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IN SILICO METHODS IN PREDICTION OF DRUG METABOLISM, MASS FRAGMENTATION, AND CHROMATOGRAPHIC BEHAVIOR

APPLICATION TO TOXICOLOGICAL DRUG SCREENING BY LIQUID CHROMATOGRAPHY/TIME-OF-FLIGHT MASS SPECTROMETRY

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Any fool can know.
The point is to understand.

Albert Einstein

CONTENTS

ABBREVIATIONS

2C-H	2,5-dimethoxyphenethylamine
2-DPMP	2-desoxypipradrol
3,4-DMMC	3,4-dimethylmethcathinone
bbCID	broadband collision-induced dissociation
CNS	central nervous system
CYP	cytochrome P450
DMPEA	3,4-dimethoxyphenethylamine
EI	electron impact
FWHM	full width half maximum
FT-ICR/MS	Fourier-transform ion-cyclotron resonance mass spectrometry
GC	gas chromatography
HHMA	3,4-dihydroxymethamphetamine
HLM(s)	human liver microsome(s)
HMA	4-hydroxy-3-methoxyamphetamine
HRMS	high-resolution mass spectrometry
ISCID	in-source collision-induced dissociation
LC	liquid chromatography
M(1-12)	metabolite (numbering 1-12)
$[M+H]^+$	protonated molecule
mDa	millidalton
MPA	methiopropamine
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass to charge ratio
NMR	nuclear magnetic resonance
NPS(s)	new psychoactive substance(s)
ppm	parts per million
PRS(s)	primary reference standard(s)
α-PVP	α-pyrrolidinovalerophenone
Q	quadrupole
QSAR	quantitative structure-activity relationship
QSRR	quantitative structure-retention relationship
QTOFMS	quadrupole-time-of-flight mass spectrometry
QTP	quetiapine
RP	resolving power
RS	resolution
t_R	retention time
TOFMS	time-of-flight mass spectrometry
UHPLC	ultra-high performance liquid chromatography

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals **I-IV**:

I Pelander A, Tyrkkö E, Ojanperä I. *In silico* methods for predicting metabolism and mass fragmentation applied to quetiapine in liquid chromatography/time-of-flight mass spectrometry urine drug screening. *Rapid Commun Mass Spectrom* 2009; 23: 506-514.

II Tyrkkö E, Pelander A, Ojanperä I. Differentiation of structural isomers in a target drug database by LC/Q-TOFMS using fragmentation prediction. *Drug Test Analysis* 2010; 2: 259-270.

III Tyrkkö E, Pelander A, Ojanperä I. Prediction of liquid chromatographic retention for differentiation of structural isomers. *Anal Chim Acta* 2012; 720: 142-148.

IV Tyrkkö E, Pelander A, Ketola RA, Ojanperä I. *In silico* and *in vitro* metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Anal Bioanal Chem* 2013; 405: 6697-6709.

ABSTRACT

Analysis of drugs in forensic and clinical toxicology has conventionally relied on the use of primary reference standards (PRSs). However, the availability of PRSs for novel pharmaceuticals, new psychoactive substances (NPSs), and their metabolites is often limited. Full metabolite data on new pharmaceutical drugs might be unpublished, and in the case of emerging NPSs, the metabolism is often unknown. Knowledge of the metabolism of these substances is important not only for toxicological risk assessment, but also in terms of analytical method development and forensic or clinical interpretation. Mass spectrometry (MS) techniques with accurate mass measurement capability allow determination of the compound's elemental composition, which facilitates structural elucidation. Computer systems, i.e. simulation *in silico*, are available to speed up and assist with the interpretation of analytical data.

In the present thesis, current *in silico* systems were evaluated for their usefulness within accurate mass-based toxicological drug screening. Different software tools were employed to predict drug metabolism, mass fragmentation and chromatographic retention. The aim was to produce supportive information for tentative compound identification without the necessity of possessing PRSs.

Human phase I metabolism of the antipsychotic drug quetiapine (QTP) and four NPSs, 2-desoxypipradrol (2-DPMP), 3,4-dimethylmethcathinone (3,4-DMMC), α-pyrrolidino-valerophenone (α-PVP), and methiopropamine (MPA), was studied using the metabolism prediction software Meteor (Lhasa Limited). Two software tools for *in silico* fragmentation - ACD/MS Fragmenter (ACD/Labs) and SmartFormula3D (Bruker Daltonik) - were used for the identification of compound-characteristic fragments in order to differentiate structural isomers and aid in the structural elucidation of metabolites. The retention time prediction software ACD/ChromGenius (ACD/Labs) was used to calculate chromatographic retention times for a large set of compounds included in a target database for toxicological drug screening.

The *in vivo* metabolites of the compounds studied were identified in human urine samples. The metabolism of the four NPSs was also studied using human liver microsomes (HLMs), in order test the ability of the *in vitro* experiments to generate the main human urinary metabolites. The metabolites predicted *in silico* were screened from the *in vivo* and *in vitro* samples, and the results were compared with the published metabolic reactions of either the compounds studied or their structural analogs.

Liquid chromatography coupled with time-of-flight MS (LC/TOFMS) or quadrupole-time-of-flight MS (LC/QTOFMS) were the analytical methods employed in this thesis. Fragmentation of the compounds was performed using either in-source collision-induced dissociation (ISCID) or a data-dependent acquisition method. Identification of the compounds was accomplished from full-scan MS data by accurate mass and isotopic pattern match comparison (SigmaFit), and structural elucidation of the analytes was carried out by identifying the characteristic product ion structures. In these experiments, high mass accuracy was obtained: the mean mass accuracy and the mean isotopic pattern match value of the analyses were below 1 mDa and 30 mSigma, respectively.

Meteor software assigned most of the main human urinary phase I metabolites of QTP, 2-DPMP, 3,4-DMMC, α-PVP, and MPA. *In silico* metabolite prediction aided in the identification of eleven previously unreported metabolites for the NPSs studied. The *in vitro* experiments produced the majority of the most abundant NPS metabolites detected *in vivo*.

Fragment assignment by ACD/MS Fragmenter and SmartFormula3D assisted in the differentiation of structural isomers and in elucidation of the metabolite structures. Together with accurate mass data, these software tools greatly facilitated the determination of fragment structure. *In silico* fragment prediction allowed the structural identification of drug metabolites without PRSs. The retention time prediction software ACD/ChromGenius, although not sufficiently accurate to be used in compound identification alone, was useful when calculating the correct compound retention order and thus to support the differentiation of structural isomers.

The software systems employed in this thesis were useful in analytical toxicology procedures, especially when applied to accurate mass data. *In silico* metabolite prediction provided a rapid technique for generating a list of possible metabolites which can readily be screened from biological samples by their accurate masses to identify the true positive metabolites. *In vitro* studies allow experimentation with biological material for metabolism studies of toxicologically relevant substances when an authentic urine sample cannot be obtained. The fragment prediction software facilitated the structural elucidation of unknown compounds without the use of PRSs. The software also aided in the differentiation of structural isomers, which cannot be accomplished by accurate molecular mass alone. Computer aided retention time calculation can offer additional information to be used with accurate mass data and information from other software systems. The *in silico* methodologies assist preliminary compound identification in cases where no corresponding PRS is obtainable. The present thesis demonstrates an integrated approach, in which the data generated *in silico* can be applied to toxicological LC/TOFMS drug screening in support of compound identification from authentic urine samples.

1 INTRODUCTION

Drug testing serves many authorities of modern society, including pharmaceutical, food, and environment regulatory agencies, health care, and crime investigation. Forensic toxicology looks for chemical proof for legal investigations mainly in the areas of post-mortem toxicology, driving under the influence of alcohol or drugs, drug-facilitated or drug-related crime, and workplace drug testing. Clinical toxicology focuses on the diagnosis and treatment of patients. Doping control laboratories analyze prohibited substances in sports, which are listed by the World Anti-Doping Agency. Laboratories performing drug testing employ similar analytical techniques and methods. An increasingly common and important technique is liquid chromatography (LC) combined with electrospray ionization mass spectrometry (MS) at atmospheric pressure [1]. LC/MS enables analysis of polar and non-volatile compounds over a wide range of molecular sizes.

Established drugs of abuse such as amphetamine, cocaine and heroin have been joined on the illicit drug market by new psychoactive substances (NPSs). Also known as designer drugs or legal highs, NPSs are intended to mimic the effects of controlled drugs. The penalties from drugs of abuse crimes are avoided by slightly modifying the chemical structure of these compounds, as the new structure leaves them outside the regulations. The number of NPSs has increased rapidly during the last few years: notifications on 237 compounds were made through the European Union's early-warning system between 2005 and 2012 [2,3]. This sets demands on laboratories testing for these compounds, as they need to develop and maintain their analytical methods to keep up with the growing variety of molecules.

The identification of drug compounds is traditionally based on comparison with reference data, such as retention time (t_R) and/or spectral data, obtained using primary reference standards (PRSs). The availability of reference material for designer drugs, novel prescription drugs and their metabolites is often limited, which hinders method development and identification. Understanding the metabolism of new drug compounds is essential for toxicological risk assessment as well as for analytical method development, as the detection of metabolites together with the parent drug compound clearly improves the reliability of the identification. Drug metabolism studies on NPSs in humans are not possible for obvious ethical reasons. Computer software tools that can predict possible metabolic routes *in silico* based on the molecular structure of a compound are widely used by the pharmaceutical industry in drug development.

Hyphenated LC/MS techniques allow fast and sensitive generation of detailed and information-rich analytical data on the compounds studied. These techniques can provide a useful way of compensating for the difficulties related to the availability of PRSs. Accurate molecular mass measurement of a substance allows the determination of its elemental composition, which facilitates the structure identification. High-resolution MS (HRMS) instruments with high resolving power improve separation of peaks with adjacent *m/z* values. Further detailed structural information about the substances studied can be produced using tandem mass spectrometry (MS/MS) to fragment the compound to its product ions. Advanced computational methods are necessary for effective data processing. *In silico* tools may help in

predicting possible product ion structures or even in calculating the compound's t_R in the chromatographic system used.

This thesis focuses on the employment of published software tools to predict drug metabolism, mass fragmentation, and chromatographic behavior *in silico* in order to tentatively identify compounds of toxicological interest without the immediate necessity of PRSs. The results are evaluated in the context of toxicological urine drug screening based on accurate mass measurement using LC/time-of-flight (TOF) MS techniques.

2 REVIEW OF THE LITERATURE

2.1 Drug metabolism

Metabolism is the major elimination pathway of xenobiotics from the human body. Drug metabolizing enzymes are present in all tissues, but mainly in the liver and intestine [4]. Drug metabolizing reactions transform lipophilic compounds to more hydrophilic form and facilitate their excretion from the body. The reactions are divided into phase I and phase II reactions [5]. Phase I reactions (functionalization reactions), such as oxidation, hydrolysis and reduction, produce or uncover a chemically reactive functional group. The most important enzymes catalyzing phase I drug metabolism reactions are cytochrome P450 (CYP) enzymes [6]. In phase II reactions (conjugative reactions) a highly polar endogenous compound, such as glucuronic acid, is attached to the parent drug or to a metabolite from phase I reactions. Phase II reactions usually result in inactive and excreted products.

Understanding drug metabolism is essential in drug discovery and development. Metabolic reactions can potentially lead to drug-drug interactions [7] or to formation of pharmacologically more active and toxic species [8]. Toxicity, which is often related to metabolism, is a significant factor in the rejection of a lead molecule during drug development and the withdrawal of new drugs [9,10]. Determination of a drug's metabolic stability, as well as identification of its major metabolites and their structural characterization, is central during the early discovery phase.

Elucidation of the main metabolism steps of drugs is also of great interest in forensic and clinical toxicology [11,12]. Numerous NPSs emerge on the illicit drug market annually [2,3]. The metabolites of these compounds may cause toxic effects, or they can have interactions with other pharmaceutical substances. Information about the chemical structure of metabolites is also crucial in the development of toxicological screening methodologies.

2.1.1 Conventional drug metabolism studies

Prior to the introduction of *in vivo* studies on animals and humans, the metabolic fate of drugs was effectively studied using simple *in vitro* systems. The *in vitro* models help to identify the main metabolites and the primary enzymes involved in the metabolic reactions [13-15]. There are several enzyme sources available for *in vitro* metabolism studies, including subcellular fractions such as human liver microsomes (HLMs), cytosol, and S9 fraction; complementary DNA-expressed recombinant isozymes; as well as hepatocytes and liver slices. HLMs afford the most convenient way to study CYP metabolism [16,17]. *In vitro* studies employing HLMs or complementary DNA-expressed CYP enzymes can predict clinical drug-drug interactions due to inhibition or induction, as well as genetic polymorphism [14,15]. Whole cell systems, i.e. hepatocytes and liver slices, give the most complete picture for hepatic metabolism, as they comprise all the metabolizing enzymes and cofactors [18,19]. Thus, they offer a more reliable *in vivo*/*in vitro* correlation than subcellular fractions. Although, the current *in vitro* models are sophisticated and well established, they cannot replace the *in vivo* metabolism studies completely [16,20]. After comprehensive metabolism studies *in vitro*, the pharmacokinetic profile and toxicity of the drug are determined in suitable animal models (rodents and non-

rodents) [14]. The metabolic profile of the drug is further recorded *in vivo* in clinical studies [21].

Identification of all the possible metabolites is the initial step in metabolite profiling, followed by structural characterization and quantitation [22,23]. The detection of metabolites is often a challenging task, as they are present only at trace levels in complex biological matrixes. Metabolism studies therefore require highly specific, sensitive and reliable analytical methods. Several analytical techniques have been applied to studies on drugs and their metabolites, among them gas chromatography (GC), LC, ultraviolet or fluorescence detection, and MS. A commonly employed method is radioactive labeling (^{14}C, ^{3}H) of the parent drugs and detection of the metabolites using radioactivity detection [24-26]. The method enables detection and quantitation of the parent drug and metabolites without PRSs. However, due to the laborious synthesis of the radiolabeled drugs and the high costs, this approach is mainly used in drug development. Currently, the most popular and widely used technique in drug metabolite identification and structure characterization is LC/MS, which offers excellent sensitivity, specificity, and high sample throughput [22,27-33]. LC/MS also allows quantitative bioanalysis of metabolites at low concentrations. Structural information on the metabolites can be obtained using MS/MS techniques. MS techniques providing accurate mass measurement and high resolving power (RP) enable effective characterization and structural elucidation of drug metabolites in complex biological matrixes [31,33]. The use of ultra-high performance LC (UHPLC) improves chromatographic resolution and decreases analysis time [34,35]. Nuclear magnetic resonance (NMR) spectroscopy coupled with LC and MS (LC/NMR/MS) provides reliable confirmation of metabolite structures [36,37]. However, NMR analysis requires a relatively large amount of sample compared to LC/MS methods. The lack of sensitivity impairs the applicability of NMR in structural characterization of minor metabolites.

Metabolism studies are mandatory for new drugs before their submission to drug regulatory authorities with a view to approval. In contrast, NPSs are distributed on the black market without pharmacology or safety testing. Hence, forensic and clinical toxicology authorities need to examine the toxic effects and pharmacokinetics, including metabolism, of NPSs. *In vitro* experiments [38] and *in vivo* animal models [11,12] are commonly used to study the metabolism of these compounds. Different MS methodologies have served in metabolite identification and characterization of NPSs. While NMR techniques are too expensive and complicated for analytical toxicology practice, accurate mass and HRMS techniques provide valuable information to aid in proposing structures for potential metabolites [31].

