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Automated High-Throughput Analysis of Tramadol and O-Desmethyltramadol in Dried Blood Spots

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Abstract

The World Anti-Doping Agency (WADA) and the International Testing Agency (ITA) recently announced the development and implementation of dried blood spot (DBS) testing for routine analysis in time for the 2022 Winter Olympic and Paralympic Games in Beijing. Following the introduction of a ban on the use of Tramadol in competition in March 2019, the Union Cycliste International (UCI) started a pilot study for the manual analysis of Tramadol in DBS for anti-doping purposes.

In this context, we present a fully automated LC-MS/MS-based method with automated sample preparation using a CAMAG DBS-MS 500 for the analysis of tramadol and its metabolite O-desmethyltramadol in DBS. The presented approach reduces manual handling in the laboratory to an absolute minimum, only requiring the preparation of calibration and quality control DBS cards. The method was developed, optimized, and validated before performing cross-validation with a liquid blood-based analysis method using authentic samples from forensic cases.

During the validation process, the method showed an extraction efficiency of 62%, linearity r^2>0.99, accuracy and precision (within ±15% and ±20% at the LLOQ) for the determination of tramadol and

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O-desmethyltramadol. Method comparison in liquid blood with 26 samples showed good agreement (90±19% for tramadol and 94±14% for O-desmethyltramadol). In conclusion, automated analysis of tramadol and O-desmethyltramadol in DBS provides a fast and accurate solution for anti-doping screening. It is suited for high-throughput analysis, having a run time of about 4 min per sample. Furthermore, with the automated approach, manual sample extraction becomes obsolete.

Keywords

Antidoping, Tramadol, Dried Blood Spots, Fully Automated Analysis

Introduction

Tramadol is a synthetic analgesic used to relieve moderate to severe acute and chronic pain [1]. The drug acts on serotonergic and noradrenergic nociception, while its major metabolite O-desmethyltramadol acts on an opioid receptor [2,3]. The standard therapeutic doses of tramadol are 50 to 100 mg orally. Tramadol shoul not be administered more frequently than four hourly with a maximum dosage of 400 mg per day. By injection, an initial dose of 100 mg is given, followed by 50 mg every 10-20 minutes up to a total dose of 250 mg [4]. Therapeutic blood levels range from 0.1-0.8 mg/L, toxic concentration from 1 to 2 mg/L, and lethal concentration are considered to be higher than 2 mg/L. Generally, therapeutic, toxic, and lethal levels of tramadol are relatively close [5]. In most countries, tramadol is a prescription-only medicine. However, it is freely available via the internet. Common side effects, mostly at the beginning of tramadol therapy, are nausea, dizziness, and vomiting [2]. Tramadol attracted the interest of anti-doping agencies because its use, and the accompanying health side effects, could be dangerous for the sports players. The narcotic has been on the WADA monitoring program since 2012. This program was designed to monitor and detect patterns of misuse in substances not included in the prohibited list, but with the possibility of being harmful in sport. In a study, the prevalence of tramadol findings was at highest in cycling [1]. Since March 1st, 2019, tramadol has been banned by the UCI for medical reasons across all cycling disciplines. This ban refers to in-competition testing to suppress exercise-induced pain. To enforce compliance with the ban, non-invasive, volumetric dried blood spot (DBS) testing is performed [6].

In this context, we developed a fully automated DBS-based LC-MS/MS method for the simultaneous analysis of tramadol and O-desmethyltramadol. The main focus was thereby put on a system offering high-throughput and minimal turnaround time. The DBS automation was realized using a CAMAG DBS-MS 500 extraction system coupled to a triple quadrupole mass spectrometer. The successful implementation of this combination for various DBS applications has been demonstrated beforehand [7].

Materials and methods

Chemicals and materials

Cis-tramadol HCl (1 mg/mL, as free base), O-Desmethyl-cis-tramadol (1 mg/mL, as free base), and the respective internal standards cis-tramadol-^{13}C, D_3 HCl (100 µg/mL, as free base) and O-Desmethyl-cis-tramadol-D_6 (100 µg/mL, as free base) were obtained from Cerilliant (Round Rock, TX, USA).

For the fully automated analysis: 2-Propanol, acetonitrile, water, and methanol, were purchased from Carl Roth (Karlsruhe, Germany) in ultra LC-MS grade quality. Formic acid, LC-MS grade quality (labeled as ULC/MS), was purchased from Biosolve BV (Valkenswaard, Netherland). BioSample TFN filter paper AutoCollect™ DBS cards were used to prepare volumetric dried blood spots (supplied by CAMAG, Switzerland).

