: 19% (THC) 36% (THC-COOH)	Precision & Accuracy Linearity	No	7d at 4°C
	Mercolini et al., 2013	LC-MS/MS	2.5-2000 ng/ml (THC) 5-2000 ng/ml (THC-OH & THC-COOH) !! [ ]'s are those in a MeOH solution spiked to the DBS	C: 10 mm ∅ punch PAPER: Whatman 903 EXTR: 1 ml MeOH RECOV: 81-86%	Intra- and interassay precision Accuracy Linearity No ion suppression	Yes (venous DBS)	3 months at RT
Cotinine	Spector et al., 2007	GC-MS	NS	¼ of filled (± 200 µl) circle ⇒ ± 50 µl EXTR: 500 µl 0.2 M NaOH	NS	Yes (newborn DBS)	NS
	Murphy et al., 2013 (cotinine and trans 3'-hydroxycotinine)	LC-MS/MS	0.3-102 ng/g cotinine	P: 3.2 or 4.6 mm ∅ PAPER: Ahlstrom 226 EXTR: 400 µl HPLC-water SPE: Oasis MCX 96-well plate	Linearity Intra-day precision	Yes (plasma)	NS
Ethylglucuronide – ethylsulfate	Hernàndez Redondo et al., 2011	LC-MS/MS	0.1-10 µg/ml	P: 5 mm ∅ PAPER: NS EXTR: methanol	Intra- and interassay precision Accuracy	No	3 weeks at 4°C 3 weeks at RT: ± 20% ↓

Analyte		Analytical Technique	LLOQ Assay Range	Extraction (C = complete DBS / P = DBS punch) Recovery	Analytical Validation	Clinical Application	Stability of DBS
Ethylglucuronide – ethylsulfate ***continued***	Hernàndez Redondo et al., 2011 *continued*				Linearity Effect of volume spotted, site of punching		
Phosphatidylethanol (Peth)	Faller et al., 2011 Faller et al., 2012	LC-MS/MS	22.7 ng/ml (PEth 18:1/18:1) 87.3 ng/ml (PEth 16:0/18:1) 50-5000 ng/ml	C: 100 µl PAPER: Whatman 903 EXTR: 400 µl 0.5M CH_3COONa (pH5) + 600 µl isopropanol + 700 µl n-hexane ⇒ LLE RECOV: 26.6-42.5% (PEth 18:1/18:1) 68.9-90.7% (PEth 16:0/18:1)	Intra- and interassay precision Accuracy Linearity No ion suppression No carry-over	Yes (subjects in alco-hol detoxification program) Cross-comparison with whole blood	> 30d at -20°C & 20°C
	Jones et al., 2011	LC-MS/MS	8 ng/ml (PEth 16:0/18:1)	P: 3 X 3.2 mm ∅ PAPER: Whatman 903 EXTR: 50 µl 2mM CH_3COONH_4/CH_3CN /Isopropanol (20:58:22) + 500 µl MeOH RECOV: 56.0-82.9%	Intra- and interassay precision Accuracy Linearity Significant matrix effect (>40%) No carry-over	Yes Cross-comparison with whole blood	No *ex vivo de novo* formation

References

Addolorata Saracino M, Marcheselli C, Somaini L, Pieri MC, Gerra G, Ferranti A, Raggi MA. (2012) A novel test using dried blood spots for the chromatographic assay of methadone. Anal Bioanal Chem 404:503–511.

Alfazil AA, Anderson RA. (2008). Stability of benzodiazepines and cocaine in blood spots stored on filter paper. J Anal Toxicol 32:511-515.

Ambach L, Hernández Redondo A, König S, Weinmann W (2013). Rapid and simple LC-MS/MS screening of 64 novel psychoactive substances using dried blood spots. Drug Test Anal Doi 10.1002/dta.1505 *Epub ahead of print*.

Clavijo CF, Hoffman KL, Thomas JJ, Carvalho B, Chu LF, Drover DR, Hammer GB, Christians U, Galinkin JL. (2011a). A sensitive assay for the quantification of morphine and its active metabolites in human plasma and dried blood spots using high-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 400:715-728.

Clavijo CF, Thomas JJ, Cromie M, Schniedewind B, Hoffman KL, Christians U, Galinkin JL. (2011b). A low blood volume LC-MS/MS assay for the quantification of fentanyl and its major metabolites norfentanyl and despropionyl fentanyl in children. J Sep Sci. 34:3568-3577.

Clavijo CF, Thomas JJ, Hammer GB, Christians U, Galinkin JL (2010). A sensitive LC-MS/MS Assay for Quantification of Methadone and Its Metabolites in Dried Blood Spots. Proceedings of the Annual Meeting of the American Society of Anesthesiologists (ASA). San Diego, CA.

Déglon J, Thomas A, Mangin P, Staub C. (2012a). Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. Anal Bioanal Chem 402:2485-2498.

Déglon J, Versace, F, Lauer, E, Widmer, C, Mangin, P, Thomas, A, Staub C. (2012b). Rapid LC-MS/MS quantification of the major benzodiazepines and their metabolites on dried blood spots using a simple and cost-effective sample pretreatment. Bioanalysis 4:1337-1350.

Faller A, Richter B, Kluge M, Koenig P, Seitz HK, Thierauf A, Gnann H, Winkler M, Mattern R, Skopp G. (2011). LC-MS/MS analysis of phosphatidylethanol in dried blood spots versus conventional blood specimens. Anal Bioanal Chem 401:1163-1166.

Faller A, Richter B, Kluge M, Koenig, P, Seitz HK, Skopp, G. (2013) Stability of phosphatidylethanol species in spiked and authentic whole blood and matching dried blood spots. Int J Legal Med. 127(3):603-610.