2.1.2 Drug metabolism *in silico*

Experimental drug metabolism studies are time-consuming and resource intensive. Computer modeling systems for prediction of drug metabolism pathways *in silico* are therefore of considerable interest. The *in silico* tools for drug metabolism prediction have advanced considerably in the recent years [39]. Modern *in silico* approaches aim to identify the metabolic liability in a molecule, predict the metabolites' chemical structures, and explain the effects of drugs on metabolizing enzymes, focusing on detection of potential inhibition and/or induction [39]. *In silico* techniques that predict xenobiotic metabolism can be categorized into global

(comprehensive) and local (specific) systems [20]. Table 1 lists some examples of currently available *in silico* tools for predicting and studying drug metabolism.

Global methods, or so-called expert systems, aim to predict the sites and the products of metabolism using known metabolic reactions [40,41]. Predictive expert systems identify functional groups in a molecule liable towards metabolism, and involve them in a suitable metabolic reaction from their knowledge-base. Examples of the expert systems META, MetabolExpert and Meteor, and their main features are listed in Table 1.

Meteor and MetabolExpert have shown relatively high prediction sensitivity. In a diverse test set of 22 compounds, Meteor predicted ~70% of the experimentally observed metabolites [42]. MetabolExpert showed similar accuracy (82%) in a study with 21 drug molecules [43]. The most criticized feature of these expert systems is that they tend to overpredict; in other words they form an enormously long list of possible metabolites [40,42,44]. Another drawback is that some expert systems combine metabolic rules from different mammalian species, and the results may not describe metabolism in the specific species studied [39]. Despite the rather low prediction precision [42], these software tools can provide suggestions for unexpected metabolites [45].

Local methods examine particular enzymes or metabolic reactions, and they can be divided into ligand-based and structure-based approaches [20,39,46]. In the ligand-based methods, such as quantitative structure-activity relationships (QSAR) and pharmacophore modeling, the focus is on the drug compound's structure. They attempt to identify the sites of metabolism and the structures of the metabolites. Ligand-based methods do not require prior knowledge about the target protein structure. In QSAR analysis, the chemical structures of the compounds are related to the molecular properties [47]. QSAR studies seek quantitative relationships between the drug compound and its activity. Pharmacophore models provide indirect information about the protein's active site based on the structural and electronic properties of its substrates [48]. Pharmacophore models give an estimate of whether a query compound is a substrate of the enzyme being studied.

Structure-based methods study the properties of the metabolizing enzyme, its interactions with xenobiotics, and the reaction mechanism [46]. Structure-based drug metabolism prediction requires experimentally determined three-dimensional structures of drug-metabolizing enzymes and ligands. These *in silico* methods employ molecular and quantum mechanics to predict drug metabolism and drug-drug interactions.

Compared with structure-based methods, the ligand-based methods provide less certain estimate of the binding site of the metabolizing enzyme [39]. Structure-based methods suffer from the difficulty of calculating the quantum mechanics variables of the ligand-enzyme interaction [49]. Therefore, a combination of global and local approaches would be the most promising system, and would provide a greater understanding of the metabolic transformations [48]. Software systems that employ a combined approach are MetaSite [50,51], SMARTCyp [52,53] and StarDrop [54], see Table 1. In a critical evaluation of Meteor, MetaSite and StarDrop, the last two were found more precise in predicting the metabolites observed *in vivo* than the expert system, as they did not suggest as many false negative metabolites [42]. Meteor software, however, showed better prediction sensitivity in being able to assign most of the metabolites detected *in vivo*. Nevertheless, despite advances in computational drug metabolism prediction methods, it is unlikely that *in silico* tools will completely replace *in vitro* and *in vivo* studies in the near future [39,46].

Table 1 *In silico* tools for drug metabolism studies.

Global	Name	Features	Ref.
Expert systems	META	Uses a selected dictionary to create metabolic paths for query molecules.	[55,56]
	MetabolExpert	Predicts likely metabolite structures. The knowledge base includes metabolic pathways for humans, animals or plants.	[57,58]
	Meteor	Metabolite predictions based on rules that account for physicochemical and structural properties. Includes two algorithms for prediction likelihood evaluation. It can be integrated with SMARTCyp, and it is compatible with certain MS instruments.	[59,60]
Local	**Name**	**Features**	**Ref.**
	ADMET Predictor Metabolite module	Calculates likelihood scores for metabolic oxidation reactions to take place at specific atomic sites. Identifies substrates for nine CYP isoforms.	[61]
	MetaDrug	Combination of QSAR modeling and metabolic rules for metabolite prediction. Estimates metabolite primarity.	[62,63]
	MetaPrint2D and MetaPrint2D-React	Predicts sites of metabolism, metabolic transformations and metabolites using a data-mining approach. Assigns a confidence score to the predictions.	[64,65]
	MetaSite	Predicts CYP-mediated metabolic transformations, estimates the primary site of metabolism, and provides the structure of the metabolites.	[50,51]
	SMARTCyp	Ligand structure-based method to predict site-specific metabolic reactivity of five major CYP enzymes.	[52,53]
	StarDrop	Predicts the relative proportion of metabolite formation at different sites on a molecule. Employs quantum mechanical approach to identify potential sites liable for CYP-mediated metabolism.	[54]

Comprehensive databases specializing in metabolism information are available. They can aid in metabolite prediction of a novel molecule using knowledge of structurally similar compounds in the database [66]. Accelrys Metabolite database [67] includes metabolic schemes from *in vivo* and *in vitro* studies compiled from the literature. Software employing data from the Accelrys Metabolite database are the Metabolite module of ADMET Predictor [61] and MetaPrint2D [64,65] (Table 1). Other large metabolite databases are DrugBank [68], Human Metabolome Database [69,70] and METLIN [71,72]. Metabolism databases have mostly been applied to metabolomics [73-75] to cover the whole metabolic process, rather than to predict certain metabolite structures.

Software packages from MS manufacturers such as MetaboLynx [76], MetWorks [77] MetabolitePilot [78] and MetaboliteTools [79] are able to predict simple metabolic reactions based on a selection of common biotransformations. They create a list of expected metabolites to be screened from the analysis data. The role of these software packages lies mostly in metabolite identification, which is discussed in Chapter 2.2.1.

In silico metabolism prediction systems have helped to identify the biotransformation products of pharmaceuticals [42,80,81]. However, prior to the present thesis, drug metabolism prediction using commercial or self-coded software has not been exploited for NPSs or other toxicologically relevant compounds.

2.2 Software tools in compound identification

LC/MS is a standard technique for detecting and identifying small molecules in complicated biological samples. Modern LC/MS instruments provide high-throughput and information-rich MS spectra with high sensitivity. Structural elucidation of compounds requires sophisticated software tools for MS data evaluation and interpretation [82-84]. In addition to MS data managing systems, the prediction of the retention behavior of a compound plays a key role for the systematic identification process [83].

2.2.1 Mass spectral data processing

Computational MS provides solutions for automated analysis of MS data [82]. There are numerous tools for interpretation of MS data obtained by different instruments, and over a range of molecular sizes. The most relevant *in silico* tools currently available for small molecule HR and accurate mass spectral interpretation are discussed here and listed in Table 2. Their applications for compound identification are further discussed in section 2.3.2. The computational methods dealing with compound identification concentrate on molecular formula assignment, *in silico* fragmentation, and mass spectral library searches.

The most basic but highly important step in identification of a compound is the determination of its molecular formula, which serves as a basis of further structural elucidation. To reduce the number of possible candidates, accurate mass measurement with adequate mass resolution is required [85]. The simplest approaches compute the elemental composition using a set of potential elements [86,87]. Combining experimental and theoretical isotopic pattern comparison with determination of the elemental composition significantly cuts down the number of possible combinations [88]. Algorithms that calculate theoretical isotopic abundances have been applied to MS since its introduction [89]. There are several approaches available, e.g. BRAIN [90], Emass [91] and IsoDalton [92], implementing different algorithms in the isotopic pattern calculations. Seven Golden Rules is a set of heuristic rules, including the Senior and Lewis rules, isotopic abundance matching filter, and element ratio rule, for elemental composition calculations [93].

Software tools that simulate mass fragmentation can be classified either as rule-based or combinatorial [82]. Rule-based systems include a knowledge base with fragmentation rules extracted from the literature. ACD/MS Fragmenter [94] and MassFrontier [95] attempt to predict possible fragments of a compound based on its molecular structure. The state-of-the-art rule-based predictor MassFrontier evaluates the accuracy and probability of the proposed fragments and assigns structures for the product ions detected in the MS/MS spectra. The software is widely used in several fields of research, such as metabolomics [96,97] and environmental analysis [98]. It has also served for structural elucidation of designer drug metabolites [99] and for determination of fragmentation pathways of doping agents [100].

Table 2 Computational tools for MS/MS mass spectral interpretation.

Molecular formula assignment	**Name**	**Features**	**Ref.**
Isotopic pattern match scoring	Seven Golden Rules	Set of heuristic rules for elemental composition calculations.	[93]
***In silico* fragmentation**	**Name**	**Features**	**Ref.**
Rule based fragment prediction	ACD/MS Fragmenter	Predicts product ions based on common MS fragmentation rules from the literature.	[94]
	MassFrontier	General reactions for fragment prediction. >30,900 fragmentation schemes; >129,000 fragmentation reactions; >151,000 chemical structures.	[95]
Combinatorial fragmentation	Fragment iDentificator (FiD)	Calculates optimal bond energies to predict the most stable fragments. Scoring function to rank competing fragmentation pathways.	[101]
	MetFrag	Compares and scores *in silico* mass spectra obtained using the bond disconnection approach with experimental spectra.	[102]
	SIRIUS^2	Isotopic pattern analysis to determine the molecular formula, and computed fragmentation tree explaining the product ion peaks.	[103,104]
MS library search	**Name**	**Features**	**Ref.**
MS/MS spectral libraries	MassBank	Open access database with ~40,000 spectra acquired on diverse types of MS instruments.	[105,106]
	METLIN	Open access database with >57,000 MS/MS spectra of >11,000 metabolites.	[71,72]
	NIST/EPA/NIH MS/MS Mass Spectral Library 2012	>9,900 ion trap spectra of >4,600 compounds; >91,000 collision-cell spectra of >3,700 compounds; NIST MS Search algorithm.	[107]
	Wiley Registry of Tandem Mass Spectral Data, MSforID	>12,000 spectra of >1,200 compounds, MSforID search algorithm.	[108]
Software for spectral library search	SmileMS	Universal spectral library search algorithm for targeted and non-targeted screening.	[109]

Instead of general fragmentation rules, combinatorial fragmenters use molecular structures and experimental MS/MS spectra to predict structure-characteristic fragmentation trees [102,110-112]. The benefit of the fragment tree approach is that it can automatically identify unknown compounds by comparing experimental MS/MS spectra with the fragment trees of reference compounds. Fragmentation pattern similarities are strongly correlated with the structural analogy of molecules. Combinatorial fragmentation systems such as MetFrag [102], SIRIUS^2 [103,104] and FiD [101] were developed by non-commercial authorities and are freely available. The performances of MetFrag [102] and FiD [101] were also compared with MassFrontier, and both combinatorial methods achieved more accurate results than the rule-based system.

Traditionally, spectral library search has been the primary approach for identification of small molecules [82,83]. There are large spectral libraries available for GC/EI mass spectra matching, e.g. Wiley Registry 10th Edition/NIST 2012 (>870,000 mass spectra) [113]. MS/MS spectral libraries for internal laboratory use have been created [114,115]; however, the rather poor reproducibility of electrospray ionization MS/MS spectra between different instruments has hindered the development of comprehensive reference spectral libraries for LC/MS data [116]. A platform-independent MS/MS spectral library with a peak-matching search algorithm MSforID was introduced by Oberacher et al. [117,118]. The library was developed on a quadrupole (Q) -TOFMS instrument using 10 different collision energies. The approach is today part of the Wiley Registry of Tandem Mass Spectral Data, which includes over 12,000 accurate mass MS/MS spectra [108]. The NIST LC/MS/MS 2012 [107] included in the Wiley/NIST 2012 library [113] covers small organic compounds and peptides, the spectra being acquired on different types of mass spectrometers. MassBank [105,106] and METLIN [71,72] are public spectral databases that allow web-based MS/MS spectra searches or comparisons. Despite the effort put into universal MS/MS spectral libraries and their search algorithms, their reliability, robustness, and transferability are still doubtful, especially for the identification of unknown compounds [119].

SmileMS software allows targeted and non-targeted screening via a spectral library search approach for LC/MS/MS data [109]. It is compatible with data acquired with most LC/MS/MS instruments (unit resolution and HR), and both commercial and in-house spectral libraries can be utilized. SmileMS can detect unknown compounds automatically with group-specific structures stored in the fragment library [120,121].

Metabolites are commonly identified by comparing and contrasting the test sample with the blank sample (negative control) [22]. However, finding traces of metabolites in complex biological matrixes is often challenging, as the metabolite ions can be masked by background noise or matrix components [29]. Methodologies for post-acquisition data mining of accurate mass and HRMS data have been developed. Mass defect filtering removes biological background ions whose *m/z* decimal portion is dissimilar (typically ±50 mDa) to that of the parent compound being studied [122,123]. An accurate mass-based isotope pattern filtering algorithm is applicable for matrix ion removal, and the method helps identification of metabolites with stable-labeled isotopes, or compounds containing natural isotopes such as those of chlorine or bromine [124]. The background subtraction approach removes interfering matrix ion signals detected in the control samples within a specified time window and mass error tolerance from the test samples [125-127]. This system does not require knowledge of the metabolite structures or fragmentation pathways. Some of the above-mentioned data processing tools have been integrated into sophisticated metabolite identification software from MS manufacturers, examples being MetaboLynx [76], MetWorks [77] and MetabolitePilot [78].

In the present thesis, *in silico* fragment prediction was applied to structural characterization of toxicologically relevant compounds and their metabolites. Fragment identification was used to resolve a previously criticized limitation related to full-scan accurate mass measurement techniques, namely differentiation between structural isomers.

2.2.2 Liquid chromatographic retention prediction

Prediction of chromatographic separation using *in silico* tools has benefited analytical method development, optimization and validation [128]. Commercial software tools that predict liquid chromatographic retention parameters include DryLab [129] and ChromSwordAuto [130]. These tools base their calculations on experimental data, and have predicted t_R for a small number of compounds in rather simple isocratic or linear gradient systems [128,131]. Their main purpose of use is thus in method development.

Quantitative structure-retention relationship (QSRR) models can give more comprehensive information about retention phenomena [132-134]. QSRR models aim at finding the relation between calculated molecular descriptors and retention. They seek to identify the most useful structural descriptors of a compound, calculate the physicochemical properties of the analytes, describe the molecular retention mechanism of a structure, and compare the separation mechanism of different chromatography columns. QSRR systems can also estimate the biological activity of drug candidates and other xenobiotics [135]. Drug compound t_R prediction in an LC system using QSRR models has been applied to retention behavior determination [136,137]. In terms of compound identification, they have been applied to proteomics [138,139] and metabolomics [140].

A critical phase in the QSRR analysis is the selection of the most representative molecular descriptors from a large collection of possibilities [132]. The models also require careful evaluation and validation with a large set of test compounds [134]. In addition, QSRR models without a commercial or open software implementation are thought to be too complicated for the majority of analysts [83].