For the manual liquid blood analysis: 1-chlorobutane, puriss p.a., was obtained from Sigma-Aldrich (St. Louis, MS, USA). Di-Natriumhydrogenphosphate anhydrous was obtained from Merck (Darmstadt, Germany). Acetonitrile, 99.9%, for HPLC gradient grade was ordered from Acros Organics (Geel Belgium). Formic acid, 98%, puriss.p.a., was ordered from Fluka (Buchs, Switzerland). De-ionized water was produced with a Milli-Q water system from Millipore (Billerica, USA).

Preparation of DBS calibrators and quality control samples

Individual calibrator and quality control DBS samples were prepared by mixing 240 µL of whole blood (collected in Vacutainer tubes from BD (Franklin Lakes, NJ, USA), containing lithium heparin as an anticoagulant) with 10 µL from individual analyte working solution containing a mixture of tramadol and O-desmethyltramadol. After spiking, the samples were shaken for one hour on an IKA KS 501 digital shaker (Staufen, Germany), operated at 300 rpm, and spotted using a volumetric pipette (Socorex Acura 825). Before the analysis, samples were let to dry at room temperature (about 21°C) for at least three hours.

Fully automated extraction of DBS samples

A DBS-MS 500 (CAMAG, Switzerland) was connected as the front end autosampler to a Shimadzu (Kyoto, Japan) LC-MS system, see Figure 1. The extraction solvent for the DBS elution consisted of a water-methanol mixture (90/10,v/v). The elution solvent and the chromatographic conditions were optimized to achieve baseline separation and nearly symmetric peak shape for both analytes, requiring higher quantities of aqueous solution to resolve O-desmethyltramadol nicely. Each DBS card was

photographed with a built-in camera before and after the extraction process, to document the samples, check for the presence of a blood spot, to center the extraction head position, and to verify where the extraction took place. Methanolic internal standard solution (20 µL spray volume, containing 25 ng/mL cis-tramadol-^{13}C, D_3, and 10 ng/mL O-Desmethyl-cis-tramadol-D_6) was sprayed in a homogenous layer of 1 cm^2 over the center of each spot (flow rate of 120 µL/min). Afterward, the internal standard solution was dried for 60 s at room temperature. In contrast to mixing the internal standard into the extraction solvent, this procedure enables compensating for extraction differences (recovery bias) [8,9]. The extraction of a 4.2 mm sub-punch from the center of the DBS was realized with an extraction volume of 20 µL at a flow rate of 30 µL/min, into a 20 µL Viper Loop from Thermo Fisher Scientific (Waltham, MA, USA). Afterward, the extraction cell and tubings were rinsed with the inbuilt wash station using two different solvents (solvent 1: 45 s with methanol/water/acetonitrile/2-propanol 25/25/25/25, v/v/v/v, solvent 2: 20 s with the extraction solution)

Liquid chromatography and mass spectrometry conditions

The Shimadzu based LC-MS system composed of two Nexera LC30 AD X2 pumps, a CTO-20AC column oven equipped with a six-port switching valve and a 20 µL stainless steel mixer, and an LCMS-8050 mass spectrometer equipped with an electrospray ionization source operated in positive, selected reaction monitoring (SRM) mode (see Table I). Interface parameters were: nebulizing gas flow 3 L/min, heating gas flow 10 L/min, drying gas flow 10 L/min interface temperature 300°C, desolvation line temperature 250°C, and heat block temperature 400°C. Chromatographic separation of the analytes was realized with a Kinetex 2.6 µm biphenyl, 100 x 2.1 mm column from Phenomenex (Torrance, CA, USA), preheated at 50°C. The column was equilibrated with a mixture of 99% A (water containing 0.1% formic acid) and 1% B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.5 mL/min. The gradient for the analytical column was as follows: From 0.1 min to 2.0 min, the % B was increased to 60%. From 2.0 to 2.5 min, the % B was increased to 95% B and a flow rate of 0.75 mL/min. At 2.5 min, the flow rate and solvent composition were returned to the starting conditions and held until 3.0 min. To prevent system and column contamination, a KrudKatcher Ultra filter from Phenomenex was connected upstream to the analytical column, and the flow to the mass spectrometer was only active from 1.6-2.35 min by switching the ten-port valve within the column oven (for a chromatogram, see Figure 2).

Method validation

Method validation experiments were performed to establish the validity of the fully automated LC-MS/MS orienting on the FDA guidelines for Bioanalytical Method Validation Guidance for Industry (May 2018)[10].