Forni S, Pearl PL, Gibson, KM, Yu Y, Sweetman L. (2013) Quantitation of gamma-hydroxybutyric acid in dried blood spots: Feasibility assessment for newborn screening of succinic semi-aldehyde dehydrogenase (SSADH- deficiency. Mol Genet Metab. 109(3):255-259.

Garcia Boy R, Henseler J, Mattern R, Skopp G. (2008). Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. Ther Drug Monit 30:733-739.

Havard G, Théberge M-C, Boudreau N, Lévesque A, Massé R (2010). Development and Validation of a Dried Blood Spot Assay for the Determination of Midazolam in Human Whole Blood by LCMSMS. Proceedings of the American Association of Pharmaceutical Scientists (AAPS) - Pharmaceutical Sciences World Congress. New Orleans, LA.

Henderson LO, Powell MK, Hannon WH, Bernert JT, Jr., Pass KA, Fernhoff P, Ferre CD, Martin L, Franko E, Rochat RW, Brantley MD, Sampson E. (1997). An evaluation of the use of dried blood spots from newborn screening for monitoring the prevalence of cocaine use among childbearing women. Biochem Mol Med 61:143-151.

Henderson LO, Powell MK, Hannon WH, Miller BB, Martin ML, Hanzlick RL, Vroon D, Sexson WR. (1993). Radioimmunoassay screening of dried blood spot materials for benzoylecgonine. J Anal Toxicol 17:42-47.

Hernández Redondo A, Schroeck A, Kneubuehl B, Weinmann W. (2013). Determination of ethyl glucuronide and ethyl sulfate from dried blood spots. Int J Legal Med

Hudson W, Yong B, Boguszewski P (2011). Analysis of Clozapine, Nortriptyline, Paroxetine and Zolpidem Using Dried Blood Spots. [Online] Available at: http://www.chem.agilent.com/Library/applications/5990-8033EN.pdf. Accessed on July, 1st, 2012.

Ingels AS, De Paepe P, Anseeuw K, Van Sassenbroeck DK, Neels H, Lambert WE, Stove CP. (2011). Dried blood spot punches for confirmation of suspected gamma-hydroxybutyric acid intoxications: validation of an optimized GC-MS procedure. Bioanalysis 3:2271-2281.

Ingels AS, Lambert WE, Stove CP. (2010). Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. Anal Bioanal Chem 398:2173-2182.

Jantos R, Skopp G. (2011). Comparison of drug analysis in whole blood and dried blood spots. Toxichem Krimtech 78:268-275.

Jantos R, Veldstra JL, Mattern R, Brookhuis KA, Skopp G. (2011a). Analysis of 3,4-methylenedioxymetamphetamine: whole blood versus dried blood spots. J Anal Toxicol 35:269-273.

Jantos R, Schumacher M, Skopp G. (2011b). Comparison of Opioid Analysis in Whole Blood and Dried Blood Spots. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA.

Jantos R, Vermeeren A, Sabljic D, Ramaekers JG, Skopp G. (2012). Degradation of zopiclone during storage of spiked and authentic whole blood and matching dried blood spots. Int J Legal Med 127(1):69-76.

Jones J, Jones M, Plate C, Lewis D. (2011). The detection of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol in human dried blood spots. Anal Methods-Uk 3:1101-1106.

Langel K, Uusivirta H, Ariniemi K, Lillsunde P. (2011). Analysis of Drugs of Abuse by GC-MS in Dried Blood Spot Sample Matrix. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA.

Lauer E, Déglon J, Versace F, Thomas A, Mangin P, Staub C. (2011). Target Screening of Drugs from Dried Blood Spot Samples Based on LC-MS/MS and On-Line Desorption. Proceedings of the 49th Annual Meeting of The International Association of Forensic Toxicologists (TIAFT). San Francisco, CA.

Marin LJ, Tyrrel-Pawlowic CA, Moroney EC, Coopersmith BI, Shet MS. (2010). Comparative analysis of buprenorphine and oxycodone in human dried blood spots (DBS) using LC/MS/MS. Proceedings of the American Association of Pharmaceutical Scientists (AAPS) - Pharmaceutical Sciences World Congress. New Orleans, LA.

Mercolini L, Mandrioli R, Gerra G, Raggi MA. (2010). Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection: a novel test for cocaine and alcohol intake. J Chromatogr A 1217:7242-7248.

Mercolini L, Mandrioli R, Sorella V, Somaini L, Giocondi D, Serpelloni G, Raggi MA. (2013). Dried blood spots: liquid chromatography-mass spectrometry analysis of Δ9-tetrahydrocannabinol and its main metabolites. J Chromatogr A 1217:33-40.

Moll V, Clavijo C, Cohen M, Christians U, Galinkin J. (2009). A Analytical Method To Determine Ketamine and Norketamine Levels in Dried Blood Spots. Proceedings of the Annual Meeting of the American Society of Anesthesiologists (ASA). New Orleans, LA.

Mommers J, Mengerik Y, Ritzen E, Weusten J, van der Heijden J, van der Wal S (2013). Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. Anal Chem Acta 774:26-32.

Murphy SE, Wickham KM, Lindgren BR, Spector LG, Joseph A (2013). Cotinine and *trans* 3'-hydroxycotinine in dried blood spots as biomarkers of tobacco exposure and nicotine metabolism. J Expo Sci Environ Epidemiol 23(5):513-518.

Saussereau E, Lacroix C, Gaulier JM, Goullé JP. (2012). On-line liquid chromatography/tandem mass spectrometry simultaneous determination of opiates, cocainics and amphetamines in dried blood spots. J Chromatogr B 885-886:1-7.