The prediction and investigation of the retention behavior of a compound are key for structural elucidation by MS coupled with chromatographic separation techniques [83]. The calculated retention index or t_R can be used as an orthogonal filter for determination of the correct molecular formula and structure. GC retention index prediction was included in the Seven Golden Rules approach [93] (see Chapter 2.2.1, and Table 2), which showed a substantial reduction in possible structures (from 36,623 to 105). Kern et al. [98] used a simple LC t_R prediction as part of a pesticide transformation product screening, which reduced the number of possible structures by 30%. No universal retention index database is available for LC systems, and thus t_R prediction is not as straightforward as for GC [83]. This thesis demonstrates an original approach for identification of drug compounds included in a toxicology database using t_R prediction.

2.3 Accurate mass-based mass spectrometry

The principle of calculating the elemental composition of a molecule from the mass of an ion, provided it is measured with sufficient accuracy, was introduced in the 1950s [141]. Mass accuracy is the difference between the theoretical and measured mass of an ion, and high mass accuracy refers to mass measurements below 5 ppm [142]. Resolution (RS) is the degree of separation of two mass spectral peaks, and RP is the capacity of a mass spectrometer to distinguish ions with close *m/z* values [85]. RP is dependent on the *m/z* value and the charge state of the ion measured, and for evaluation between different mass analyzers the *m/z* value of

the reported RP should be specified. Two different definitions are used to describe RS and these are explained in detail in Table 3. The 10% valley definition is used with magnetic sector instruments, and the peak full width at half maximum (FWHM) definition is employed with quadrupole, ion trap, TOF, and Fourier-transform ion-cyclotron resonance (FT-ICR) techniques [143]. A high RP in MS is essential to separate adjacent mass peaks, which is often required in the analysis of complex biological matrixes. Mass analyzers capable of a routine broadband RP of ≥20,000 are classified as HRMS [142]. HRMS produces narrower mass peaks, and reduces the ambiguity related to the determination of elemental composition. Modern HRMS instruments provide a mass accuracy of <5 ppm routinely, and even a mass accuracy of <1 ppm has become common [85]. However, accurate mass measurement is attainable without high RP [144], and accurate mass measurement techniques and HRMS are presented here in parallel. Table 3 lists the key terms related to these methodologies.

Table 3 Terms related to HR and accurate mass MS [145].

Term	Definition
Accurate mass	The experimentally determined mass of an ion.
Exact mass	Summation of the masses of the most abundant isotope of each element (monoisotopic mass).
Mass accuracy	The difference between the measured and theoretical value of the mass of an ion.
Resolution (RS)	Measure of separation between two mass spectral peaks, expressed as $(m/z)/\Delta(m/z)$, where the observed (m/z) value is divided by the smallest difference $\Delta(m/z)$ for two ions that can be separated.
• 10% valley	A value for two peaks of equal height in a mass spectrum that are separated by a valley of no more than 10% of the peak height.
• FWHM	A value for a single peak, $\Delta m/z$ is the peak full width at half maximum.
Resolving power (RP)	The ability of a mass spectrometer to provide a certain value of mass resolution.

HRMS is an increasingly popular analytical technique in studies of small molecules in biological samples [31,146-149] and in the environmental sciences [150,151]. It is a central technique in pharmaceutical drug development [33,152] and metabolism studies [29-31,33], and has been applied in clinical and forensic toxicology [146,148] and doping control [153]. Combined with LC, HRMS instruments provide fast and sensitive detection of compounds with a large diversity in molecular size [85]. HRMS instruments are commonly coupled with a Q or ion trap [154-157] to perform MS/MS analysis and attain detailed structural information. The key factors in achieving good mass accuracy are ion abundance, peak shape, RP, and mass scale calibration [158,159]. Accurate mass measurement, high RP and the isotopic pattern fit of an ion increase certainty when calculating a compound's elemental composition [88,93,160,161]. HRMS and accurate mass instruments that are compatible with LC and used for biochemical analyses are TOFMS, orbital trapping instruments (orbitrap), and FT-ICR/MS [85].

In a TOF mass analysis, the ions are accelerated with an electrostatic field and follow a given flight path to the detector [162]. The mass of the ion is determined by the flight time, the length of the flight path and its kinetic energy. TOF instruments are capable of fast and sensitive full-scan data acquisition, and the RP has been shown to be independent of the acquisition rate [163]. Standard TOF instruments with an orthogonal ion accelerator have a high RP (~20,000 at m/z ~900 for TOFMS, and ~40,000 at m/z ~900 for QTOFMS instruments) and mass accuracy (<5 ppm for TOFMS, and <2 ppm for QTOFMS) [164]. Co-eluting isobaric compounds

with a mass difference below the instrument's RP will therefore form a sum peak, which leads to large mass errors and false molecular formula assignment [161]. This increases the risk of false negative results when complex biological matrixes are analyzed [163]. Modern TOFMS instruments provide an even higher RP of >40,000 and a mass accuracy below 1 ppm [35,164].

Orbitraps operate on the basis of harmonic ion oscillations in an electrostatic field with a frequency characteristic of their *m/z* values [165]. Orbitraps can operate with an RP up to 240,000 (*m/z* 400) and <1 ppm mass accuracy [164]. However, the RP of orbitraps depends crucially on the acquisition rate, and 1 Hz data collection time is needed for maximum RP [35,161]. This is too slow for modern UHPLC instruments with a column particle size of <2 μm which are capable of producing narrow (2-4 s) chromatographic peaks [35].

In FT-ICR/MS the charged ions in a magnetic field move in a circular oscillation at an *m/z*-specific cyclotron frequency, and this signal can be converted to a mass spectrum [166]. In terms of RP and mass accuracy, FT-ICR/MS instruments are the most powerful available. An FT-ICR/MS instrument can operate at an RP of 2,500,000 (*m/z* 400) and a mass accuracy <1 ppm [164]; however, when coupled with LC an RP of 50,000-100,000 is achieved [167]. In terms of routine analytical toxicology practice, FT-ICR/MS instruments are very expensive and complicated [146], although they are widely used in metabolomics [167] and proteomics [168].

2.3.1 Accurate mass-based toxicological drug screening

In analytical toxicology, numerous toxicologically significant compounds need to be detected and identified routinely within a limited turnaround time and with high reliability. Traditionally, systematic toxicological screening analyses are based on GC/MS, with compound identification relying on a comparison with reference data [146,149,153,169,170]. Since the introduction of accurate mass-based drug screening by LC/TOFMS in toxicology [144,171-173] more than a decade ago, the technique has successfully been adopted by laboratories in the fields of clinical and forensic toxicology, as well as doping control [146,149,153,169,170]. The technique is suitable for a broad variety of specimens, including urine [144], blood [174] and vitreous humor [175]. In toxicological drug screening, the methods exploiting HRMS and accurate mass measurement techniques are based on TOFMS and orbitrap instruments. The TOFMS databases cover several hundreds or even thousands of drug compounds and their metabolites [114,176,177], while databases for orbitraps include a maximum of 320 compounds [178].

Target screening methods for qualitative analysis by accurate mass and HRMS most commonly rely on full-scan data acquisition followed by a search through an in-house database of exact monoisotopic masses and t_R [144,172-174,179-181]. Comparison of theoretical and measured isotopic patterns provides additional information. To support compound identification, the respective formulae of known metabolites are included in the database [144,173]. Collision-induced dissociation (CID), either in the ion source (in-source collision-induced dissociation, ISCID) [176], or in the collision cell (broadband, bbCID) [182], can be used to generate structural information on the target compounds. In data-independent acquisition mode all ions are fragmented in every second scan with the same collision energy. Diagnostic product ion data can be added to the database to support parent compound identification [100,176,182-184].

Another compound detection and identification approach for toxicological drug screening by accurate mass and HRMS is spectral library search [114,185,186]. MS/MS spectra are produced using data-dependent acquisition, where fragmentation is performed for targeted compounds or for ions exceeding an abundance threshold. Broecker et al. [114] introduced a library search method including CID mass spectra of >2,500 toxicological compounds and metabolites, measured with an LC/QTOFMS instrument. An advanced search algorithm included in the Wiley Registry of Tandem Mass Spectral Data, MSforID [108] strives for platform independence and instrument universality [185-187]. The library includes about 10,000 spectra for 1,200 substances, and has been found to be both robust and sensitive in identification of toxicologically relevant compounds in human body fluids [186]. Nevertheless, identification of compounds not included in the library is not possible. However, despite promising developments in sophisticated search algorithms, LC/MS spectral libraries cannot perform with the same universality and robustness as those of GC/MS [188].

Polettini et al. [177] introduced a method for toxicological drug screening by LC/TOFMS in which the reference database of approximately 50,500 toxicologically relevant substances was a subset derived from the PubChem Compound Database. The latter database includes pharmaceutical and illicit drugs, as well as other poisons, and contains around 6,000 phase I metabolites. Due to the size of the database, the number of possible candidates with identical molecular formulae ranged from 1 to 39, precluding explicit compound identification. The number of hits was reduced by half using the "metabolomics" approach, in which the isomers were differentiated by identifying their metabolites [189]. This approach presumably works well for unusual toxicological cases, where the answer needs to be sought outside the repertoire of conventional drug screening methods [146]. The use of very large databases in daily analytical toxicology may be impractical because of the excessive number of false-positive findings.

2.3.2 Compound identification without primary reference standards

The poor availability of PRSs delays the analysis of drugs, for which identification is traditionally based on comparison of chromatographic retention and spectra between the analyte and the standard [190]. This problem especially relates to rare and new drug substances and to drug metabolites [191].

NPSs, or so-called designer drugs, comprise a variety of compounds that are intended to mimic the effects of existing controlled drugs [2,192]. These substances are of special interest among drug users, as they are not controlled under international drug laws. The main groups of NPSs that are followed by the EU early-warning system are phenethylamines, cathinones, piperazines, tryptamines, synthetic cannabinoids, as well as a large group of plant-derived and synthetic compounds outside the above-mentioned categories [2]. The pharmacological and toxic effects and pharmacokinetics, including metabolism, of NPSs in humans is often incomplete or even unknown [11]. Data on the metabolism of designer drugs is required, as it is a prerequisite for development of toxicological urine drug screening procedures, as well as for toxicological risk assessment [191,193]. The lack of PRSs hinders not only the detection of NPSs, but also the identification of metabolites of pharmaceuticals, as detailed information about the human urinary metabolites is not always available [194]. Knowledge of the biotransformation products of toxicologically relevant compounds is essential, as the detection of the metabolites

in conjunction with the parent drug confirms the identification [120,144]. Numerous drugs are excreted mainly as their metabolites [195], and for some drugs detection of the metabolites proves illicit drug use [196].

One of the major advantages of accurate mass and HRMS-based screening is the complete collection of full-scan data, which allows retrospective data-mining of formerly unknown compounds [146,153,169,170,184]. Accurate mass MS is a powerful tool for elucidating the structure of small molecules (<2,000 Da) [83]. However, even a very high mass accuracy (<1 ppm) alone is not enough to restrict the molecular formulae to a single possibility, when the size and complexity of the molecule increase [88]. Large public web-based compound databases, for example PubChem and ChemSpider, or the specific drug and metabolism databases HMDB and DrugBank, make it possible to search for exact masses or molecular formulae. Even so, the database search is likely to result in several structural isomers for a molecular formula [84]. Orthogonal filters, such as isotopic pattern filter [88], heuristic rules for elemental composition calculation [93] and t_R and mass fragmentation prediction [84], are applied to narrow down the number of compound candidates. Nevertheless, the assignment of the most likely structure to a molecular formula is the most challenging phase in the identification procedure [84].

The compound database and MS/MS spectra database search approaches allow the identification of the compounds included in the datasets; however, this limits the detection of unknown compounds and metabolites. This restriction can be overcome using fragmentation trees and automated fragmentation pattern similarity comparison [102,110-112]. This method enables deduction of the compound class of an unknown, and identification of substances not included in any databases. Thus, fragmentation tree systems are of special interest within metabolomics. Wolf et al. [102] exported fragment tree data for reference compounds from large public databases. However, unambiguous compound identification could not be achieved, because with a large dataset the result list contains many structurally similar compounds. Rasche et al. [110] used a fragment tree system with a relatively small database, and reliably identified the molecular formulae of 35% of the unknown metabolites. The system was less successful for compounds lacking similar molecules in the database and substances with poor MS/MS spectral data.

In forensic and clinical toxicology practice the focus is on a limited number of relevant compounds. The large database search approaches are rarely used in this context, as the number of false-positive findings increases with the size of the target database [146]. Wissenbach et al. [120] successfully used the SmileMS software for non-targeted screening of drugs-of-abuse metabolites. The identification of unknown compounds was based on detection of group-specific structures from unit resolution multiple stage MS^2 and MS^3 data. Accurate mass MS/MS analysis enables determination of the product ion's elemental composition. *In silico* fragment prediction to assign product ion structures facilitates the structural elucidation of the parent compound. Fragment structure prediction combined with accurate mass data has helped in metabolite structure characterization [28,197,198]. The employment of *in silico* tools with accurate mass and moderate RP TOFMS data for compound identification and structural characterization in a forensic toxicology context has been studied and is further discussed in this thesis.

3 AIMS OF THE STUDY

The aims of the study were:

- To evaluate the ability of metabolite prediction software to predict the human phase I metabolism of toxicologically relevant compounds (**I**, **IV**).
- To employ two software tools for mass fragmentation *in silico* in order to identify characteristic fragments of compounds for structural determination of drug metabolites and differentiation of structural isomers (**I**, **II**, **IV**).
- To assess the capacity of liquid chromatography retention time prediction software to calculate retention times for compounds included in a large target database for toxicological drug screening (**III**).
- To obtain additional information by *in silico* predictions for tentative compound identification without primary reference standards (**I**-**IV**).
- To demonstrate the benefits of different *in silico* methods when applied to toxicological drug screening by liquid chromatography/time-of-flight mass spectrometry (**I**-**IV**).

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

All solvents and reagents were of analytical, LC or LC/MS grade. Pharmaceutical purity standards were obtained from several different suppliers. 2-Desoxypipradrol (2-DPMP), 3,4-dimethylmethcathinone (3,4-DMMC), and methiopropamine (MPA) were seized material obtained from the Finnish National Bureau of Investigation or from the Finnish Customs (**IV**). HLMs and NADPH regenerating systems A and B were provided by BD Biosciences (Woburn, MA, USA) (**IV**).

4.1.2 Urine samples

Urine samples were either collected at autopsies (**I**, **IV**) or they were clinical toxicology cases investigated at our laboratory (**IV**). The urine samples examined were tested positive for the compounds studied in our routine drug screening by LC/TOFMS [144,176]. Drug-free urine was used in metabolism studies (**I**) as a pseudo-reference sample.

4.2 Sample preparation

4.2.1 Urine samples

Urine samples (1 mL) were hydrolyzed with β-glucuronidase, and mixed-mode solid phase extraction was used for sample preparation (**I**, **IV**).

4.2.2 *In vitro* incubations

Phase I metabolism *in vitro* of 2-DPMP, 3,4-DMMC, α-PVP, and MPA was studied using HLMs (**IV**). The reaction mixture consisted of NADPH regenerating systems A and B in 100 mM phosphate buffer at pH 7.4 as described in the general assay of BD Biosciences. The drug concentration was 100 μM, and the protein concentration was 2.0 mg/mL. The incubation time was 4 hours at 37°C. A blank sample without the drug, a biological control sample without either HLMs or coenzymes, and a chemical control sample without HLMs and coenzymes were prepared in addition to the test samples.

4.3 Liquid chromatography/mass spectrometry

The LC/MS instrumentations, including the columns and mobile phase components used in the study are listed in Table 4. The LC instruments included a vacuum degasser, a binary pump, an autosampler and a column oven. LC separations were performed in stepwise gradient mode at 40°C.