Sensitivity

The limit of detection (LOD) was set according to a signal-to-noise ratio ≥3 for all the monitored transitions (quantifier and qualifier) and evaluated by series of dilution. The LLOQ was set in agreement with the therapeutic blood level of tramadol (0.1-0.8 mg/L) and was tested for the accurate determination of its concentration with accuracy and precision within ±20% [11].

Selectivity

The selectivity of the method was tested by analyzing blank DBS samples from six different subjects (4 male, 2 female), with and without the spray addition of internal standard, and comparing them to blank blood samples spiked at the LLOQ (25 ng/mL for tramadol and 20 ng/mL for O-desmethyltramadol).

Calibration and linearity

The linearity of the signal response was assessed by analyzing tramadol and O-desmethyltramadol in DBS calibration samples on three separate days, measuring a calibration at the beginning and the end of each batch. For tramadol, eight levels of DBS calibration samples covering a range from 25-1000 ng/mL (25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL) were measured together with the respective internal standard. For O-desmethyltramadol, seven levels of DBS calibration samples covering a range from 20-400 ng/mL (20, 40, 80, 160, 240, 320, and 400 ng/mL) were measured together with the respective internal standard. Concentrations were determined using the LabSolution Browser Data Analysis Tool, fitting the calibration curve in linear regression with a weighing of 1/x.

Accuracy and precision

Accuracy and precision were determined by replicate analysis (n=6) in DBS from separate quality control (QC) cards in three different batches on three separate days. Tramadol QC samples were measured at the lower limit of quantitation (LLOQ, 25 ng/mL), at medium (200 ng/mL), and at high (600 ng/mL) concentrations. O-desmethyltramadol QC samples were analyzed at the LLOQ (20 ng/mL), at medium (80 ng/mL), and at high (240 ng/mL) concentrations. The accuracy was measured as the closeness of the target analyte concentration and expressed as percent difference (%bias). Precision was expressed as %CV of the method. Acceptance criteria were ±15% for both accuracy and precision at all concentrations except for the LLOQ, where ±20% are acceptable.

Matrix effect and ion suppression

Matrix effect interferences were assessed by the injection of matrix extracts from three different blank blood samples spiked with different spike solutions to result in different tramadol (100 ng/mL and 600 ng/mL) and O-desmethyltramadol concentrations (40 ng/mL and 240 ng/mL).. Each sample was prepared in triplicate by extracting a single 20 µL DBS spot in the DBS MS-500 elution solution (10/90, methanol/water) for half an hour while shaking at 300 rpm on an IKA KS 501 digital shaker. Afterward, the extracts were evaporated to dryness in a heating block set at 50°C, with a gentle stream of nitrogen, and reconstituted in 500 µL of elution solvent containing ISTD and analyte. For the matrix effect, the internal standard corrected analyte area was compared to samples containing no matrix. Ion enhancement and ion suppression were investigated by comparing the analyte area without any internal standard correction. As a pure solvent solution spreads differently on filter paper than blood samples, an evaluation of the fully automated method's recovery was not possible with the fully automated, sub-punch based approach.

Carry-over

Carry-over was assessed by the analysis of the highest calibrator in triplicate (1000 ng/mL), followed by a blank blood sample.

Extraction efficiency

The extraction efficiency was established by a sixfold extraction and analysis of the same spot. Thereby the total analyte signal was summed and compared to the signal intensity obtained during the first extraction to report the method's extraction efficiency.

Stability

The stability of the DBS samples was assessed by storing DBS samples spiked at 400 ng/mL, packed in a minigrip bag together with drying agent (silica gel pack), for one month at room temperature and in the freezer at -20°C. A comparison between freshly prepared DBS samples and the stability samples was performed in triplicate.

Hematocrit effect

For the evaluation of the hematocrit dependency of the analysis, DBS with artificial hematocrit values were prepared: Freshly collected blank blood was centrifuged (10 min with an RCF of 1300) to separate red blood cells and plasma. Blood and plasma were mixed to create a range of blood hematocrit levels (20, 30, 40, 50, 60, and 70 %). Afterward, the blood samples were spiked with different spike solutions to result in different tramadol (100 ng/mL and 400 ng/mL) and O-desmethyltramadol concentrations (40 ng/mL and 160 ng/mL). DBS were prepared from these solutions (20 µL) and analyzed in triplicate.

Spot size effect

To evaluate the effect of spot size on the analysis result, blood was spiked at 200 ng/mL and spotted on DBS cards using different volumes (7.5 µL 10 µL, 15 µL, 20 µL). Each sample volume was analyzed in triplicate. The recommendation for the maximum volume loaded per sample area of AutoCollect™ DBS cards is 20 µL.