Skopp G (2007). Blood spot analysis. Joint Meeting of the ICADTS and TIAFT. Seattle, WA.

Sosnoff CS, Ann Q, Bernert JT, Jr., Powell MK, Miller BB, Henderson LO, Hannon WH, Fernhoff P, Sampson EJ. (1996). Analysis of benzoylecgonine in dried blood spots by liquid chromatography--atmospheric pressure chemical ionization tandem mass spectrometry. J Anal Toxicol 20:179-184.

Spector LG, Hecht SS, Ognjanovic S, Carmella SG, Ross JA. (2007). Detection of cotinine in newborn dried blood spots. Cancer Epidemiol Biomarkers Prev 16:1902-1905.

Thomas A, Déglon J, Steimer T, Mangin P, Daali Y, Staub C. (2010). On-line desorption of dried blood spots coupled to hydrophilic interaction/reversed-phase LC/MS/MS system for the simultaneous analysis of drugs and their polar metabolites. J Sep Sci 33:873-879.

Thomas A, Geyer H, Schänzer W, Crone C, Kellmann M, Moehring T, Thevis M. (2012). Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. Anal Bioanal Chem 403:1279-1289.

Appendix 2 Overview per patient of the sodium oxybate dose which was taken at bedtime, the measured GHB concentrations, the time between sodium oxybate intake and DBS collection, the number of usable DBS and remarks. If at least 2 DBS on a DBS card had acceptable quality (№ usable DBS ≥ 2), analysis was performed in duplicate (n= 2 DBS) and the result of the 2 measurements is reported (table GHB conc (µg/ml)). The remaining DBS were kept for eventual re-analysis. If only one DBS was considered suitable for analysis (№ usable DBS =1), that DBS was analyzed, and only that single result is given (table GHB conc (µg/ml)). If there were no DBS with acceptable quality provided (№ usable DBS =0), no analysis could be performed.

Patient 1 4.50 g (53.6 mg/kg b.w.)	**GHB conc (µg/ml)**	**Δ time (min) sodium oxybate intake-DBS collection**	**№ usable DBS**	**Remarks**
Day 1	32.6 35.2	20	4	
Day 2	42.9 41.9	15	3	
Day 3	39.1 40.9	20	4	
Day 4	19.9 28.3	30	4	
Day 5	25.7	20	2	At morning: 20.2 µg/ml
Day 6	68.1 61.2	20	4	
Day 7	51.0 44.0	20	4	
Patient 2	**GHB conc (µg/ml)**	**Δ time (min) sodium oxybate intake-DBS collection**	**№ usable DBS**	**Remarks**
Series 1 3.75 g (75.0 mg/kg b.w.)				
Day 1	54.9	17	1	
Day 2	20.7	25	1	
Day 3	103.8	20	1	
Day 4			0	
Day 5	49.1	17	1	
Day 6	19.2	15	1	
Day 7	112.7	18	1	

Series 2 **3.75 g** **(75.0 mg/kg b.w.)**				
Day 1	49.2 57.5	20	4	Long sleep, slept well 1.16 am until 4.53 am
Day 2	48.7 50.1	25	4	Slept normal 0.30 am until 3.25 am
Day 3	201	15	1	Short sleep, tired again shortly after – 5.00 am until 6.30 am **DBS collected after 2nd dose**
Day 4	132 117	20	3	Slept not enough and badly 0.20 am until 2.45 am
Day 5	152 136	20	3	Slept not enough and badly 1.10 am until 3.50 am
Day 6	150 173	20	2	Slept not enough and badly 0.05 am until 2.30 am
Day 7	55.5	15	1	Slept not long, but good 3.50 am until 6.30 am **DBS collected after 2nd dose**
Patient 3	**GHB conc (µg/ml)**	**Δ time (min) sodium oxybate intake-DBS collection**	**№ usable DBS**	**Remarks**
Series 1 **4.50 g** **(41.3 mg/kg b.w.)**				
Day 1	86.7	30	1	
Day 2	88.1 82.7	30	2	
Day 3	51.8 53.4	30	4	
Day 4	43.0 41.7	30	4	
Day 5				Stayed unexpectedly with his parents
Day 6	68.2 63.0	30	3	
Day 7	56.4 57.5	30	4	
Series 2 **4.50 g** **(40.9 mg/kg b.w.)**				
Day 1	82.7 74.2	20	3	
Day 2	64.0 54.5	20	3	
Day 3	81.8 81.7	20	4	
Day 4	61.9 61.1	20	4	
Day 5	9.10 8.17	20	4	Unexplainable low GHB conc
Day 6	86.5 94.9	20	4	
Day 7				Used all the material

SUMMARY

The aim of this work was to quantify the low molecular weight compound and drug of abuse gamma-hydroxybutyric acid (GHB) in various biological matrices using gas-chromatography coupled to mass spectrometry (GC-MS). The structure of GHB requires chemical modification and we chose to derivatize GHB to enhance volatility, to improve the chromatographic properties and to increase detection sensitivity. Since derivatization typically results in an additional step during sample treatment, we aimed to develop one-step sample preparation procedures based on direct derivatization techniques.

In **PART I** of this thesis**,** a brief background on GHB is provided, including an overview of its chemical properties, metabolization, use, abuse, effects, adverse effects and current legal status (**Chapter I.A**). **Chapter I.B** provides an in-depth overview of screening and confirmation techniques used for GHB in biofluids.