Table 4 LC/MS instrumentation used in studies **I-IV**.

Paper	LC	MS	Column	Mobile phase	MS/MS
I	1100[a]	micrOTOF[b]	Luna C18[d] 100×2 mm (3 µm)	5 mM NH_4OAc, 0.1% FA + ACN (1)	ISCID
II	1200[a]	micrOTOF-Q[b]	Luna PFP[d] 100×2 mm (3 µm)	2 mM NH_4OAc, 0.1% FA + MeOH (2)	AutoMS(n), unselective
III	1200[a]	micrOTOF-Q[b]	C18 and PFP	(1) and (2)	none
IV	UPLC[c]	micrOTOF-Q[b]	PFP	(2)	AutoMS(n), precursor

[a]Agilent Technologies (Santa Clara, CA, USA), [b]Bruker Daltonik (Bremen, Germany), [c]Waters (Milford, MA, USA), [d]Phenomenex (Torrance, CA, USA)
ACN acetonitrile, *FA* formic acid, *MeOH* methanol, *NH_4OAc* ammonium acetate

The TOF instruments were coupled with an orthogonal electrospray ionization source. The nominal resolution of the instruments was 10,000 FWHM (at *m/z* 922). The instruments were operated in positive ion mode with an *m/z* range of 50-800. External instrument calibration was performed with sodium formate solution using ten cluster ions ($Na(NaCOOH)_{1\text{-}10}$) with exact masses between 90.9766 and 702.8635, and the same ions were used for post-run internal mass calibration for each sample. Mass fragmentation was carried out using ISCID (**I**), or either an unselective (**II**) or a selected precursor (**IV**) AutoMS(n) method.

4.4 Software

4.4.1 Data analysis

DataAnalysis software (Bruker Daltonik, Bremen, Germany) was used for processing of the sample analysis data (**I**-**III**: version 4.0; **IV**: version 4.1). MetaboliteDetect 2.0 (Bruker Daltonik, Bremen, Germany) was employed in finding quetiapine (QTP) metabolites in human urine (**I**) with a relative intensity threshold of 30%. The software subtracts the blank sample data from the test sample data, and then lists the molecular formulae calculated for all peaks detected. Thus, information on both predicted and unexpected metabolites is obtained. An automatic reverse database search [173] for assigning designer drug metabolites in human urine and HLM incubation samples was carried out with TargetAnalysis 1.2 (Bruker Daltonik, Bremen, Germany) (**IV**). The selected metabolite identification criteria were: peak area counts of 2,000, mass tolerance of ±3 mDa, and isotopic pattern match value, mSigma threshold of 200. The SigmaFit (Bruker Daltonik, Bremen, Germany) algorithm compares the theoretical [199] and measured isotopic patterns, and calculates a match factor based on the deviations of the signal intensities [200]. The better the isotopic match, the lower the SigmaFit value.

4.4.2 Metabolism

The metabolism of QTP (**I**) and the NPSs, 2-DPMP, 3,4-DMMC, α-PVP and MPA (**IV**), was predicted using Meteor software (versions 10.0.2 (**I**) and 14.0.0 (**IV**), Lhasa Limited, Leeds, UK). Meteor is a rule-based expert system which predicts the metabolism of a compound by comparing the substructures to structure-metabolism rules included in its knowledge base. The

possibility of the metabolic reactions is evaluated, and more improbable metabolites are rejected. The prediction parameters used for Meteor in this study (**I**, **IV**) included phase I reactions in mammals, while the maximum number of metabolic steps was set at four, and the reaction likelihood level was either plausible or equivocal. The predicted metabolic reactions were compared with the published reactions of the drugs (**I**, **IV**) or their structural analogs (**IV**).

4.4.3 Mass fragmentation

ACD/MS Fragmenter software (Advanced Chemistry Development, Toronto, Canada) was employed to predict mass fragmentation (**I, II**: version 11.01; **IV**: 12.01). This software generates fragments for a molecule using fragmentation rules known in the literature. ACD/MS Fragmenter provides possible fragment structures with calculated exact masses and detailed fragmentation routes. Positive mode atmospheric pressure ionization, as well as fragment reactions including heterolytic and homolytic cleavages, neutral losses, and hydrogen rearrangements, were the fragmentation parameters selected in this study. Experimental spectra of the compounds studied were compared with the predicted fragments to identify the characteristic fragment structures.

SmartFormula3D is a mass spectra interpretation tool included in DataAnalysis (Bruker Daltonik, Bremen, Germany). The software was used to differentiate structural isomers (**II**), and to obtain additional structural information on designer drug metabolites (**IV**). SmartFormula3D calculates molecular formulae for possible fragments and precursor ions from experimental accurate mass and isotopic pattern match results. It includes an algorithm that calculates whether or not a product ion formula is a subset of the precursor ion. Product ions that cannot be fragmented from the precursor ion, and precursor ions that cannot be comprised of the product ions observed, are excluded.

4.4.4 Chromatographic retention

ACD/ChromGenius 12.00 software (Advanced Chemistry Development, Toronto, Canada) was used to calculate t_R for compounds included in a database for toxicological urine drug screening (**III**). Two t_R knowledge bases, PFP and C-18, were created from the LC/QTOFMS in-house toxicology databases of approximately 500 compounds. ACD/ChromGenius software used chemical structures and calculated physicochemical properties to predict t_R for compounds. Each compound is compared to the 30 most similar structures in the knowledge base to search for the physical properties that correlate with t_R. The predicted t_R of the compounds in the PFP and C18 knowledge bases, as well as the calculated retention order of the 118 structural isomers in the PFP knowledge base, were compared with the experimental values.

5 RESULTS AND DISCUSSION

5.1 Metabolite prediction

Table 5 presents the generalized phase I metabolic reactions employed by the Meteor software for metabolite prediction of QTP (**I**) and the four NPSs studied: 2-DPMP, 3,4-DMMC, α-PVP, and MPA (**IV**). The total number of metabolites generated *in silico* and the prediction likelihood levels of Meteor for each reaction are also presented. The metabolism of 2-DPMP, 3,4-DMMC, α-PVP, and MPA was also predicted from the published reactions of their structural analogs. Studies of the metabolism of 2-DPMP had not previously been reported. Its metabolites were concluded from the metabolism of phencyclidine-structured designer drugs [12]. 3,4-DMMC metabolites [201] were completed by comparison with the metabolism of β-keto-structured cathinones [12,202]. α-PVP metabolites identified in rats [203] were supplemented with metabolic information on other pyrrolidinophenone-derived drugs [11,12]. In addition to the normetabolite of MPA [204], its metabolism was predicted by using methamphetamine [205] and thiophene-structured compounds [206] as model compounds. The main metabolic pathways of QTP are sulfoxidation, hydroxylation, oxidation to the corresponding carboxylic acid, *N*-dealkylation, and *O*-dealkylation [207]. Meteor software did not predict hydroxy metabolites, which were therefore added manually to the list of metabolites and applied to the MetaboliteDetect software.

Table 5 Metabolic reactions employed in metabolite prediction by Meteor software and based on the analogous reactions found in the literature (*) (**I**, **IV**).

QTP (n=14 / *11)	**2-DPMP** (n=42 / *14)	**3,4-DMMC** (n=69 / *11)	**α-PVP** (n=15 / *23)	**MPA** (n=21 / *13)
**Dealkylation*[a]	**Dealkylation*[b]	**Dealkylation*[a]	**Dealkylation*[a]	**Dealkylation*[b]
**Oxidation*[a]	Deamination[c]	**Hydroxylation*[a,b]	**Dehydrogenation*[b]	Deamination[b]
Sulfonation[a]	Decarboxylation[c]	**Oxidation*[b]	**Hydroxylation*[b]	Decarboxylation[c]
**Sulfoxidation*[a]	**Dehydrogenation*[b]	**Reduction*[a]	**Oxidation*[b]	*Hydroxylation[b,c]
	Hydrolysis[c]		*Reduction*[b]	Oxidation[b]
	**Hydroxylation*[b,c]			Reduction[b]
	**Oxidation*[a,b]			
Hydroxylation*	Reduction[b]			*Sulfoxidation**

*Published analogous reactions, **Not predicted by Meteor
[a]Probable likelihood level, [b]Plausible likelihood level, [c]Equivocal likelihood level
Total number of predicted metabolites are in brackets. Identified metabolic reactions are in italics.

5.1.1 Quetiapine metabolism

Twelve phase I metabolites were detected and identified for QTP in ten autopsy urine samples by LC/TOFMS using MetaboliteDetect software and manual inspection (**I**). The metabolites identified were *N*-desalkyl-QTP (M1), *O*-desalkyl-QTP (M2), QTP-sulfoxide (M3), OH-QTP (M4), QTP-acid (M5), *N*-desalkyl-OH-QTP (M6), *O*-desalkyl-OH-QTP (M7), *O*-desalkyl-QTP-

sulfoxide (M8), *O*-desalkyl-QTP-acid (M9), QTP-sulfoxide acid (M10), OH-QTP-sulfoxide (M11) and *N*-desalkyl-OH-QTP-sulfoxide (M12). Contrary to what was reported in the original publication **I,** QTP did not metabolize by *N*-dealkylation and sulfoxidation. This structure was later found to be an ISCID fragment of the *O*-dealkyl-sulfoxy metabolite (M8). Eleven metabolites for QTP have been described in the literature [207-210]. However, no detailed structural information about QTP metabolites is available. The metabolic reactions participating in formation of metabolites the M1-M12 are listed in Table 6. The metabolites identified here were formed via the known metabolic reactions [207]. Figure 1 shows the positions at which the metabolic reactions took place in the QTP molecule.

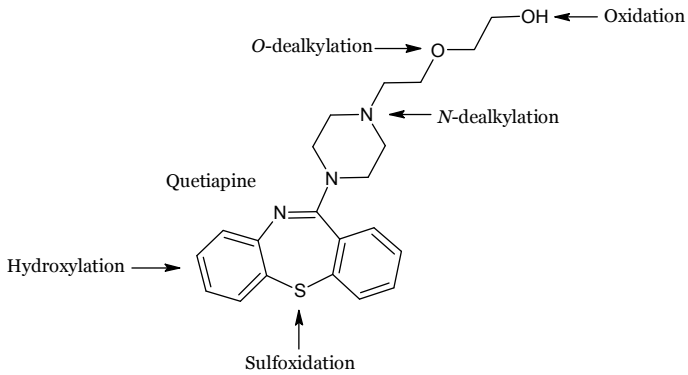

Figure 1 Main metabolic steps for QTP in human (**I**)

Meteor predicted 14 metabolites for QTP, and seven were detected in the urine samples. The five metabolites identified, but not predicted by Meteor under the chosen reasoning constraints, were hydroxylated species. Aromatic hydroxylation is one of the main metabolic routes of QTP, and the OH- and *N*-desalkyl-OH- metabolites are pharmacologically active [207]. Therefore, missing an important metabolic route was considered a significant drawback in the performance of the Meteor software.

5.1.2 Designer drug metabolism

The human urinary phase I metabolic reactions identified for 2-DPMP, 3,4-DMMC, α-PVP, and MPA are listed in Table 6 (**IV**). The metabolic reactions that took place in the *in vitro* experiments by HLMs are also presented.

2-DPMP was metabolized extensively by oxidative metabolic reactions. From the ten 2-DPMP urine samples studied, six phase I metabolites were identified: OH-DPMP (M1 and M2), oxo-DPMP (M3), OH-oxo-DPMP (M4), di-OH-oxo-DPMP (M5) and di-OH-carboxy-DPMP (M6). These were formed via aromatic and aliphatic hydroxylation and dehydrogenation reactions, as well as oxidation after opening of the piperidine ring structure. The proposed *in vivo* human phase I metabolism of 2-DPMP is presented in Figure 2. The aromatic and aliphatic hydroxy metabolites (M1 and M2) were present in relatively high abundance in the urine samples studied, indicating that hydroxylation is the main phase I metabolic route for 2-DPMP.

The *in vitro* experiments produced aliphatic OH-DPMP (M2) and oxo-DPMP (M3), and the aromatic OH-DPMP (M1) was detected at trace levels.

Table 6 Phase I metabolic reactions of QTP, 2-DPMP, 3,4-DMMC, α-PVP and MPA identified *in vivo* in human urine (**I**, **IV**).

	QTP	**2-DPMP**	**3,4-DMMC**	**α-PVP**	**MPA**
M1	***N*-dealkylat.**	**Hydrox. (arom.)**	****N*-demethyl.**	***Reduction**	****N*-demethyl.**
M2	***O*-dealkylat.**	*Hydrox. (ali.)	***Reduction**	***Hydrox.**	
M3	**Sulfoxidation**	***Hydrox. (ali.)** **Dehydrogen.**	***N*-demethyl.** **Reduction**	***Hydrox.** **Dehydrogen.**	
M4	Hydrox.	2 × Hydrox. (ali.) 1 × Dehydrogen.	***Hydrox.**	***Reduction** **Hydrox.** **Dehydrogen.**	
M5	**Oxidation**	3 × Hydrox. (ali. & arom.) 1 × Dehydrogen.	***N*-demethyl.** **Hydrox.**	*Degradation of pyrrolidine ring	
M6	*N*-dealkylat. Hydrox.	2 × Hydrox. (arom.) Ring opening Oxidation	***Reduction** **Hydrox.** **Oxidation**	*Hydrox. Dehydrogen. Ring opening Oxidation	
M7	*O*-dealkylat. Hydrox.			**Hydrox.** **Oxidation**	
M8	***O*-dealkylat.** **Sulfoxidation**				
M9	***O*-dealkylat.** **Oxidation**				
M10	**Sulfoxidation** **Oxidation**				
M11	Hydrox. Sulfoxidation				
M12	*N*-dealkylat. Hydrox. Sulfoxidation				

Metabolites predicted by Meteor are presented in bold
*Metabolites found *in vitro*
arom. aromatic, *ali.* aliphatic, *dealkyl.* dealkylation, *demethyl.* demethylation, *dehydrogen.* dehydrogenation, *hydrox.* hydroxylation

Meteor software predicted two of the six metabolites identified (Table 6). The reason for this relatively poor prediction accuracy was that Meteor did not suggest an aliphatic hydroxylation reaction, which was involved in the formation of OH-DPMP (M2), OH-oxo-DPMP (M4), and di-OH-oxo-DPMP (M5). Meteor predictions at equivocal likelihood level proposed hydroxylation at carbon atoms in the piperidine ring only as a reaction intermediate in the lactam structure (oxo-2-DPMP; M3) formation. The software predicted correctly the oxidative *N*-dealkylation, resulting in piperidine ring opening, and the subsequent oxidation of the primary alcohol to the corresponding carboxylic acid. However, it failed to predict the final structure of the aromatic di-OH-carboxy-DPMP (M6), as sequential hydroxylation reactions in the aromatic rings were not predicted at equivocal likelihood level.

Three 3,4-DMMC-positive autopsy urine cases were studied, and six phase I metabolites (Table 6) were identified: dimethylcathinone (DMC; M1), β-OH-DMMC (M2), β-OH-DMC (M3), OH-methyl-DMMC (M4), OH-methyl-DMC (M5) and β-OH-carboxy-DMMC (M6). Meteor software predicted all the identified metabolites, and the main *in vivo* metabolic events were detected *in vitro* as well. Figure 2 shows the suggested main metabolic routes for 3,4-DMMC. DMC (M1), β-OH-DMMC (M2), and β-OH-DMC (M3), which were formed via *N*-demethylation and reduction, and their combination, were consistent with the metabolites reported for 3,4-DMMC [201]. Here, OH-methyl-DMMC (M4) and β-OH-carboxy-DMMC (M6) were identified, metabolites that had previously been published as putative [201]. *N*-demethylation and hydroxylation produced the novel metabolite OH-methyl-DMC (M5). An MS/MS spectrum of M5 could not be generated, and an accurate mass and isotopic pattern match solely determined identification of this compound.