Cross-Validation to the analysis of authentic samples in liquid blood

A total of 26 authentic samples, which were found to be positive for tramadol (>LOD and <1000 ng/mL) during the routine forensic casework at the Institute of Forensic Medicine Bern, were spotted as DBS and reanalyzed using fully automated DBS analysis to perform cross-validation. The authentic blood samples were obtained between October 2018 until August 2019 and analyzed after the reception. DBS analysis of all the samples took place in February 2020. Before the DBS analysis, the samples were stored in liquid form in the freezer at -20°C. For the analysis of tramadol and O-desmethyltramadol in liquid blood, a fully validated method was used with a five-point calibration from 50-1000 ng/mL. In brief, 200 µL of blood was extracted with 1 mL 1-chlorobutane together with a phosphate buffer (pH 9, 5.0 g Na_2HPO_4 in 500 mL water) and internal standard. After vortexing for 5 min, the sample was centrifuged at 13'000 rpm. The supernatant was transferred into a glass vial, evaporated to dryness in a heating block set at 50°C, with a gentle stream of nitrogen, and reconstituted in 200 µL of reconstitution solvent (water/acetonitrile, 5/95 +0.1% formic acid). Chromatographic separation took place by injecting 20 µL onto a Synergi Polar RP 4 µm, 50 x 2.0 mm column from Phenomenex (Torrance, CA, USA). Mass spectrometric analysis was performed on a Sciex 3200 QTrap (Toronto, Canada), operated in positive electrospray, SRM mode.

Results

Method validation

Sensitivity

The LOD for tramadol was observed at 2 ng/mL, the LOD for O-desmethyltramadol at 5 ng/mL. To ensure proper quantification of concentations below and within the therapeutic range while maintaining the accuracy and precision within ±20%, the LLOQ for tramadol was set to 25 ng/mL and for O-desmethyltramadol to 20 ng/mL.

Selectivity

The analysis of blank DBS samples from six different subjects showed noise signal intensities <1000 in the retention time region of tramadol and <250 in the retention time region of O-desmethyltramadol for the SRM 1 transitions. After the addition of internal standard, minor SRM 1 signal intensities occurred: For tramadol up to 2'200 counts and for O-desmethyltramadol, up to 600 counts were observed. At the LLOQ, 380'000 counts for tramadol and 125'000 counts for O-desmethyltramadol were found.

Calibration and linearity

Tramadol and O-desmethyltramadol in DBS showed a good linear correlation. The following coefficient of determination (r^2) values were found throughout the three validation runs: Tramadol SRM 1 0,99398±0,00337 (range: 0,99013-0,99641), tramadol SRM 2 0,99404±0,00285 (range: 0,9908-0,99612), O-desmethyltramadol SRM 1 0,99196±0,00233 (range: 0,9906-0,99466), and O-desmethyltramadol SRM 2 0,99297±0,00147 (range: 0,99135-0,9942).

Accuracy and precision

The accuracy (%bias) and precision (%CV) determined from six replicates of each of the three different QC samples on three separate days were both <15%, except for the LLOQ, where <±20% is tolerable, see Table II.

Matrix effects and ion suppression

Post-extraction spiking of matrix extracts revealed that the average matrix effect (%ME) in comparison to a neat solution was 99±3% at K3 and 96±8% at K6 for tramadol and 103±2% at K3 and 101±5% at K6 for O-desmethyltramadol. Thus, the investigated matrix effects can be considered as negligible when compensated with deuterated internal standards. Ion suppression was investigated by comparing the analyte area without any internal standard correction: For tramadol a response of 66±7 % at K3 and 66±7% at K6 and for O-desmethyltramadol a response of 73±9% at K3 and 81±14% at K6 was observed.

Carry-over

Carry over analysis after triplicate analysis of K7 showed an average carry-over of about 0.4%, representing 6-8 % of the tramadol signal area and 2-3% of the O-desmethyltramadol signal area observed at the LLOQ. The analysis of a blank sample reduces carry-over after the highest calibrator sample.

Extraction efficiency

Extraction efficiency was established at the high QC concentration (600 ng/mL tramadol and 240 ng/mL O-desmethyltramadol), being 62% with the first extraction, and 16% with the second extraction.

Stability

After a month of storage, DBS samples showed no decrease in concentration, independent of storage in the freezer or at room temperature. The average concentrations obtained for tramadol were 98% when stored at room temperature and 95% when stored in the freezer. For O-desmethyltramadol 97% when stored at room temperature and 90%, when stored in the freezer, were observed.