In the second part (**PART II**) of this work, the development of an accurate and sensitive method for the GC-MS-based determination of GHB in dried whole blood samples is presented. A dried blood spot (DBS) is capillary whole blood obtained by a finger or heel prick and collected on a filter paper. DBS sampling has generally been used for newborn screening. However, more recently this alternative sampling strategy is increasingly receiving interest in the context of e.g. therapeutic drug monitoring (TDM), (pre)clinical studies, pharmacokinetics and toxicology. An overview of the use of DBS in toxicology, with a focus on the determination of drugs of abuse, is presented in **Chapter II.A**. The DBS sampling technique ensures an easy and rapid collection of a representative sample without specific handling or storage requirements. This is of interest for GHB, which is rapidly metabolized following ingestion (hence, rapid sampling is an advantage) and which is subject to storage issues (*de novo* formation).

Since GHB requires chemical modification prior to GC analysis and derivatization is generally experienced as laborious and tedious, we opted to directly derivatize GHB in DBS, setting-up a quick and efficient sample treatment procedure. As summarized in **Chapter II.B**, if derivatization of the DBS sample is necessary, a DBS sample treatment procedure generally starts with extraction, followed by evaporation of (an aliquot of) the extraction solvent under a stream of nitrogen before adding the derivatization reagent(s). Then, the excess reagent is removed and finally the

redissolved or reconstituted derivatized extract is ready to be injected. Modifications of this general scheme have been described, e.g. the sample can be injected directly after the derivatization step. Another convenient DBS treatment procedure is obtained by direct derivatization, thus by applying extraction solvents and derivatization reagents simultaneously to the DBS, or even by adding only the derivatization reagents "on spot" without the use of any extraction solvent. The latter approach was chosen for method development.

Chapter II.C.1 and **II.C.2** describe the optimization of the complete procedure for quantitative analysis of GHB in DBS, with special attention to the sample treatment, followed by method validation. First, DBS of 50 µl were prepared and, after addition of internal standard GHB-d6, these were directly derivatized ("on spot") using 100 µl of a freshly prepared mixture of trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB-OH) (2:1). Following drying and reconstitution in ethylacetate, the derivatized extract was injected into a GC-MS, operating in the electron impact mode (EI), with a total run time of 12.3 min. Method validation included the evaluation of linearity, precision, accuracy, sensitivity, selectivity and stability. A weighting factor of $1/x^2$ was chosen and acceptable intra-batch precision, inter-batch precision and accuracy were seen. The linear calibration curve ranged from 2 to 100 µg/ml, with a limit of detection of 1 µg/ml.

As we also wished to collect DBS in a real-life setting, a more convenient approach than the use of precision capillaries is the collection of the drops of blood directly on the filter paper. The adjustment of the first method to enable the analysis of a fixed area (6-mm DBS punch) instead of a fixed volume (50-µl DBS) is presented in **Chapter II.C.3**. Punching out a disc requires the investigation of the impact of additional parameters such as the influence of the volume spotted, of the punch localization and of the hematocrit (Ht). Method validation included the evaluation of linearity, precision, accuracy, sensitivity, dilution integrity, selectivity and stability. The best blood volume spotted was between 20 and 35 µl, regardless of the Ht of the blood sample. Furthermore, a homogenous distribution of GHB in DBS was demonstrated. The 6-point calibration curve ranged from 2 to 100 µg/ml with a limit of detection of 1 µg/ml. QC samples (2, 10 and 100 µg/ml) were prepared separately in whole blood with low (0.38), intermediate (0.45) and high (0.50) Ht. A weighting factor of $1/x^2$ was chosen and overall acceptable precision (% RSD between 3.8 and 14.8) and accuracy were obtained (% bias between 1.2 and 12.2). GHB appeared to be stable in DBS stored at RT for at least 148 days. In 24 cases, a suspected GHB-intoxication was successfully

confirmed by DBS analysis, suggesting the routine applicability of the DBS sampling technique for GHB analysis in toxicological cases.

Chapter II.C.4 presents an exploratory study set up to measure GHB concentrations in DBS collected by narcoleptic patients who use the sodium salt of GHB (sodium oxybate, Xyrem®). The applicability of the developed DBS-based GC-MS method was evaluated, as well as the feasibility of the sampling technique in an ambulant setting. Therefore, 7 narcoleptic patients being treated with sodium oxybate at the department for Respiratory Diseases of Ghent University Hospital were asked to collect DBS approximately 20 min after the first sodium oxybate intake during a maximum of 7 consecutive days. Using an automatic lancet, patients pricked their fingertip and collected blood drops on a DBS card. The DBS cards were sent to the laboratory by regular mail and, before analysis, were visually inspected to record DBS quality (large enough, symmetrically spread on the filter paper with even coloration on both sides of the filter paper). In total, 5 series of DBS were obtained from 3 patients. Analyzing the DBS in duplicate resulted in acceptable within-DBS card precision and DBS with acceptable quality were obtained by patients without supervision.

The third part of this work (**PART III**) describes the development of a headspace-trap (HS-trap) GC-MS method to determine GHB in various biological fluids. Following a brief description of headspace injection techniques, with a focus on headspace-trap and its applications found in literature (**Chapter III.A**), the development of the HS-trap method is presented (**Chapter III.B**). Following optimization of headspace conditions and trap settings, validation was performed. Although sample preparation only consists of the addition of salt and derivatization reagents directly to a 100 µl-sample in a HS-vial, adequate method sensitivity and selectivity was obtained. Calibration curves ranged from 5 to 150 µg/ml GHB for urine, from 2 to 150 µg/ml for plasma, and from 3.5 to 200 µg/ml for whole blood. Acceptable precision and accuracy (<13 % bias and imprecision) were seen for all quality controls (lower limit of quantification-level, low, medium, high), including for the supplementary serum- and lyzed blood-based QC's, using calibration curves prepared in plasma or whole blood, respectively.