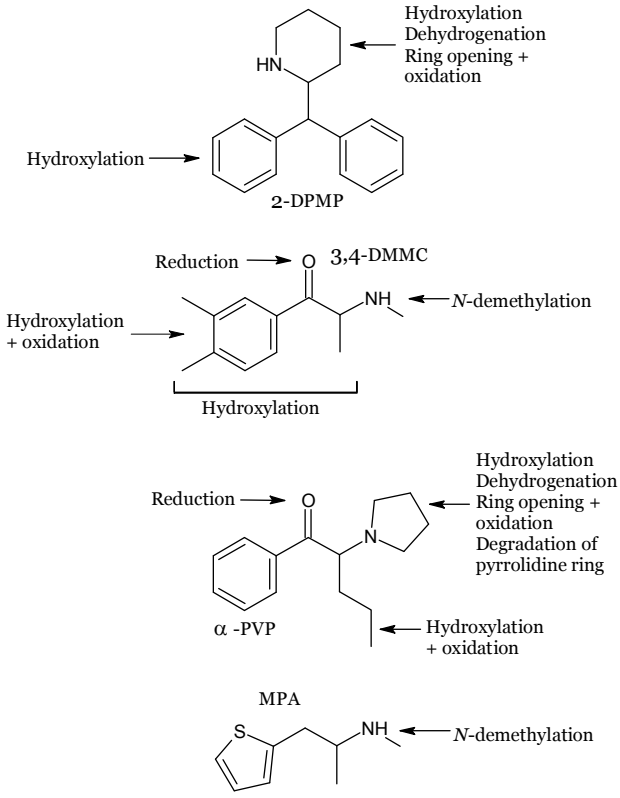

Figure 2 Main metabolic steps for 2-DPMP, 3,4-DMMC, α-PVP, and MPA in human (**IV**)

α-PVP was extensively metabolized in man, and seven phase I metabolites: β-OH-α-PVP (M1), OH-propyl-α-PVP (M2), oxo-α-PVP (M3), β-OH-oxo-α-PVP (M4), *N*,*N*-bisdealkyl-α-PVP (M5), α-PVP-*N*-butylic acid (M6), and α-PVP-propanoic acid (M7), were identified in the eight urine samples studied. Figure 2 and Table 6 show the metabolic reactions involved in the metabolism of α-PVP. Reduction of the β-ketone to the corresponding alcohol formed β-OH-α-PVP (M1), which was the most abundant metabolite of α-PVP both *in vivo* and *in vitro*. OH-

propyl-α-PVP (M2), oxo-α-PVP (M3), and *N,N*-bisdealkyl-α-PVP (M5) had earlier been identified in rat urine [203]. Oxo-α-PVP (M3) was further metabolized by reduction as well as pyrrolidinone ring opening and oxidation to produce the respective metabolites β-OH-oxo-α-PVP (M4) and α-PVP-*N*-butylic acid (M6). Reduction of the β-ketone (β-OH-α-PVP, M1; and β-OH-oxo-α-PVP, M4), and oxidation of the propyl side chain (α-PVP-propanoic acid, M7) were previously unreported metabolic routes for α-PVP. Four novel phase I metabolites: M1, M4, M6, and M7, were identified. The main metabolic reactions of α-PVP identified *in vivo* also took place in the *in vitro* experiments. Meteor software predicted correctly five of the seven metabolites identified for α-PVP, and, most important, it helped in identification of a new main metabolic route: reduction of the β-ketone.

MPA is known to be metabolized to a minor extent in humans [204], which supports the identification of only one metabolite, the *N*-desmethyl metabolite (M1), for MPA (Table 6, and Figure 2). In addition to nor-MPA, traces of hydroxy metabolites could be seen *in vitro*, which, however, were not detected in human urine samples. Meteor predicted the *N*-demethylation reaction as well.

5.2 Mass fragmentation *in silico*

Fragment identification *in silico* was employed in structural elucidation of the metabolites of QTP, 2-DPMP, 3,4-DMMC, α-PVP and MPA (**I**, **IV**), and in differentiation of their isomeric metabolites, such as the hydroxy and sulfoxy metabolites of QTP and aromatic and aliphatic OH-DPMP (**I**, **IV**). *In silico* fragment assignment was also used to differentiate between the structural isomers found in a large target database for toxicological drug screening (**II**).

ACD/MS Fragmenter predicted approximately 30 to 250 fragments per compound, depending on size and structure (**II**). The software listed several possible fragment structures with calculated monoisotopic masses under the respective nominal mass. SmartFormula3D suggested 1-4 formulae as a precursor ion and 2-15 formulae for the respective product ion. The software provides a sum formula for the product ions identified, and thus the results do not enable fragment structure determination alone. Neither of the software tools estimated differences in ion abundances, and thus these were not used in compound identification.

5.2.1 Differentiation of structural isomers

ACD/MS Fragmenter and SmartFormula3D helped in the differentiation of 111 structural isomers belonging to an in-house toxicology database (**II**). For 80% of the compounds studied three characteristic fragments could be identified, and 82% of the fragments were identified by both software tools. Fragment identification assisted in differentiation of 82% (91 compounds in 38 isomer groups) of the structural isomers. Ten isomer pairs, i.e. diastereomers or position isomers, fragmented identically, and therefore mass fragmentation *in silico* did not benefit the differentiation of these compounds. Eight of these pairs could be separated by proper chromatography, however. Protriptyline and nortriptyline, as well as *cis*-3-methylfentanyl and *trans*-3-methylfentanyl, remained inseparable both by chromatography and by fragmentation.

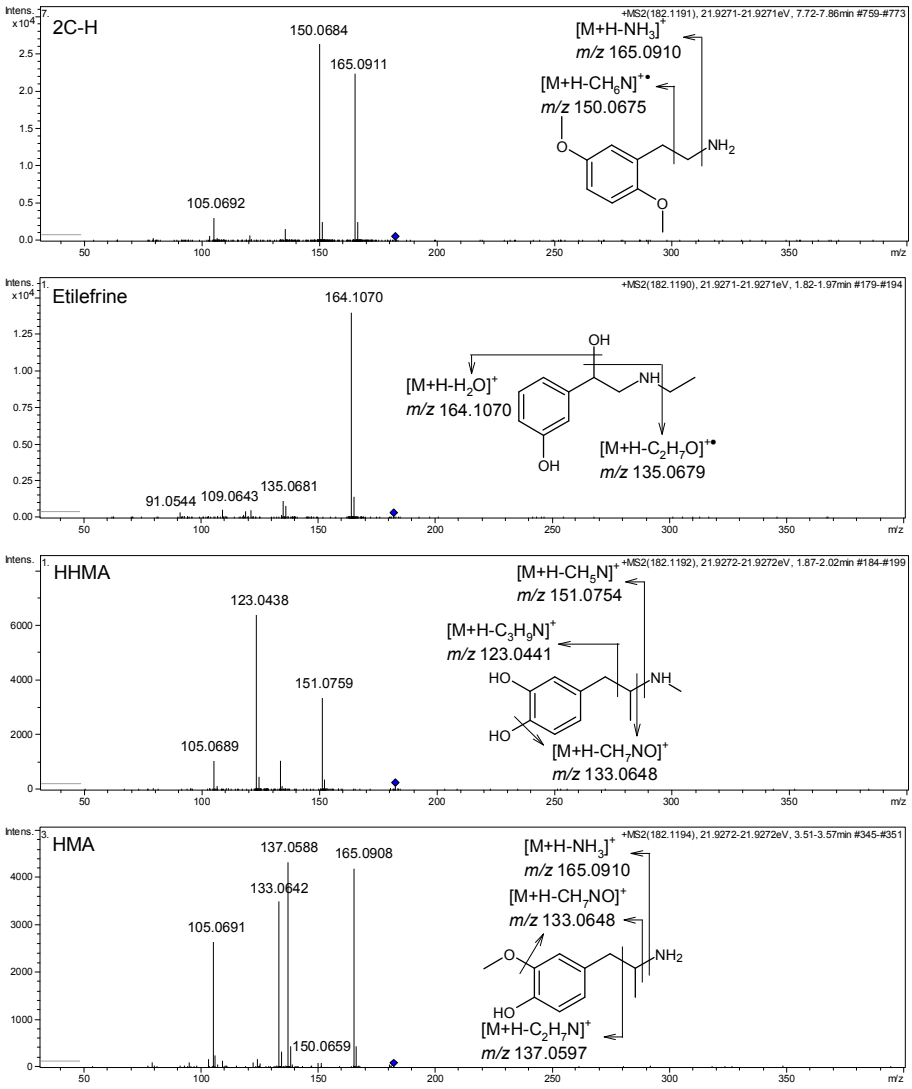

Figure 3 MS/MS spectra and fragmentation schemes of structural isomers: 2C-H, etilefrine, HHMA, and HMA, with the molecular formula $C_{10}H_{15}NO_2$ corresponding to $[M+H]^+$ at 182.1176. The proposed fragmentation schemes are based on fragment identification *in silico* (**II**)

Figure 3 exemplifies differentiation of four phenethylamine-structured isomers with a molecular formula of $C_{10}H_{15}NO_2$ using *in silico* fragment identification. Both ACD/MS Fragmenter and SmartFormula3D identified the product ions presented, except for the 2,5-dimethoxyphenethylamine (2C-H) product ion with an exact mass of 105.0699 ($[M+H-C_2H_7NO_2]^+$), which was proposed solely by SmartFormula3D. The structure of this fragment was therefore not verified. All the fragments were identified within a mass error of ±1 mDa from the exact monoisotopic mass. Product ions with exact masses of 150.0675 for 2C-H and 135.0679 for etilefrine resulted from loss of radical cations. Other fragmentation reactions were neutral losses. Etilefrine and 3,4-dihydroxymethamphetamine (HHMA) did not differ in their chromatographic retention times (Δt_R 0.07 min), and thus in an actual toxicology case in which

both compounds are positive, a sum spectra would be seen. However, characteristic fragments would allow identification of both compounds even from the same spectrum.

Fragment prediction with ACD/MS Fragmenter helped in differentiation of metabolites with an identical molecular formula (**I**). The hydroxy and sulfoxy metabolites of QTP (M3 and M4), as well as *O*-desalkyl-OH-QTP (M7) and *O*-desalkyl-QTP-sulfoxide (M8) (Table 6) share the same molecular formula, and consequently could not be separated by TOFMS data only. The spectra from these isomeric metabolites, obtained by ISCID analysis, showed compound-characteristic fragments. The product ions formed after cleavage of the sulfoxy group ($[M+H-OS]^+$) from the sulfoxy metabolites unambiguously distinguished them from the hydroxy metabolites.

2-DPMP metabolites formed via aromatic hydroxylation (M1) and hydroxylation at the piperidine ring (M2) could be differentiated using ACD/MS Fragmenter (**IV**). A loss of water was detected in the spectra of M2, which was not seen in the spectra of the aromatic hydroxylated metabolite M1. A compound-characteristic fragment of M1 - a benzylphenol structure ($C_{13}H_{11}O$ at exact mass *m/z* 183.0804) - proved that the hydroxylation took place at the aromatic part of the molecule.

The α-PVP metabolites OH-propyl-α-PVP (M2) and β-OH-oxo-α-PVP (M4) have the molecular formula $C_{15}H_{21}NO_2$ ($[M+H]^+$ at 248.1645) (**IV**). The product ions identified, formed via the loss of the hydroxypropyl group ($[M+H-C_3H_7O]^+$) from M2, and fragmentation of the pyrrolidinone ring ($[M+H-C_4H_7NO]^+$) from M4, verified the different structures.

5.2.2 Metabolite structure identification

In silico fragment prediction was used for QTP (**I**), 2-DPMP, 3,4-DMMC, α-PVP and MPA (**IV**) metabolite structure identification without the respective PRSs. One to four characteristic fragments were identified for 11 QTP metabolites from the ISCID data using ACD/MS Fragmenter. The low intensity of the *O*-desalkyl-QTP-acid (M9) did not allow fragment identification, and thus verification of the structure would require MS/MS analysis. Meteor software suggested a sulfone structure, corresponding to the molecular formulae of OH-QTP-sulfoxide (M11) and *N*-desalkyl-OH-QTP-sulfoxide (M12). This could be excluded as the characteristic product ion from sulfoxy cleavage was identified.

The structures of the 2-DPMP, 3,4-DMMC, α-PVP and MPA metabolites detected in the human urine samples and *in vitro* experiments were confirmed by comparing the mass spectra of the metabolites with the product ions identified for the parent compounds using ACD/MS Fragmenter and SmartFormula3D (**IV**). The fragmentation of the metabolites mainly followed the path of the parent compound. Fragment prediction was employed to determine the site of the metabolic reaction in the molecule. Structural elucidation by the fragment prediction software supported the identification of 11 previously unreported designer drug metabolites. ACD/MS Fragmenter aided in, for example, identification of the novel metabolites α-PVP-*N*-butylic acid (M6) and α-PVP-propanoic acid (M7). Product ions identified from the loss of acetic acid and the loss of aminobutyric acid proved the structure of α-PVP-*N*-butylic acid (M6), which was formed by pyrrolidinone ring opening and oxidation. An oxidation reaction was found to take place at the propyl side chain (M7), as the loss of propanoic acid was detected. The software helped to differentiate between aromatic and aliphatic hydroxylations; however, the exact site of

the hydroxylation could not be identified in the cases of the OH-DPMPs (M1 and M2), and OH-methyl-DMMC (M4).

5.3 Liquid chromatographic retention prediction

The correlation between the experimental and the calculated t_R in the PFP knowledge base was satisfactory (r^2=0.8533) (**III**). The prediction accuracy of the control knowledge base C18 was practically the same (r^2=0.8497). The C18 control knowledge base was created to confirm the prediction capacity of ACD/ChromGenius, and the results were not used for compound retention order determination. The mean and median absolute errors of the compounds in the PFP knowledge base were 1.12 min, and 0.84 min, respectively, in a 20 min analysis time. Figure 4 presents the distribution of t_R errors in the PFP knowledge base. For 17% of the compounds the calculated t_R differed by ±2% from the experimental value, and for 58% the t_R error was within ±10%. The ACD/ChromGenius calculations were no higher or lower than the experimental t_R, and for most compounds (57%) the t_R error was less than ±1.00 min. Compounds that were structurally very distinctive, such as hydroxychloroquine (Δt_R 6.10 min) and amiodarone (Δt_R 4.83 min), showed poor correlations between experimental and calculated t_R. Different drug compound categories showed variations in prediction accuracy. The calculations were more precise for the structurally consistent groups phenethylamines, and tri- and tetracyclic central nervous system (CNS) drugs than for opioids, which have greater structural variety. Due to relatively large absolute t_R errors, the results of ACD/ChromGenius were useless for compound identification alone.