Hematocrit effect

Over the range of 20-60%, there was no issue of HCT-based area bias when analyzing 4.2 mm diameter DBS sub-punches using internal standard spray application for the two respective concentrations of tramadol (100 ng/mL and 400 ng/mL) and O-desmethyltramadol (40 ng/mL and 160 ng/mL). The observed average concentrations obtained at different hematocrit concentrations strongly agreed with the concentrations obtained at an average hematocrit concentration of 40%, see Figure 3. At a hematocrit concentration of 70%, a substantial decrease in the measured concentration was observed. The average spot size at a total volume of 20 µL decreased from 54.5 mm^2 at a hematocrit of 20% to 45 mm^2 at a hematocrit of 70%.

Spot size effect

Compared to the concentration obtained with a 20 µL spot, the smallest spot size of only 7.5 µL showed an average reduction in the mean concentration of 17±12% for tramadol and of 10±15% for O-desmethyltramadol. A spot of 7.5 µL equals a spot diameter of only 5.16±0.098 mm. Potentially the complete moistening from the top to the bottom of the DBS paper over the whole extraction area of 4.2 mm is reduced when using such small sample volumes. Between 10, 15, and 20 µL DBS spots, no difference in the average sample concentration was found for tramadol (within 3%) and O-desmethyltramadol (within 6%).

Cross-validation to the analysis of authentic samples in liquid blood

A comparison between the concentrations obtained using liquid blood analysis and fully automated DBS analysis showed good agreement between the two methods. For tramadol, the linear regression analysis of 26 samples was y=1.0804X+9.5565 with a coefficient of determination (r^2) of 0.9383, see Figure 4. The 23 samples which were within or very close to the calibration range of the liquid blood method (ranging from 48-956 ng/mL) showed an average agreement of 90±19%. For O-

desmethyltramadol, the linear regression analysis of 26 samples was y=1.0807X+0.0858 with a coefficient of determination (r^2) of 0.9818, see Figure 4. The 9 samples which were within or very close to the calibration range of the liquid blood method (ranging from 49-387 ng/mL) showed an average agreement of 94±14%.

Discussion

The aim of this study was the development of a method for the fully automated sample preparation, extraction, and online LC-MS/MS analysis of tramadol and its metabolite O-desmethyltramadol. In contrast to only analyzing tramadol, the method presented here has a distinct advantage, as the presence of the metabolite allows confirming the positive tramadol finding within the run. The automated system enables the screening of a large set of DBS samples. With its short sample turnover time of about 4 minutes per sample, it permits analyzing more than 300 samples within 24 hours. Showing good selectivity and sensitivity, the method is ideally suited for tramadol screening in the anti-doping environment. Such anti-doping programs to screen for tramadol in DBS have been reported beforehand, in press statements of the Union Cycliste International (UCI) in collaboration with the Research and Expertise in anti-Doping sciences (REDs) from the University of Lausanne[12]. Thereby, the UCI has carried out 143 tests on 117 riders at 11 events on the UCI WorldTour Calendar. All tests came back negative. Sampling was performed by the use of DBS Systems sampling kits (HemaXis), however, methodic details about the instrumental analysis procedure remain unknown.

During the method validation process, the automated extraction proved to be a fast and reliable method for the determination of DBS samples. The application proved to be linear, accurate, and precise. Hematocrit studies showed minimal differences over the range of 20%-60%. These minor differences are likely owed to the spray application of the internal standard compensating for hematocrit based recovery bias, as reported previously by Abu-Rabie et al. [13].

Performing cross-validation with authentic blood samples, it could be shown that the tramadol concentrations determined by the fully automated system are comparable to the measurement in liquid blood. However, as DBS sample preparation is performed fully automated, manual laboratory work is reduced to an absolute minimum with this novel method. If the analysis is performed quantitatively, only the calibrator and quality control samples must be prepared manually. If the analysis is performed qualitatively (screening), no manual sample preparation at all is required. Concerning the carry-over of up to 0.4%, we recommend the reanalysis of tramadol positive samples with concentrations close to the lower limit of reporting, if preceded by a sample with a high concentration. Another option is the extraction of a blank sample after each authentic blood sample. For anti-doping

screening for tramadol, carry-over issues are negligible, as positive findings for banned substances are rare. In an analysis of 9851 urine samples assessed in the Madrid Doping Control Laboratory before the ban of tramadol, between 2013 and 2017, the number of tramadol positive findings was found to be 1.4% in all sports, whereby 65.2% of these findings were related to cycling [1]. A drawback of the method is the fact that the implementation of O-desmethyltramadol requires a high water content within the extraction solvent. This is owed to the observation that if the organic content is too high, the peak shape of O-desmethyltramadol becomes distorted. As this high aqueous content leads to an increase in matrix component elution, it is accompanied by shorter maintenance intervals of the DBS autosampler (cleaning of the extraction head) when compared to organic solvent elution.