To conclude (**PART IV**), procedures to determine GHB in microvolumes (≤ 100 µl) of biofluids have successfully been developed, validated and applied. The novel approach of direct "on spot" derivatization, followed by analysis with GC-MS, proved to be reliable, fast and applicable in routine toxicology for the analysis of both volumetrically applied DBS as for analysis of discs

punched out from DBS. Furthermore, results of an exploratory study in patients treated with Xyrem® (the sodium salt of GHB, sodium oxybate) demonstrated the acceptable precision of the complete procedure, from sampling at home to quantitative analysis in the laboratory. Given the intra- and inter-individual variability in clinical effects seen with sodium oxybate, the easy adaptation of DBS sampling opens the possibility of following up GHB concentrations in patients in real-life settings in future studies. A second one-step procedure, using HS-trap, has been successfully developed and validated to determine GHB in various biofluids. Combining this relatively novel and fully automated headspace technique with "in-vial" methylation of GHB allowed for a straightforward approach. One single method could be used for all biofluids (urine, plasma, serum, whole blood or lyzed blood). Moreover, our approach involves mere addition of all reagents and sample into one vial. Incurred sample reanalysis demonstrated assay reproducibility, while cross-validation with another GC-MS method demonstrated that our method is a valuable alternative for GHB determination in toxicological samples, with the advantage of requiring only 100 µl and minimal hands-on time, as sample preparation is easy and injection automated.

SAMENVATTING

Dit werk had als doel om gamma-hydroxyboterzuur (GHB), een laag-moleculair-gewichtscomponent die misbruikt wordt als drug, op te sporen en te kwantificeren in verscheidene biologische matrices, hierbij gebruik makend van gas chromatografie gekoppeld aan massa spectrometrie (GC-MS). Gezien GHB chemisch gemodificeerd dient te worden om de vluchtigheid, chromatografische eigenschappen en detectiegevoeligheid te verbeteren, kozen we voor derivatisatie van GHB. Het includeren van een derivatisatiestap betekent echter veelal een verlenging van de staalvoorbereiding. Daarom was het ook onze doelstelling één-staps staalvoorbereidingsprocedures te ontwikkelen door het gebruik van directe derivatisatie.

In het eerste deel van dit werk, ***PART I***, wordt algemene informatie omtrent GHB weergegeven. Meer bepaald worden in ***Chapter I.A***. de chemische eigenschappen, metabolisatie, gebruik, misbruik, effecten en neveneffecten kort beschreven. Vervolgens wordt informatie gegeven over de wetgeving omtrent GHB. Het tweede hoofdstuk van dit deel, ***Chapter I.B***, geeft een gedetailleerd overzicht van screenings- en bevestigingstechnieken toegepast voor GHB in biologische vloeistoffen.

Het tweede deel, ***PART II***, beschrijft de ontwikkeling van een accurate en gevoelige methode voor de bepaling van GHB in gedroogde bloedspots (*DBS*) gebruik makend van GC-MS. Een gedroogde bloedspot is capillair bloed verkregen door de vingertip of de hiel te prikken en de bloeddruppels te verzamelen op een filterpapier. Deze manier van staalafname wordt reeds een 50-tal jaar gebruikt voor de screening bij pasgeborenen op zeldzame metabolische stoornissen (*newborn screening*). Meer recent is er een toegenomen interesse om deze alternatieve staalafname ook te gebruiken in bijvoorbeeld therapeutische drug monitoring, in (pre)klinische studies, voor farmacokinetiek en in de toxicologie. Een overzicht van het gebruik van DBS in het domein van de toxicologie, met bijzondere aandacht voor de bepaling van drugs in DBS, wordt gegeven in ***Chapter II.A***. Deze wijze van staalafname maakt een eenvoudige en snelle afname van een representatief staal mogelijk, vaak zonder specifieke behandelings- of bewaringsvereisten. Dit is in het bijzonder van belang voor GHB. Het wordt snel gemetaboliseerd na orale inname en bijgevolg biedt een snelle en eenvoudige staalafname voordelen. Bovendien is GHB gevoelig voor wijzigingen in concentratie tijdens bewaring (*de novo* vorming).

Zoals vermeld vereist de structuur van GHB een chemische modificatie om GC analyse mogelijk te maken. Omdat implementatie van derivatisatie bij staalvoorbereiding in het algemeen een significante verhoging van de werklast met zich meebrengt, kozen we voor een directe derivatisatie van GHB in DBS. Deze aanpak resulteerde in een snelle en efficiënte staalvoorbereiding. Zoals beschreven in ***Chapter II.B*** begint, indien derivatisatie van de componenten die men wenst te bepalen in DBS nodig is, de staalvoorbereiding in het algemeen met een extractie, gevolgd door het droogdampen van (een deel van) het extractiesolvent m.b.v. stikstof alvorens de gewenste derivatisatiereagentia toe te voegen. Vervolgens wordt de overmaat reagens verwijderd en kan het staal, na (her)oplossen van het residu in een geschikt solvent, geïnjecteerd worden. Verschillende wijzigingen van deze algemene procedure zijn beschreven, zoals o.a. directe injectie van het staal na derivatisatie. Ook het simultaan toevoegen van extractiesolventen en derivatisatiereagentia aan de DBS of zelfs van de derivatisatiereagentia alleen ("*on spot*" zonder het gebruik van extractiesolventen), vereenvoudigt de staalvoorbereiding. Deze laatste strategie werd gevolgd bij onze methodeontwikkeling