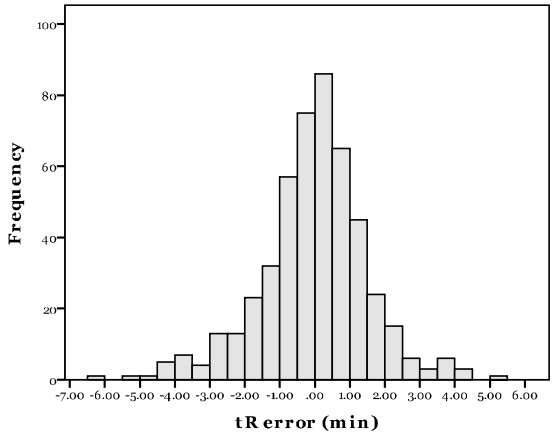

Figure 4 Distribution of t_R errors in the PFP knowledge base (**III**)

Despite the large absolute errors in calculated t_R, ACD/ChromGenius proved its feasibility in predicting the compound elution order. The software calculated the correct retention order for 68% of the structural isomer groups in the PFP knowledge base. The retention order of the isomers was more correctly calculated for compounds with adequate prediction accuracy: eight of the nine isomer groups with tri- and tetracyclic CNS drugs were correctly predicted.

Phenethylamine derivatives constitute potential isomeric NPSs [211], in which the substituent and its position vary. The benefit of the ACD/ChromGenius software lies in the retention order calculation for different phenethylamines. An example of a correctly predicted elution order for five phenethylamine isomers: etilefrine, HHMA, 4-hydroxy-3-methoxyamphetamine (HMA), 3,4-dimethoxyphenethylamine (DMPEA), and 2C-H, is presented in Table 7. The predicted t_R indicates the compound elution order compared with other possible substances.

Table 7 Five isomeric phenethylamine derivatives in the PFP knowledge base: etilefrine, HHMA, HMA, DMPEA, and 2C-H, with experimental and calculated t_R, and their absolute errors (**III**).

Formula	Compound	Experimental t_R	Calculated t_R	Absolute t_R error
$C_{10}H_{15}NO_2$	Etilefrine	1.82	2.35	0.53
$[M+H]^+$ 182.1176	HHMA	1.87	2.91	1.04
	HMA	3.34	3.61	0.27
	DMPEA	5.08	5.18	0.10
	2C-H	7.63	5.54	2.09

5.4 Software tools applied to accurate mass data

Identification of the compounds by LC/TOFMS (**I**) and LC/QTOFMS (**II-IV**) was based on t_R repeatability, mass accuracy and isotopic pattern match value (SigmaFit). The mean mass accuracy and the mean SigmaFit value were less than 1 mDa, and 30 mSigma, respectively, by both TOFMS and QTOFMS instruments. The excellent mass accuracy with adequate instrument resolution enabled the determination of molecular formulae for both parent compound and its product ions. *In silico* fragment structure identification assisted the differentiation of isomeric drug metabolites (**I, IV**) and the structural isomers from a toxicology database (**II**), as compounds with identical elemental composition are inseparable from full-scan accurate mass data only. Combining accurate mass data and fragment prediction facilitated the elucidation of the structure of the metabolites (**I**, **IV**).

Both ACD/MS Fragmenter and SmartFormula3D enable exploitation of accurate mass data in the interpretation of their fragment assignments. The exact mass information provided by these software tools made it easy to distinguish between product ions formed via even and odd electron cleavages. ACD/MS Fragmenter allowed an exact monoisotopic mass to be calculated for the proposed fragments, which facilitated the selection of the respective product ion from the experimental MS spectrum. The software also provides a visual inspection of the proposed fragment structure, which simplifies determination of the correct product ion configuration. ACD/MS Fragmenter was found to be useful with ISCID data as well, which demonstrates its benefits over library spectra comparison. The list of predicted fragments, however, included many potential false-positive predictions, and thus is not useful for mass fragmentation studies alone without experimental data to compare with.

SmartFormula3D proved to be an effective tool for assigning possible product ions for the parent compound from the TOFMS data. However, it does not offer any information about fragment structure, and therefore, when the structure of an unknown compound needs to be

determined, its usefulness is limited. The software would most likely benefit rapid identification of compound-characteristic fragments from MS/MS data obtained by PRSs.

ACD/MS Fragmenter and SmartFormula3D showed some lack of robustness in their performance, as they did not identify all the product ions seen in the spectra, and a few dissimilarities were observed in their results. Therefore, the results from *in silico* fragment assignment should always be checked carefully to confirm that the proposed structures are logical. Nevertheless, the results of ACD/MS Fragmenter and SmartFormula3D support each other's fragment proposals.

The software gave information neither about the charge distribution nor the location of the radical site in the proposed product ion. They did not estimate the probability or the ion abundance of the proposed fragments. Identification of all the possible product ions seen in the mass spectrum is necessary when studying metabolism of a new drug candidate where determination of the detailed structure of each metabolite is crucial. However, in terms of differentiating between regioisomers, identification of 1-3 compound-characteristic fragment structures is sufficient. In drug metabolism studies with forensic toxicology cases, the aim is usually to identify the main metabolites to be used as supporting information along with the parent compound. In cases, where qualitative metabolite identification is pursued, ACD/MS Fragmenter and SmartFormula3D provide valid structural information.

5.5 Preliminary compound identification

In silico tools used for prediction of drug metabolism (**I**, **IV**), mass fragmentation (**I**, **II**, **IV**), and chromatographic retention (**III**), combined with LC/accurate mass data, assisted with compound identification. The software performed well in both studies carried out with PRSs as well as in metabolite identification with authentic biological samples. The software solutions employed in metabolite identification, MetaboliteDetect (**I**) and TargetAnalysis (**IV**), served as rough screening tools.

The *in vitro* metabolism experiments using HLMs produced eight of the 12 most abundant *in vivo* phase I designer drug metabolites detected in human urine (**IV**). For some of the metabolites that showed probable stereoisomerism *in vivo*, such as metabolite β-OH-oxo-α-PVP (M4), the *in vitro* incubations generated only one of the diastereomers. The incubations with HLMs produced a few designer metabolites of minor abundance that were not detected *in vivo*. Thus, when extrapolating the data from *in vitro* studies to be used in metabolite identification *in vivo*, the differences in metabolism should be considered [15,38]. Despite these dissimilarities, the *in vitro* experiments served as biological material to be used with *in silico* metabolism studies when an authentic human urine sample is unavailable. To carry out an *in vitro* study, however, requires PRSs for parent compounds.

Meteor software provided literature-based justification for the predicted metabolic reactions, which made the interpretation of the results explicit. The prediction likelihood level was found to be a useful indicator in estimating the probability of the metabolic reaction: all the metabolites identified were formed via reactions at either probable or plausible level. The inability of the software to predict some of the hydroxylation reactions, one of the major metabolic routes for QTP and 2-DPMP, indicates a lack of sensitivity in its performance. Meteor was able to predict the missing metabolic routes when less stringent likelihood level settings

were used. This, however, resulted in a large number of false-positive predictions, which impairs the usefulness of the results when the aim is to identify the main metabolic products. The tendency towards overprediction and low prediction precision of Meteor has been reported elsewhere as well [42]. The reason for the less successful predictions for 2-DPMP, compared with other NPSs studied, might be that the Meteor knowledge base contains very few metabolic reactions of structurally similar compounds. Therefore, metabolite predictions by Meteor for structurally novel drug compounds should be reviewed critically. In addition, the prediction results should always be compared with the published metabolic reactions of the structural analogs, and completed with the missing metabolites, if possible.

Meteor was no more accurate in metabolite prediction than manual deduction based on the metabolic reactions of the structural analogs. Nevertheless, the software served as a valuable and time-saving tool for creating list of possible metabolites for toxicologically interesting drug compounds. The molecular formulae presented and the exact monoisotopic masses of the proposed metabolites were easily transferred into a spreadsheet form. This list of possible metabolites can be exploited in an automated database search for accurate mass data. The database used for routine urine drug screening could be subsequently complemented with the tentatively identified metabolite formulae with the aim of facilitating drug identification in authentic human urine samples.

MetaboliteDetect and TargetAnalysis software tools were utilized in metabolite identification. MetaboliteDetect turned out to be unsuitable for screening metabolites in complex biological samples such as urine. A pooled pseudo-reference urine sample was used instead of a blank reference. Subtraction of the background ions from the test sample ions did not clean up the chromatogram substantially because of the relatively difficult sample matrix of post-mortem urine. The QTP urine samples also included several other toxicological findings which interfered with metabolite identification. The identification capacity of MetaboliteDetect software varied from 40% to 100% for the QTP metabolites. Manual detection proved to be more accurate, as the variation in metabolite identification was between 80% and 100%. The main reason for the poor identification capacity of MetaboliteDetect was that only a relative intensity threshold could be selected as an identification parameter. Therefore, when the total ion current was high, it missed even relatively abundant metabolites.

TargetAnalysis, which is designed for automated database searches, identified metabolites reliably. However, it does not include any add-ons for higher-grade metabolite identification, such as mass defect filtering or spectra comparison, and therefore is not the software of choice for unknown metabolite detection. Even a relatively small-scale metabolite screening aiming to find the main phase I metabolites of four drug compounds (**IV**) was time consuming, as the creation of the list of possible metabolites predicted by Meteor could not be automated. For metabolism studies with a considerable number of possible target compounds, such as those in metabolomics, more advanced metabolite screening tools are necessary.

ACD/MS Fragmenter and SmartFormula3D software were found to be useful for fragment structure determination. This was shown by identifying characteristic fragments for 111 compounds using PRSs (**II**). The study introduced a practical approach for preliminary compound identification even without PRSs. This was demonstrated by identifying drug metabolites for which no reference material was available (**I**, **IV**). Fragment prediction *in silico* worked similarly by excluding the false metabolite structures. For some of the potential

metabolites Meteor software predicted several structures fitting the same molecular formula. For example, eight structural isomers were predicted *in silico* for β-OH-carboxy-DMMC (M6) ($C_{12}H_{17}NO_2$; $[M+H]^+$ 224.1281) (**IV**). However, identification of the product ion, corresponding to a methylbenzoic acid structure ($C_8H_9O_2$; at exact mass *m/z* 137.0597), allowed elimination of the proposed dihydroxy metabolites.

Prediction of the correct compound elution order provides valuable information for the separation of structural isomers (**III**). Two isomer pairs: 2,5-dimethoxyamphetamine (2,5-DMA) and 3,4-dimethoxyamphetamine (3,4-DMA) ($C_{11}H_{17}NO_2$; $[M+H]^+$ 196.1332), and 4-isopropylthio-2,5-dimethoxyphenethylamine (2C-T-4) and 4-propylthio-2,5-dimethoxy-phenethylamine (2C-T-7) ($C_{13}H_{21}NO_2S$; $[M+H]^+$ 256.1366), had identical fragments, and thus could not be separated by fragment prediction alone (**II**). However, successful retention order prediction made it possible to differentiate between these isomers. The predicted t_R, combined with information produced by other *in silico* software tools employing accurate mass data, completed the compound identification.

It was shown that the software employed in predicting metabolism and chromatographic behavior was most accurate for compounds with a chemical structure similar to other compounds in the database. QTP, a dibenzothiazepine derivative [207], has a tricyclic structure, which is common to several drug compounds with pharmacological effects on the CNS [212]. Meteor software proposed correctly four of the five main metabolic reactions of QTP. For the 47 tri- and tetracyclic CNS drugs in the PFP knowledge base, the median absolute error of the ACD/ChromGenius predictions was 0.62 min. Nine isomer groups included a tri- or a tetracyclic compound, and the retention order was predicted correctly in eight cases. Retention order prediction using ACD/ChromGenius also provided important information for identifying designer drugs with a phenethylamine structure. The successful predictions related to phenethylamines are of special interest to a forensic toxicologist, as NPSs are often chemical modifications of amphetamines and cathinones: nineteen new designer drugs with a phenethylamine structure were notified to the EU early-warning system in 2012 [3].

6 GENERAL DISCUSSION

Computational systems that aid in the identification of small molecules have advanced and the number of different methods has increased rapidly during recent years. Their role is becoming increasingly important as improved analytical techniques offer a vast amount of data in a shorter turn-around time. The bottleneck in studies of unknown compounds is the amount of data that a researcher can handle, not the sample throughput time. There is therefore a pressing need for validated and robust computational methods.

Computational approaches to predicting drug metabolism are common practice in the early drug development process. The number of different *in silico* tools for metabolite prediction has increased rapidly during the preparation of the present thesis, which demonstrates the growing interest in this approach. Many of those *in silico* systems that predict ligand-enzyme interactions are without doubt too complicated to implement in analytical toxicology practice. However, *in silico* tools together with *in vitro* experiments provide useful information to support *in vivo* metabolism studies [20]. Computational metabolite prediction was introduced in forensic toxicology practice in this thesis. Meteor software provided sensitive metabolite prediction, and allowed identification of new metabolites that were not previously detected *in vivo* in rats. It is known that species differences, especially in CYP-related metabolism, can be quite large [213,214]. Therefore, conclusions about the metabolic fate in humans should not be based on animal tests alone.

Recently, *in silico* metabolite prediction has been applied to synthetic cannabinoids [215]. In a preliminary study, the MetaSite software showed promising results by predicting the main *in vitro* metabolites of the target compounds. Peters et al. [216] used a modified version of MetaboLynx software to predict and identify steroid metabolites and their designer modifications in spiked urine samples. The screening method proved successful; however, it was not tested on authentic urine samples, in which the concentrations of the metabolites can be very low. Nevertheless, *in silico* methods that predict metabolite structures would certainly be useful for screening metabolites of toxicological interest in biological samples. The proposed metabolic reactions can also complement the known metabolic reactions, even if the exact predicted structures cannot be detected. Systems that can predict drug effects on metabolizing enzymes, especially on CYP isozymes, could aid in toxicological risk assessment.

Predictive tools for drug metabolism studies have advanced considerably although there is still much room for further development and enhancement [39]. It has been claimed that computational methods cannot yet replace human expertise [20]. In the present thesis Meteor software showed the potential to be beneficial in forensic toxicology practice. However, conclusions about its feasibility with a wide variety of drug compounds would require further studies. A comparative study of the performances of different *in silico* drug metabolism systems for toxicologically relevant compounds would provide more reliable information about the advantages of this type of software.

As controlled *in vivo* metabolism studies of NPSs on humans are out of the question, *in vitro* experiments and *in vivo* animal studies have been employed with these compounds [38]. *In vivo* animal studies require special facilities, and cannot be performed in the majority of analytical

toxicology laboratories, which is why the use of *in vitro* metabolism studies has increased in popularity [38]. Metabolite characterization studies using different *in vitro* systems have been applied to compounds such as cathinones [99], synthetic cannabinoids [217], tryptamines [218] and phenethylamines [219]. Another approach employing *in vitro* studies in a toxicology context is to examine CYP-mediated metabolism. In these initial activity screening assays the goal is to determine the main enzymes involved in the elimination of the drug, and investigate possible drug-drug interactions and genetic variability in pharmacokinetics [220]. None of the current *in vitro* experimental systems predict *in vivo* metabolite pattern perfectly; however, relatively robust and reliable extrapolations regarding qualitative human metabolism can be made on the basis of appropriate *in vitro* studies [20]. Furthermore, *in vitro* samples serve as a specimen for screening *in silico* metabolites in cases where no authentic human urine sample is available.

In vitro metabolism studies are limited to compounds for which reference material is available. Such studies have been used to produce metabolite standards to compensate for the absence of PRSs [221-223]. However, the procedures involved require dozens of milligrams of the parent compound to produce a reasonable amount of metabolites. In the case of novel designer drugs or expensive standards, the method is impractical or even impossible.