Conclusion

A novel workflow for the high-throughput determination of tramadol and O-desmethyltramadol in DBS was developed, validated, and successfully applied to authentic samples. The analytical process is fully automated and ideally suited for anti-doping screening. DBS automation using the DBMS-500 autosampler permits sample identification by a barcode label on the DBS card—with a link to a laboratory information system (LIMS).

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Ethical Statement

The use of anonymized biological material does not fall under the scope of the Human Research Act (Art. 2 HRA) in Switzerland. Samples used during this project were collected for the determination of tramadol and O-desmethyltramadol, based on the enforcement of the Swiss Road Traffic Act and anonymized DBS cards were generated afterward. The DBS cards analyzed in the CAMAG DBS Laboratory in Muttenz could not be attributed to a specific person.

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Table 1 Parameters used for the selected reaction monitoring (SRM) of tramadol and O-desmethyltramadol

Analyte	Precursor [m/z]	Product [m/z]	Dwell time [ms]	Q1 pre-bias [V]	CE	Q3 pre-bias [V]	RT [min]
Tramadol SRM 1	264.1	58.25	10	-20	-17	-20	2.15
Tramadol SRM 2	264.1	264.1	10	-20	-5	-20	
O-desmethyltramadol SRM 1	250.2	58.15	10	-17	-23	-22	
O-desmethyltramadol SRM 2	250.2	250.2	10	-17	-5	-22	
Tramadol-13C, D3	268.1	58.3	10	-19	-40	-23	1.87
O-desmethyltramadol-D6	256.2	64.15	10	-17	-25	-27	

Table II Intra-assay and inter-assay accuracy and imprecision determined during three validation runs with six quality control samples at each concentration

Analyte	Concentration [ng/mL]	Intra-assay accuracy [%,n=6]	Inter-assay accuracy [%,n=3]	Intra-assay precision [%CV,n=6]	Inter-assay precision [%CV,n=3]
Tramadol SRM 1	25	113-118	116	1-3	2
	200	91-100	95	1-11	7
	600	93-106	98	5-6	5
Tramadol SRM 2	25	114-118	116	2-4	3
	200	88-92	90	4-10	7
	600	86-104	96	6-9	7
O-desmethyltramadol SRM 1	20	88-108	99	2-10	5
	80	87-95	91	7-11	9
	240	98-102	101	9-12	10
O-desmethyltramadol SRM 2	20	97-115	108	3-7	5
	80	86-95	90	7-10	9
	240	95-100	97	4-9	7

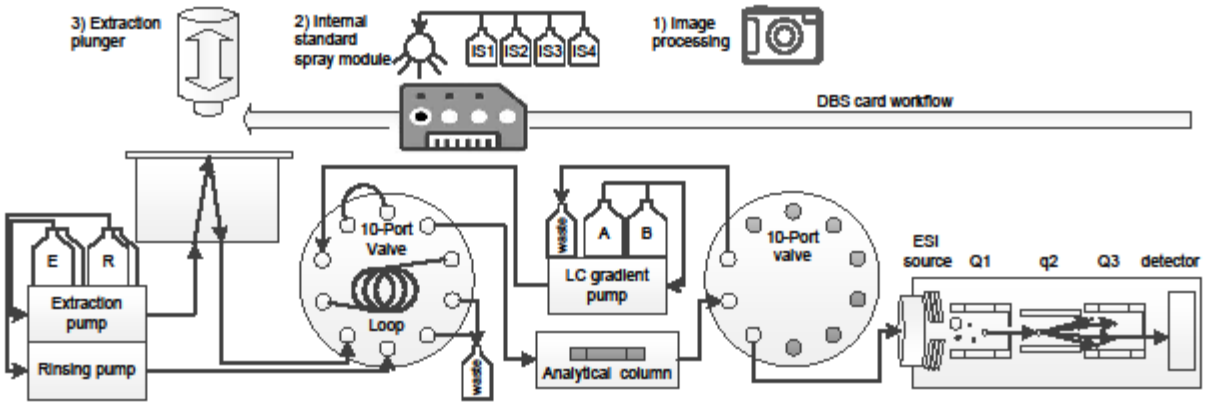

Figure 1 Hardware scheme for the experimental setup. The online connection of a CAMAG DBS MS-500 autosampler to an LC-MS/MS system is shown.