Chapter II.C.1 en ***II.C.2*** beschrijven de optimalisatie van de volledige procedure voor de kwantitatieve bepaling van GHB in DBS, met speciale aandacht voor de staalvoorbereiding, gevolgd door methode validatie. Eerst werden DBS van 50 µl bereid en na de toevoeging van de interne standaard GHB-d6, werden deze direct gederivatiseerd ("on spot") m.b.v. 100 µl van een vers bereid mengsel van trifluoroazijnzuur anhydride (TFAA) en heptafluorobutanol (HFB-OH) (2:1). Na drogen en (her)oplossen in ethylacetaat werd het gederivatiseerde extract geanalyseerd met GC-MS, in de elektron impact *mode* (EI), met een totale looptijd van 12,3 min. Voor methode validatie werden lineariteit, precisie, accuraatheid, gevoeligheid, selectiviteit en stabiliteit geëvalueerd. Een weegfactor van $1/x^2$ werd gekozen en aanvaardbare intra- en inter-batch precisie werden bekomen samen met voldoende accuraatheid. De lineaire calibratiecurve had een bereik van 2 tot 100 µg/ml, met een detectielimiet van 1 µg/ml.

Omdat we ook op een meer praktische manier DBS wilden verzamelen, werd de eerste methode aangepast om ook bloeddruppels (rechtstreeks verzameld van de vingertip op een filterpapier) te kunnen analyseren op de aanwezigheid van GHB. Bijgevolg diende onze methode aangepast te worden zodat een bepaalde oppervlakte (schijfje met 6 mm diameter) geanalyseerd kon worden in plaats van een vast volume (50 µl). Dit wordt beschreven in ***Chapter II.C.3***. Analyse van een schijfje

uit een volledige DBS vereist bijkomend de evaluatie van de impact van parameters zoals de invloed van het DBS volume, de plaats van afzonderen en het hematocriet (Ht) op het bekomen resultaat. Voor methode validatie werden lineariteit, precisie, accuraatheid, mogelijkheid tot verdunnen, gevoeligheid, selectiviteit en stabiliteit geëvalueerd. Het optimale volume bloed aangebracht op het filterpapier ligt tussen 20 en 35 µl, ongeacht de hematocrietwaarde van het staal. Bovendien werd een homogene verdeling van GHB in DBS aangetoond. De calibratiecurve, opgebouwd uit 6 punten, had een bereik van 2 tot 100 µg/ml met een detectielimiet van 1 µg/ml. Stalen voor de kwaliteitscontrole (QC stalen) (2, 10 en 100 µg/ml) werden afzonderlijk klaargemaakt in bloed met laag (0,38), intermediair (0,45) en hoog (0,50) Ht. Een weegfactor van $1/x^2$ werd gekozen en in het algemeen werden een aanvaardbare intra- en inter-*batch* precisie en accuraatheid bekomen. GHB was stabiel in DBS bewaard bij kamertemperatuur gedurende 148 dagen. Tevens werd een vermoedelijke GHB-intoxicatie bevestigd in 24 patiënten gebruik makende van DBS analyse, wat de routinematige toepasbaarheid van een DBS staalafname voor de analyse van GHB in toxicologische casussen aantoont.

Chapter II.C.4 beschrijft een exploratieve studie die werd opgezet om GHB concentraties te bepalen in DBS aangemaakt door patiënten die het natrium zout van GHB (natrium oxybaat, Xyrem®) gebruiken voor de behandeling van narcolepsie met kataplexie. De toepasbaarheid van onze nieuw ontwikkelde GC-MS methode en de geschiktheid van de DBS staalafnametechniek in ambulante omstandigheden werden geëvalueerd. Aan 7 patiënten behandeld op de afdeling longaandoeningen van het UZ Gent werd gevraagd DBS aan te maken ongeveer 20 min na de eerste inname van natrium oxybaat en dit gedurende maximum 7 opeenvolgende dagen. M.b.v. een automatisch lancet konden de patiënten hun vingertip prikken om vervolgens bloeddruppels te verzamelen op een DBS kaart. Deze DBS kaarten werden verstuurd naar het laboratorium en werden vóór analyse geïnspecteerd op kwaliteit (groot genoeg, symmetrische spreiding en roodkleuring aan beide zijden van het papier). In totaal werden 5 reeksen DBS verkregen van 3 patiënten. Het analyseren van de DBS in duplicaat resulteerde in aanvaardbare binnen-DBS kaart precisie en DBS met aanvaardbare kwaliteit werden aangemaakt door de patiënten zonder enige supervisie.

Het derde deel van dit werk (***PART III***) beschrijft de ontwikkeling van een *headspace-trap* (*HS-trap*) GC-MS methode voor de bepaling van GHB in verschillende biologische vloeistoffen. Na een korte

beschrijving van de bestaande *HS* injectie technieken, met de focus op *HS-trap* en diens gepubliceerde toepassingen (***Chapter III.A***), wordt de ontwikkeling van de *HS-trap* methode beschreven (***Chapter III.B***). Na optimalisatie van de *HS* condities en *trap* instellingen werd de methode gevalideerd. Hoewel de staalvoorbereiding enkel de directe toevoeging van zout en derivatisatiereagentia aan 100 µl van een staal in een *HS-vial* omvat, werden adequate methode gevoeligheid en selectiviteit verkregen. Calibratiecurves hadden een bereik van 5 tot 150 µg/ml GHB voor urine, van 2 tot 150 µg/ml voor plasma, en van 3,5 tot 200 µg/ml voor bloed. Aanvaardbare precisie en accuraatheid werden verkregen voor alle QC stalen (niveau van de kwantificatielimiet, laag, medium, hoog), inclusief voor QC stalen bereid in serum en gelyseerd bloed, waarvoor calibratiecurves aangemaakt in respectievelijk plasma of bloed werden toegepast.