MS instruments with high mass accuracy, moderate or high RP, and sufficient sensitivity facilitate the identification and structural characterization of toxicological compounds and their metabolites in complex biological matrixes [31,33,146]. Sophisticated computational systems are indispensable for effective data processing, and numerous applications are available. The identification of toxicologically relevant compounds has conventionally been based on comparison with reference GC/EI spectra. Despite the recent advances in development of universal MS/MS spectral libraries, there are doubts about their reliability and transferability [187]. Compound identification by spectral library search does not enable identification of unknown substances such as metabolites. MS/MS data for non-targeted compounds of low abundance is also lost in data-dependent acquisition analysis. The novel fragmentation tree approach [102,110-112] may aid in solving this limitation, but the further validation of these software systems is required [110]. Screening against very large databases using orthogonal filters for accurate mass and HRMS data has been used in determining the elemental composition of unknowns [96,224-226]. The method was proposed as a systematic work flow for untargeted screening, for instance in the environmental sciences [84]. Recently, the rapidly growing open access compound and spectral databases have attracted criticism about the reliability of their contents [227,228]. The authors highlight the responsibility of the analyst for ensuring that the data are faultless and high of quality.

The above-mentioned approaches are particularly popular in the area of metabolomics, where the aim is to identify and quantify all metabolites in a given biological context [229]. Nevertheless, these systems seem too laborious for routine use in forensic toxicology [146]. From the toxicology point of view, more useful computational tools for accurate mass data are those that allow identification of the main metabolites of the target compound. Mass defect filtering enables fast screening of possible metabolites, and this approach has helped in identifying metabolites of synthetic cannabinoids in *in vitro* samples [217]. MetaboLynx software has served in the identification of steroid metabolites in spiked urine samples [216]. However, with the exception of the present study, automated metabolite detection software tools have not been applied to post-mortem cases. Post-mortem urine samples from forensic

toxicology investigations are very heterogeneous in quality, as they often contain interfering compounds such as other drugs and their metabolites, or substances formed in putrefaction reactions. As found here, such software systems are probably better suited for *in vitro* metabolism studies or for *in vivo* samples from controlled metabolism studies. The most frequently used approach to screening metabolites from HR accurate mass MS data is to generate a list of possible metabolites manually, and search for them in authentic biological samples [31].

Accurate mass data alone does not allow the structure of an unknown compound to be characterized with certainty. However, MS/MS analysis allows determination of the elemental composition of the product ions, and can be used in structural characterization of the parent compound. As shown in the present thesis, mass spectral interpretation tools and *in silico* fragment prediction software assist in matching the product ions to the correct parent compound structure. This provides enough information to establish the observed metabolic reaction of a parent compound and to differentiate between structural isomers. Mass spectral interpretation and structural determination of unknown compounds is without any doubt possible without software assistance; however, it does enable spectral processing to be performed within a reasonable time scale even for analysts with less experience. The software used here worked well for a heterogeneous range of compounds, and can therefore be used without prior knowledge of the fragmentation behavior of the substance. The combination of accurate mass measurement and fragment prediction allows the fragment structure to be determined. This provides a more universal identification approach for unknown compounds than a spectral library comparison. An untargeted screening method for drugs of abuse and antidepressant metabolites by SmileMS software worked well with unit resolution MS data, where detection was based on known fragment structures recorded in the library [120,121]. However, the approach does not allow identification of structurally unique compounds that are not included in the dataset. On the other hand, ISCID analysis does not produce pure spectra for a suitable spectral library comparison. However, ACD/MS Fragmenter and SmartFormula3D enabled characteristic fragment identification even from a mixed spectrum. These fragment prediction software tools are useful in creating a database with qualifier ions for screening and confirmation of targeted compounds in a single analytical run [176,182]. Accurate mass analysis with ISCID or bbCID using data-independent acquisition also allows a retrospective fragmental data investigation of unknown compounds without the need for reanalysis.

The predicted t_R can be used as a powerful orthogonal filter to cut down the number of possible chemical structures [83,84,98,230], and it plays an important role in data mining procedures such as in metabolomics [140]. Retention time prediction proved to be useful in the differentiation of structural isomers, but the system suffered from a severe lack of robustness and reliability. No comprehensive conclusions about the benefits of retention time prediction with ACD/ChromGenius or other software tools in forensic toxicology can be drawn based on this thesis, as the results have been achieved using an in-house database. The results for ACD/ChromGenius need careful validation for each single LC method, which makes the use of the software rather inflexible. In a recently introduced application, a QSRR method was used to predict UHPLC t_R of drug compounds to be used in toxicological drug screening [231]. However, the dataset of 175 compounds in this study seems rather limited if t_R is to be predicted for structurally novel compounds. Currently, no commercial software with a user-friendly interface

is available with which to perform reliable and robust LC t_R calculations for small molecules. Computational methods for processing the data from MS analyses have advanced enormously during the last decade. Whether similar progress will occur in the future in LC data processing remains to be seen.

The predictive software systems used in this thesis had moderately user-friendly interfaces, which made them easy to employ in routine laboratory work. The mathematical basis behind the predictions of Meteor software was quite well explained by Lhasa Limited, which made the evaluation of the results reliable. ACD/Labs did not provide the full algorithms for their software ACD/MS Fragmenter and ACD/ChromGenius. This, especially in terms of retention time prediction, made a comprehensive analysis of the software performance impossible. The full algorithm in commercial predictive systems is rarely freely available, which makes the comparison between different software tools difficult.

The *in silico* tools employed in the present thesis were from several manufacturers, and were found beneficial more or less as independent systems. Therefore, most of the data entry, transfer and processing had to be done manually. In many cases this was the most time consuming part of the study. In order to operate as time-saving and work-enhancing tools, the software should be platform-independent. Flexible data transfer between the MS instrument, the data processing systems for compound identification, and the different predictive software tools, would greatly improve the analysis and identification of small molecules.

Based on the findings in this thesis, a systematic workflow for metabolism studies employing different *in silico* systems and accurate mass data is presented in Figure 5. The workflow describes a procedure starting from prediction of the possible metabolites, followed by the identification and structural elucidation of the metabolites in biological samples. In the present thesis, the focus was on identification of phase I metabolites, although the suggested workflow would function for investigation of phase II metabolites as well. In the final step, the proposed metabolite formulae, along with the product ion data and possible retention time information are added to the target drug screening database. The workflow demonstrated can be applied to predictive software other than that employed in this thesis. This method helps in the identification of the parent compound when an authentic urine sample turns up. The role of metabolite prediction and fragment prediction software is central in the workflow presented, as they definitely speed up the identification and structural verification of the proposed metabolites. Retention order prediction may be useful in a case where isomeric metabolites cannot be differentiated explicitly from each other based on their MS/MS spectra. The workflow functions even if an authentic urine sample is not available. However, in such cases, the metabolites should be considered as tentative propositions.

Careful selection of the most convenient software to be used in metabolism studies based on the workflow presented here is necessary. The software tools chosen in the present thesis functioned well individually; however, when combined they were too rigid for systematic large-scale metabolism studies. A more advanced data processing tool for compound identification and software compatible with the analysis data would have made metabolite characterization more effective.

The metabolites found in these studies were detected using the present LC/TOFMS urine drug screening method of our laboratory. Even more metabolites might have been detected with a more selective and sensitive method validated separately for each of the compounds studied.

However, the results achieved here were directly applicable to the routine drug screening method.

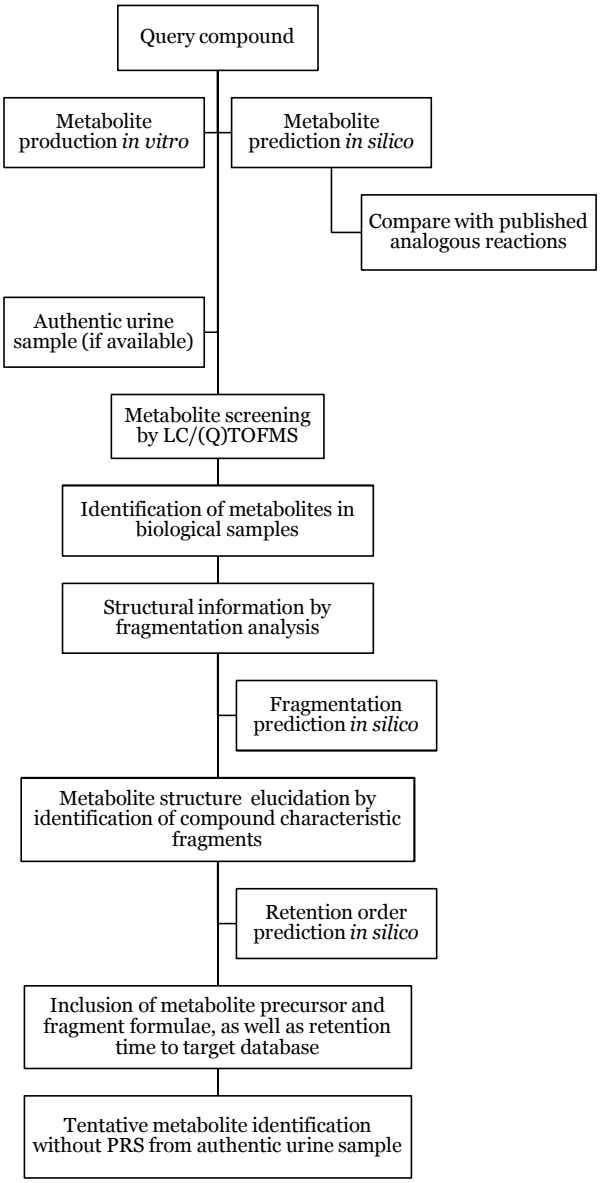

Figure 5 Systematic workflow for metabolism studies for toxicologically relevant compounds utilizing *in silico* systems and accurate mass measurement

Because of the legal consequences, an unambiguous identification of substances is of high priority in forensic and clinical toxicology. A false report may lead to incorrect convictions or to a patient's misdiagnosis, with the result that the laboratory may be perceived as unreliable [232]. Laboratory guidelines and requirements for compound identification are available for doping analysis [233] and forensic toxicology [234]. These regulations, however, cannot be

directly applied to HR and accurate mass data, as their requirements for mass accuracy and resolution (two decimal places and 10,000 FWHM, respectively) are not parallel with the performance of modern HRMS instruments. The EU Council Directive for food safety control [235] stipulates a minimum of four (4.0) identification points for animal and meat residue LC/MS analysis. HRMS provides 2.0 points for a precursor ion and 2.5 points for a product ion. In addition, a relative t_R error of 2.5% is allowed. EU Reference Laboratories for Residues of Pesticides published a document (SANCO/12495/2011) [236] that includes a mass accuracy tolerance of <5 ppm and accepts compound identification with two diagnostic product ions. Yet, the document suggests ion ratio tolerances that are unsuitable for full-scan data acquisition [237]. Rivier [238] has reviewed and summarized the guidelines for different LC/MS techniques to be used in forensic toxicology and doping analysis ten years ago. Analytical techniques, especially in the area of HRMS, have advanced, affording more informative data for compound identification today. Up-to-date MS identification guidelines in forensic toxicology case work are published by the Australian/New Zealand Specialist Advisory Group in Toxicology [239]. However, these criteria for compound identification strongly rely on the use of PRSs. Nielen et al. [160] proposed an identification criterion for screening and confirmation analysis that combines accurate mass (≤5 mDa) and mass resolution (RP 10,000-20,000 FWHM). They also commented on the identification of unknowns, and suggested that the proposed structures should be confirmed with either NMR or HRMS capable of RP ≥70,000 FWHM. Despite this, the present instructions related to compound identification by accurate mass do not take into account isotopic pattern determination, which was found crucial for calculating the molecular formula [88]. The current advanced methodologies in chromatography, i.e. UHPLC, provide enhanced separation capacity, repeatability and stability, which might impose demands on new t_R criteria as well.

These criteria cannot be applied to compound identification as such when PRSs are not available, and therefore the results should be treated as tentative. However, the reliable characterization of novel compounds is important, as an increasing number of new designer drugs is announced annually. The current *in silico* methods are not yet sufficiently validated to produce robust data that could be regarded as an extra identification point. Nevertheless, the present thesis clearly shows that computational methods do provide additional data to be used in preliminary identification for toxicologically relevant compounds. The information achieved using different *in silico* tools is straightforward to apply in accurate mass-based urine drug screening.

7 CONCLUSIONS

Computational methods for studying drug metabolism, mass fragmentation and chromatographic behavior proved to be feasible in analytical toxicology practice. The software was of particular benefit when applied to accurate mass data.

In silico prediction of drug metabolism with Meteor software was a rapid way to create a list of possible metabolites to be screened from biological samples. The software was found most accurate for compounds, such as phenethylamines or tricyclic CNS drugs, which were structurally most similar to the compounds used to compile the Meteor knowledge base (**I**, **IV**). The use of *in silico* metabolite prediction also enabled the identification of an unpublished metabolic route and detection of unreported metabolites. Meteor software showed a tendency towards overprediction, and therefore the results need to be verified against biological samples. The *in vitro* experiments provide material for metabolite screening when no authentic urine sample is available.

Fragmentation identification *in silico* aided the structural characterization of isomeric compounds and drug metabolites (**I**, **II**, **IV**). The combination of two software solutions ACD/MS Fragmenter and SmartFormula3D, which use a different approach in fragment assignment, produced reliable information for structural elucidation. These software tools, together with accurate mass fragmentation data, enabled determination of the product ion structure. The method allows the identification of structurally novel compounds, such as designer drug metabolites, that are not present in any compound or spectral database.

Retention time calculation can be used as additional information in differentiating between structural isomers (**III**), which are inseparable by accurate mass determination alone. Within the database used, the ACD/ChromGenius software was most accurate for drugs with a phenethylamine structure. Therefore, the *in silico* t_R prediction can provide valuable information for the differentiation of novel designer drugs.

The software employed in the present thesis can be used to produce information for tentative compound identification when no PRSs are available. The data obtained using these *in silico* systems can further be applied to the toxicology database for accurate mass-based urine drug screening to facilitate compound identification in authentic cases.

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In silico methods for predicting metabolism and mass fragmentation applied to quetiapine in liquid chromatography/time-of-flight mass spectrometry urine drug screening

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Current *in silico* tools were evaluated for their ability to predict metabolism and mass spectral fragmentation in the context of analytical toxicology practice. A metabolite prediction program (Lhasa Meteor), a metabolite detection program (Bruker MetaboliteDetect), and a fragmentation prediction program (ACD/MS Fragmenter) were used to assign phase I metabolites of the antipsychotic drug quetiapine in the liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) accurate mass data from ten autopsy urine samples. In the literature, the main metabolic routes of quetiapine have been reported to be sulfoxidation, oxidation to the corresponding carboxylic acid, *N*- and *O*-dealkylation and hydroxylation. Of the 14 metabolites predicted by Meteor, eight were detected by LC/TOFMS in the urine samples with use of MetaboliteDetect software and manual inspection. An additional five hydroxy derivatives were detected, but not predicted by Meteor. The fragment structures provided by ACD/MS Fragmenter software confirmed the identification of the metabolites. Mean mass accuracy and isotopic pattern match (SigmaFit) values for the fragments were 2.40 ppm (0.62 mDa) and 0.010, respectively. ACD/MS Fragmenter, in particular, allowed metabolites with identical molecular formulae to be differentiated without a need to access the respective reference standards or reference spectra. This was well exemplified with the hydroxy/sulfoxy metabolites of quetiapine and their *N*- and *O*-dealkylated forms. The procedure resulted in assigning 13 quetiapine metabolites in urine. The present approach is instrumental in developing an extensive database containing exact monoisotopic masses and verified retention times of drugs and their urinary metabolites for LC/TOFMS drug screening.