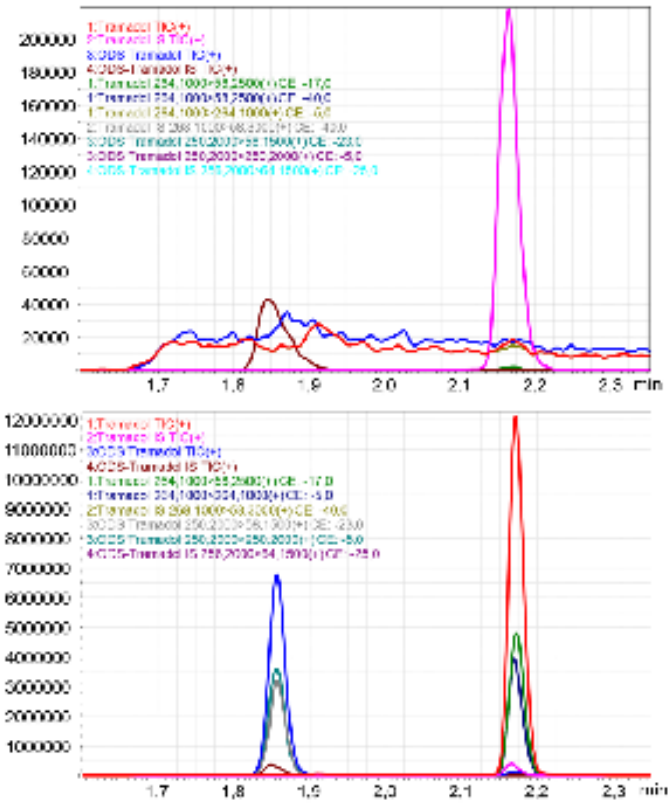

Figure 2 blank blood sample sprayed with internal standard depicted in the upper pane. Blank blood sample sprayed with internal standard and spiked with 200 ng/mL of tramadol and 80 ng/mL of O-desmethyltramadol depicted in the lower pane.