Tot slot (***PART IV***) kan er geconcludeerd worden dat in dit werk procedures voor de bepaling van GHB in microvolumes (≤ 100 µl) van biologische vloeistoffen werden ontwikkeld en gevalideerd. De nieuwe aanpak van "*on spot*" derivatisatie, gevolgd door analyse met GC-MS, bleek betrouwbaar, snel en toepasbaar in routine toxicologie voor de analyse van GHB in zowel volumetrisch aangemaakte DBS als in 6-mm schijfjes uit DBS. Daarenboven tonen de resultaten aan dat de volledige procedure – van de staalafname thuis tot en met de kwantitatieve analyse in het laboratorium – met aanvaardbare precisie kan worden uitgevoerd. Gezien de intra- en inter-individuele variatie in klinische effecten bij het gebruik van natrium oxybaat, zou de introductie van staalafname via de DBS techniek in de toekomst de opvolging van GHB concentraties mogelijk kunnen maken in ambulante omstandigheden.

Een tweede één-staps procedure voor de bepaling van GHB in verschillende biologische vloeistoffen werd succesvol ontwikkeld en gevalideerd gebruik makend van de *HS-trap* injectietechniek. Het combineren van deze relatief nieuwe en volledig automatische *HS* techniek met "*in-vial*" methylering van GHB, resulteerde in een uiterst eenvoudige procedure. Eenzelfde methode kon gebruikt worden voor alle onderzochte biologische vloeistoffen (urine, plasma, serum, bloed en gelyseerd bloed). Bovendien houdt onze aanpak enkel de toevoeging in van alle reagentia aan één vial. Herhaalde analyse van stalen toonde de reproduceerbaarheid aan van de ontwikkelde methodologie, terwijl cross-validatie met een andere GC-MS methode aangaf dat onze methode een waardig alternatief is voor de bepaling van GHB in toxicologische stalen. Deze methode heeft bovendien als voordelen dat er slechts 100 µl staal nodig is en dat ze minimale

manuele handelingen vereist, waarbij eenvoudige staalvoorbereiding en automatische injectie mogelijk zijn.

CURRICULUM VITAE

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Education

1997-2003: Latin-Maths high school degree at Sint-Bavo Humaniora, Ghent

2003-2008: Pharmacist degree obtained at Ghent University (distinction)

Work Experience

2008-2012: Ph.D. student at the Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University (Prof. Willy Lambert / Prof. Christophe Stove)

Extra-scientific contributions

- *Education*
  - Support of the practical excercises Toxicology given during the 1st semester of the 2nd Master in Pharmaceutical Care or Drug Development
  - Support of the Masterthesis of the 1st Master in Pharmaceutical Care or Drug Development (5 students)
  - Lessons in gas chromatography for students Biotechnology (evening course, CVO-IVV)
  - Support of demonstrations for *Chemical Criminalistics* (students Criminology)
- Laboratory & Department
  - Support in specific forensic cases
  - Representative of the Academic Assisting Staff at Departmental meetings
- Course followed
  - Basis of Gas Chromatography, Interscience, Breda, The Netherlands, Nov 18-20, 2008

2013-……: Stagiair-assistant juridical expert at the National Institute of Criminalistics and Criminology, Federal Public Service Justice in Brussels

Membership of scientific organizations

TIAFT (The International Association of Forensic Toxicologists)

BLT (Belgium Luxembourg Toxicological Society)

BGFW (Belgian Society of Pharmaceutical Sciences)

A1-publications (status 12/09/2013 - Web of Science)

- Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. Ingels ASME, Lambert WE, Stove CP; 2010. Analytical and Bioanalytical Chemistry, 398(5): 2173-2182.
  (IF (2010): 3,841; IF (2012): 3,659; 1st Quartile (9/75) of 'Chemistry, Analytical'; Times Cited: 10)

- Dried blood spot punches for confirmation of suspected GHB-intoxications: validation of an optimized GC-MS procedure. Ingels AS, De Paepe P, Anseeuw K, Van Sassenbroeck D, Neels H, Lambert W, Stove C; 2011. Bioanalysis, 3(20): 2271-2281.
  (IF (2011): 3,223; IF (2012): 3,253; 1st Quartile (15/75) of 'Chemistry, Medicinal'; Times Cited: 8)

- Dried blood spots in toxicology: from the cradle to the grave? Stove CP, Ingels ASME, De Kesel PMM, Lambert WE; 2012. Critical Reviews in Toxicology, 42(3): 230-243.
  (IF (2012): 6,253; 1st Quartile (5/85) of 'Toxicology'; Times Cited: 9)

- Feasibility of following up gamma-hydroxybutyric acid concentrations in sodium oxybate (Xyrem®)-treated narcoleptic patients using dried blood spot sampling at home. An exploratory study. Ingels ASME, Hertegonne K, Lambert WE, Stove CP; 2013. CNS Drugs, 27(3): 233-237.
  (IF (2012): 4,826; 1st Quartile (24/260) of 'Pharmacology & Pharmacy'; Times Cited: 0)

- Determination of gamma-hydroxybutyric acid in biofluids using a one-step procedure with "in-vial" derivatization and headspace-trap gas chromatography-mass spectrometry. Ingels ASME, Neels H, Lambert WE, Stove CP; 2013. Journal of Chromatography A, 1296: 84-92.