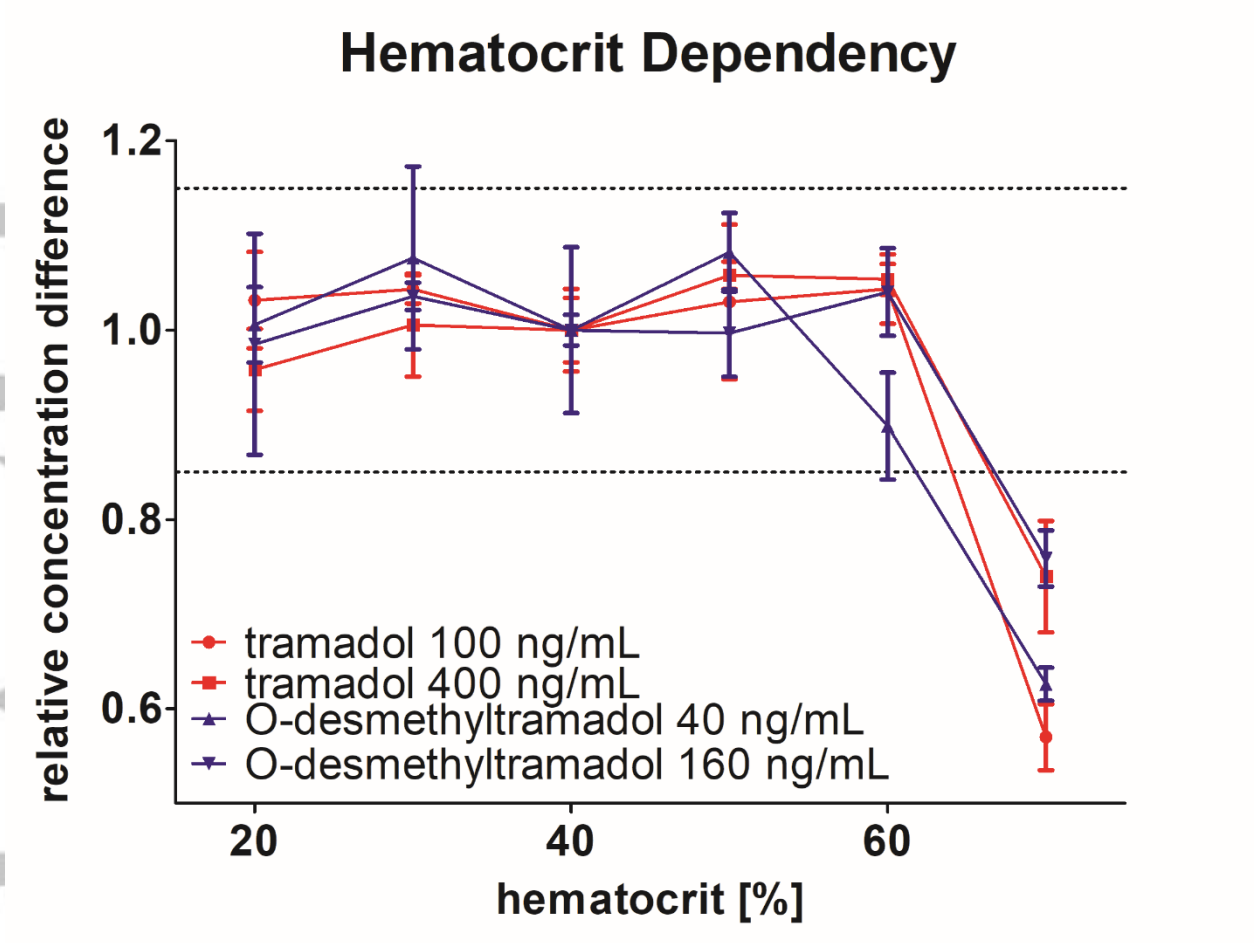

Figure 3 Hematocrit dependency of DBS measurements of tramadol and O-desmethyltramadol investigated by mixing RBC with plasma. The results obtained at a hematocrit of 40% were set to having a relative concentration difference of 1.0

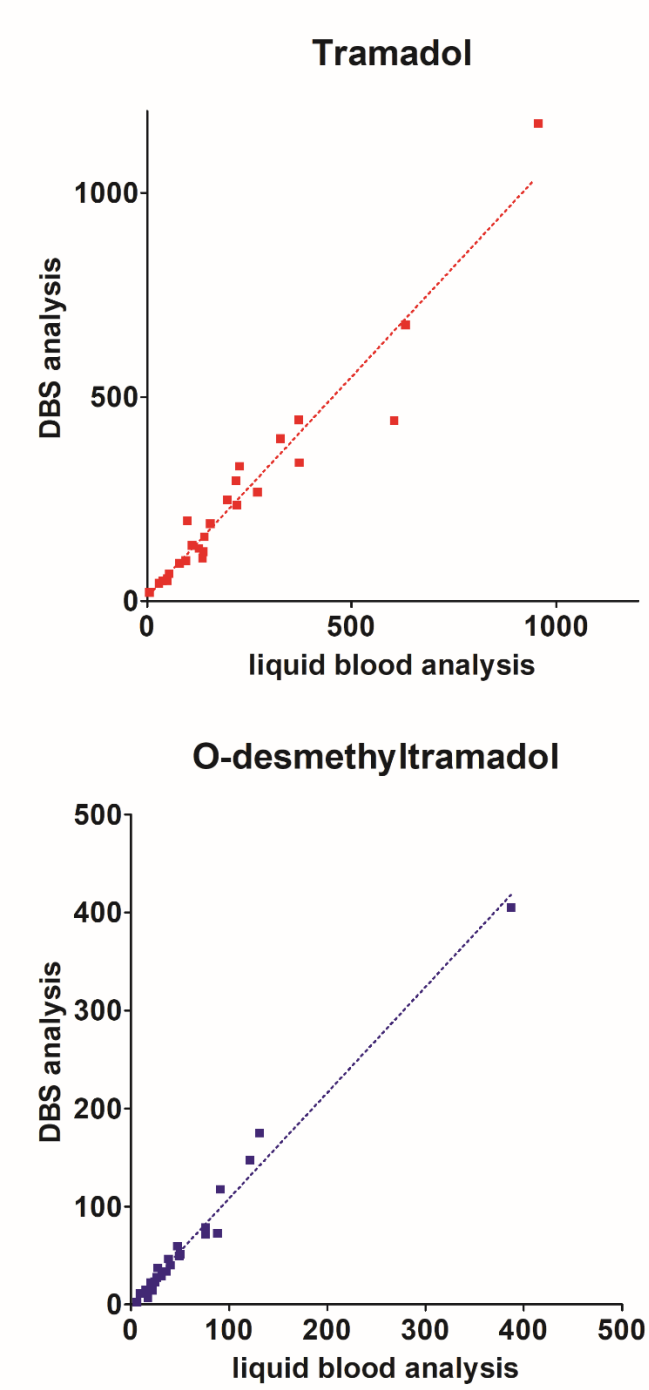

Figure 4 Cross-validation study comparing fully automated DBS analysis and manual liquid blood analysis.