(IF (2012): 4,612; 1st Quartile (6/75) of 'Chemistry, Analytical'; Times Cited: 0)

- Quantitation of gamma-hydroxybutyric acid in dried blood spots in newborn screening. Stove CP, Ingels ASME, Lambert WE; 2013. Molecular Genetics and Metabolism, 110: 195. (Letter to the Editor)
  (IF (2012): 2,834; 2nd Quartile (47/121) of 'Medicine, Research & Experimental'; Times cited: 0)

Book chapter

Derivatization techniques in dried blood spot analysis. In "Dried Blood Spots: Applications and Techniques". Ingels ASME, Sadones N, De Kesel PMM, Lambert WE, Stove CP. Eds. Li W and Lee M, John Wiley & Sons, Chapter 27, *in press*.

International scientific journals (non-peer reviewed)

Dried blood spot analysis spreads to more applications. Ingels ASME, Lambert WE, Stove CP; 2012. Clinical and Forensic Toxicology News, March 2012: 2-3.

Congress participation (personal or in collaboration)

2009

- Poster presentation at the 47th Annual meeting of The International Association of Forensic Toxicologists (TIAFT) Geneve, Swiss, 23rd – 27th of August, 2009 Determination of GHB and related compounds in dried blood spots (Ann-Sofie ME Ingels, Willy E Lambert, Christophe P Stove; presenting author: Christophe P Stove) Abstract in Ann. Toxicol. Anal., 21, S1-76 (2009)

2010

- Poster presentation at the Joint meeting of the Belgium Luxembourg Toxicological Society (BLT) and Belgian Society of Emergency and Disaster Medicine (BESEDIM) Common and uncommon poisoning in the emergency room Brussels, Belgium, 13th of April, 2010 Determination of GHB in dried blood spots using a simple GC-MS method (Ann-Sofie ME Ingels, Willy E Lambert, Christophe P Stove)

- Poster presentation at the European Bioanalysis Forum (EBF) workshop: Connecting strategies on dried bloodspots Brussels, Belgium, 17th and 18th of June 2010 Determination of GHB in dried blood spots using a simple GC-MS method (Ann-Sofie ME Ingels, Willy E Lambert, Christophe P Stove)

- Poster presentation at the 48th Annual meeting of The International Association of Forensic Toxicologists (TIAFT) Joint meeting with the Society of Toxicological and Forensic Chemistry (GTFCh) Bonn, Germany, 29th of August – 2nd of September, 2010 Determination of GHB in dried blood spots using a simple GC-MS method with direct "on spot" derivatization (Ann-Sofie ME Ingels, Willy E Lambert, Christophe P Stove) Abstract in Toxichem. Krimtech., 77, 28-29 (2010)

- Oral presentation at the Belgium Luxembourg Toxicological Society (BLT) meeting Echternach, Grand Duchy of Luxembourg, 1th and 2nd of October 2010 Determination of gamma-hydroxubutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization: validation and application (Ann-Sofie ME Ingels, Willy E Lambert, Christophe P Stove; presenting author: Christophe P Stove)

2011

- Oral presentation at the 2011 Joint Society of Forensic Toxicology (SOFT) The International Association of Forensic Toxicologists (TIAFT) International Conference & Expo on Forensic and Analytical Toxicology San Francisco, United States, 25th – 30th of September, 2011 Determination of gamma-hydroxybutyric acid in dried blood spots with "on spot" derivatization and GC-MS: method development, validation and application (Ann-Sofie ME Ingels, Peter De Paepe, Kurt Anseeuw, Diederick van Sassenbroeck, Hugo Neels, Willy E Lambert, Christophe P Stove)

- Poster presentation at the 12th international congress of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) Stuttgart, Germany, 2nd – 6th of October, 2011 Determination of gamma-hydroxybutyric acid in dried blood spots with "on spot" derivatization and GC-MS: method development, validation and application (Ann-Sofie ME Ingels, Peter De Paepe, Kurt Anseeuw, Diederick van

Sassenbroeck, Hugo Neels, Willy E Lambert, Christophe P Stove; presenting author Christophe P Stove) (Best poster in Clinical Toxicology) Abstract in Ther. Drug Monit., 33, 474 (2011)

- Oral presentation at the 12th international congress of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) Stuttgart, Germany, 2nd – 6th of October, 2011 DBS in toxicology: "on spot" derivatization and GC-MS determination Application on DBS from GHB patients (Workshop New developments in dried blood spot sampling (DBS) in TDM) (Ann-Sofie ME Ingels, Peter De Paepe, Kurt Anseeuw, Diederick van Sassenbroeck, Hugo Neels, Willy E Lambert, Christophe P Stove; presenting author Christophe P Stove)

2012

- Oral presentation at the 16th forum of Pharmaceutical Sciences of the Belgium Society of Pharmaceutical Sciences, Blankenberge, Belgium, 7th and 8th of May, 2012 Determination of gamma-hydroxybutyric acid in dried blood spots of patients taking Xyrem®: Application of a validated GC-MS method (Ann-Sofie ME Ingels, Katrien Hertegonne, Willy E Lambert, Christophe P Stove)

2013

- Oral presentation at the 51th Annual meeting of The International Association of Forensic Toxicologists (TIAFT) Funchal, Madeira, Portugal, 2nd – 6th of September, 2011 One for all, all in one: Determination of gamma-hydroxybutyric acid in biofluids using "in-vial" derivatization and headspace-trap gas chromatography mass spectrometry (Ann-Sofie ME Ingels, Willy E Lambert, Hugo Neels, Christophe P Stove; presenting author Christophe P Stove) Abstract in abstract book, OA6, p69.