There is growing interest today in the use of accurate mass methods in various fields of small molecule analysis, including pharmaceutical chemistry,[1] drug metabolite research,[2] pesticide monitoring,[3] and analytical toxicology.[4] This is partly due to the fact that current liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) instruments provide a robust and cost-effective means for acquiring accurate masses in complex biological samples on a routine basis. The advantages of modern LC/TOFMS include high speed, good sensitivity, sufficient resolution, and mass accuracy similar to that of more expensive accurate mass instruments.[5]

Our group has for several years been developing automated LC/TOFMS methods for toxicological urine drug screening,[6–8] street drug analysis,[9] and doping control.[10] We have established a screening approach that involves accurate mass measurement in a biological matrix combined with a reverse search based on a large target database of exact monoisotopic masses. Entries in the database, representing the elemental formulae of reference substances and their metabolites, are compared with the measured masses for protonated molecules $[M+H]^+$. Dedicated software has been developed to perform the automated data analysis. After mass scale calibration of the data, extracted ion chromatograms (EICs) are created in a 0.002 m/z mass window for the $[M+H]^+$ ion of each molecular formula included in the database. Peak detection and identification criteria are applied according to mass accuracy, isotopic pattern match (SigmaFit), area and retention time, if available. Lastly, an MS Excel-based result report is created.[8]

Poor accessibility of reference standards for new drugs, designer drugs and metabolites hinders the analysis of these compounds by conventional techniques, which essentially rely on reference standards.[9] In our LC/TOFMS method, the current in-house database for toxicological urine screening includes 830 masses comprising a wide variety of medicinal and illicit substances, whereas retention time information obtained with reference standards is available only for 50% of

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these compounds. Applying known urinary metabolic patterns greatly facilitates substance identification as drug metabolites support the parent drug finding and as a number of compounds are excreted solely as metabolites. Unfortunately, sufficient information on human urinary metabolites does not always exist in the literature, neither for old nor new drugs.

Another analytical challenge concerns compounds with identical molecular formulae as these compounds cannot be differentiated by accurate mass only in the absence of reference standards. Collision-induced dissociation (CID), performed either in-source (ISCID)[3] or preferably in tandem mass analyzers,[11] would allow differentiation of most unresolved combinations, but the interpretation of electrospray mass spectra requires significant expertise, and automated methods would reduce time and labour costs for analytical toxicology laboratories. To meet these challenges, *in silico* tools have been made generally available for predicting both drug metabolism in connection with MS experiments and electrospray MS fragmentation.[12,13]

Generally, drug screening by the LC/TOFMS approach described above is fairly straightforward. However, to fully utilise the information-rich capability of the method, an analyst should be able to assign undetected metabolites, previously known or unknown, in the MS acquisition data related to casework, and further, to include the spectral and chromatographic data for these compounds in the target database to aid identification in other cases. Traditionally, this has only been realisable in the basic research departments of universities and pharmaceutical companies, which are capable of obtaining the appropriate reference standard by synthesis.

The present study examines current representatives of generally available software to assess their potential for predicting metabolism and MS fragmentation in the context of analytical toxicology practice. This includes identification of as many metabolites as possible without the corresponding reference substances in order to use metabolic patterns as supporting information for the parent identification, and to exclude unidentified peaks from the raw data obtained. A metabolite prediction program (Lhasa Meteor), a metabolite detection program (Bruker MetaboliteDetect), and a fragmentation prediction program (ACD/MS Fragmenter) are used to assign phase I metabolites of the antipsychotic drug quetiapine in LC/TOFMS data from autopsy urine samples. Quetiapine was selected as the study compound as it is a common finding in forensic toxicology casework, it is extensively metabolised,[14] and detailed information about the chromatographic behaviour of the metabolites is not available in the literature.

EXPERIMENTAL

Materials

HPLC grade acetonitrile was purchased from Rathburn (Walkerburn, UK) and β-glucuronidase from Roche (Mannheim, Germany). All the other solvents and reagents were of analytical reagent grade from Merck (Darmstadt, Germany). Water was purified with a DirectQ-3 instrument (Millipore, Bedford, MA, USA). Isolute HCX-5 (130 mg, 10 mL) mixed-mode solid-phase extraction (SPE) cartridges were from Biotage (Hengoed, UK). The mixed-mode phase included C-4 carbon chains combined with sulfonic acid functionalities.

Sample preparation

Urine samples were collected at autopsy, and sample preparation was carried out according to the laboratory's routine procedures, as described earlier.[7] Ten successive cases containing quetiapine, based on the LC/TOFMS results, were selected for this study.

Urine samples (1 mL) were hydrolysed with β-glucuronidase for 2 h in a water bath at 56°C. Then, 10 μL of dibenzepin internal standard solution (10 μg/mL in methanol) and 2 mL of pH 6 phosphate buffer were added to the hydrolysed urine sample. The SPE procedure was performed as follows. The SPE cartridges were conditioned with 2 mL of methanol, followed by 2 mL of water and 3 mL of pH 6 phosphate buffer. The sample was added, followed by washing with 1 mL of pH 6 phosphate buffer and drying for 5 min. The cartridge was further washed with 1 mL of 1 M acetic acid and again dried for 5 min. The acidic/neutral fraction was eluted with 3 mL of ethyl acetate/hexane (25:75, v/v), and the cartridge was dried for 2 min. The cartridge was washed with 1 mL of methanol and dried for 2 min. The basic fraction was eluted with 3 mL of freshly made ethyl acetate/ammonia (98:2, v/v), 25% ammonia solution in water. The combined effluents were evaporated to dryness at 40°C, reconstituted with 150 μL of acetonitrile/0.1% formic acid (1:9, v/v) and analysed by LC/TOFMS.

A pooled pseudo-reference urine sample to be used in metabolism studies was prepared as follows. Ten autopsy urine samples from five male and five female cases were hydrolysed and pooled. Based on LC/TOFMS, the samples were either drug-free or contained caffeine and/or nicotine only. Two 1 mL aliquots were prepared as described above. The parallel samples were checked for an identical reference profile by LC/TOFMS, and only one of them was used as the reference sample.

Another ten samples not containing quetiapine were analysed for the ions of the assigned quetiapine metabolites in order to evaluate potential matrix effects, and all of the samples proved to be free from interference.

Liquid chromatography/time-of-flight mass spectrometry

LC/TOFMS analysis was performed as described earlier,[8] with minor modifications. The liquid chromatograph was a 1100 series instrument (Agilent Waldbronn, Germany) including a vacuum degasser, autosampler, binary pump, and column oven. Separation was performed in gradient mode with a Luna C-18(2) 100 × 2 mm (3 μm) column and a 4 × 2 mm pre-column at 40°C (Phenomenex, Torrance, CA, USA). Mobile phase components were 5 mM ammonium acetate in 0.1% formic acid and acetonitrile. The flow rate was 0.3 mL/min. The proportion of acetonitrile was increased from 10% to 40% at 10 min, to 75% at 13.5 min, to 80% at 16 min, and held at 80% for 5 min. The post-time was 6 min and the injection volume 10 μL.

The mass analyser was a Bruker micrOTOF mass spectrometer with an electrospray ionisation (ESI) source

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and a six-port divert valve (Bruker Daltonics, Bremen, Germany). The instrument controls were performed with HyStar 3.2 and micrOTOFcontrol 2.2 software (Bruker Daltonics). The nominal resolution of the instrument was 10 000. The instrument was operated in positive ion mode with m/z range of 50–800. The capillary voltage was 4500 V, the capillary exit was 85 V, and skimmer 1 was 35 V. The nebuliser gas pressure was 1.6 bar, and the drying gas flow was 8 l/min. The drying temperature was 200°C. The spectra average was set to 2, and the summation was 12 000, corresponding to 0.6 s sample time. The transfer time was 40 μs, and the hexapole RF was 45 Vpp.

ISCID was used in the experiments with ACD/MS Fragmenter software. The instrument parameters were as described above, except for the capillary exit and skimmer 1, which were set to 180 and 60 V, respectively.

Software

Meteor 10.0.2 software by Lhasa Ltd. (Leeds, UK) is a knowledge-based expert system for metabolism prediction. In the prediction process, the software utilises structure-metabolism rules stored in the knowledge base, with analysis of the likelihood of a particular prediction based on global lipophilicity-metabolism relations, and selection between potentially competing biotransformations.[15] The metabolic reactions are reported with detailed route information, and the predicted metabolites are categorised as *plausible* or *probable*. Meteor was used in this study to predict quetiapine metabolism. The default values of prediction parameters were used, excluding phase II metabolites, as hydrolysed urine samples were used in this study.

MetaboliteDetect 2.0 by Bruker Daltonics is a metabolite detection program designed for use in metabolism studies for which an authentic blank reference sample is available. The software subtracts the *blank reference sample data* from the *data collected after drug exposure*, referred to hereafter as *reference* and *sample*, respectively. In an ideal case, the subtraction produces a clean result chromatogram containing only a few peaks. Subsequently, the software lists the molecular formulae generated for all peaks detected, the difference between the measured and theoretical mass, and the SigmaFit value. Information on both expected metabolites and other findings, classified as unexpected metabolites, is thus obtained. As the samples in this study were collected at autopsies, authentic blank reference urine samples were not available and therefore a pooled pseudo-reference urine sample was used instead. The main parameters used were as follows: *Calculate Difference* was used at the *Expose* mode with a ratio of 5; *Detect Masses* applied the EIC at the *Simple Peak Detection* mode; *Noise PC* was 2; and *Intensity Threshold* was 30%.

ACD/MS Fragmenter 11.01 by Advanced Chemistry Development (Toronto, Canada) is a new fragmentation prediction program based on the established MS fragmentation rules from the literature. The software generates a tree-structured presentation of the predicted fragments according to the ionisation mode and the number of fragmentation steps selected. When fragments detected in the experimental data are selected from the tree, the software provides detailed information on the routes of fragmentation and all possible structure candidates for a specific mass. The exact masses of the fragments are provided automatically. API positive mode ionisation was selected for the prediction, and the number of fragmentation steps was set to 5.

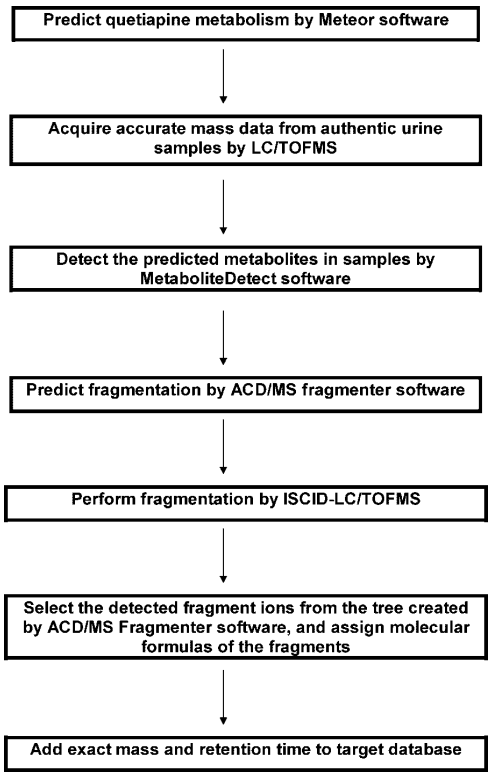
Study design

The study design is presented in Scheme 1.

RESULTS AND DISCUSSION

Prediction of metabolism by Meteor software

Meteor software predicted 14 metabolites of quetiapine overall. All these were categorised as probable, and the plausible ranking was given only to the minor moieties that were cleaved. Of the 14 metabolites, eight were detected by LC/TOFMS in the autopsy urine samples by using MetaboliteDetect software and manual inspection. The main reason for this difference was the fact that Meteor did not predict any hydroxy metabolites of quetiapine. Instead, Meteor suggested further oxidation of sulfoxides to sulfones. In the literature, the main metabolic routes of quetiapine have been reported to be sulfoxidation, oxidation to the corresponding carboxylic acid, *N*- and *O*-dealkylation and hydroxylation.[16] Consequently, the hydroxy metabolites were added manually to the list of metabolites used in the further stages of the study (Table 1, Fig. 1). Four third-stage



Scheme 1. A generalised presentation of the study design.

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Table 1. Quetiapine and 13 metabolites detected by the MetaboliteDetect software in ten autopsy urine samples. All average values were calculated from absolute values

Compound	Molecular formula	$[M+H]^+$	RT min	STDEV min	Mass error average ppm	Mass error average mDa	Sigma Fit average	Detected by Metabolite Detect	Detected manually	Predicted by Meteor
Quetiapine	C21 H25 N3 O2 S	384.1740	11.29	0.03	1.86	0.72	0.007	100%	100%	
M1	C17 H17 N3 S	296.1216	10.95	0.04	1.70	0.50	0.008	90%	100%	yes
M2	C19 H21 N3 O S	340.1478	11.07	0.02	1.78	0.52	0.010	60%	100%	yes
M3 peak 1	C21 H25 N3 O3 S	400.1689	8.68	0.03	2.15	0.96	0.010	80%	100%	yes
M3 peak 2	C21 H25 N3 O3 S	400.1689	8.87	0.02	2.40	0.96	0.011	80%	100%	yes
M4	C21 H25 N3 O3 S	400.1689	6.73	0.02	1.22	0.67	0.011	60%	100%	no
M5	C21 H23 N3 O3 S	398.1533	11.80	0.01	1.65	0.66	0.018	70%	80%	yes
M6	C17 H17 N3 O S	312.1165	4.64	0.09	1.67	0.52	0.010	100%	100%	no
M7 peak 1	C17 H17 N3 O S	312.1165	8.42	0.05	1.24	0.39	0.010	100%	100%	yes
M7 peak 2	C17 H17 N3 O S	312.1165	8.60	0.03	1.56	0.49	0.010	90%	100%	yes
M8	C19 H21 N3 O2 S	356.1427	5.88	0.03	1.93	0.69	0.007	70%	90%	no
M9 peak 1	C19 H21 N3 O2 S	356.1427	8.40	0.02	1.18	0.42	0.009	50%	100%	yes
M9 peak 2	C19 H21 N3 O2 S	356.1427	8.61	0.04	1.36	0.48	0.014	40%	100%	yes
M10	C19 H19 N3 O2 S	354.1271	7.76	0.01	2.23	0.79	0.004	40%	90%	yes
M11 peak 1	C21 H23 N3 O4 S	414.1482	9.31	0.01	1.86	0.77	0.024	80%	80%	yes
M11 peak 2	C21 H23 N3 O4 S	414.1482	9.49	0.01	1.38	0.57	0.021	70%	80%	yes
M12 peak 1	C21 H25 N3 O4 S	416.1639	6.09	0.16	1.91	0.80	0.007	70%	100%	no
M12 peak 2	C21 H25 N3 O4 S	416.1639	6.50	0.02	0.56	0.23	0.027	40%	90%	no
M13	C17 H17 N3 O2 S	328.1114	5.17	0.04	2.21	0.67	0.006	90%	100%	no

metabolites predicted by Meteor were not detected in the samples, probably due to their low concentrations.

The known main metabolites, excluding hydroxylated products, were included in the eight both predicted by Meteor and detected in the samples. The five metabolites not predicted by Meteor were all hydroxy derivatives. Several papers have been published about quetiapine pharmacokinetics, and most of them mention that 11 metabolites have

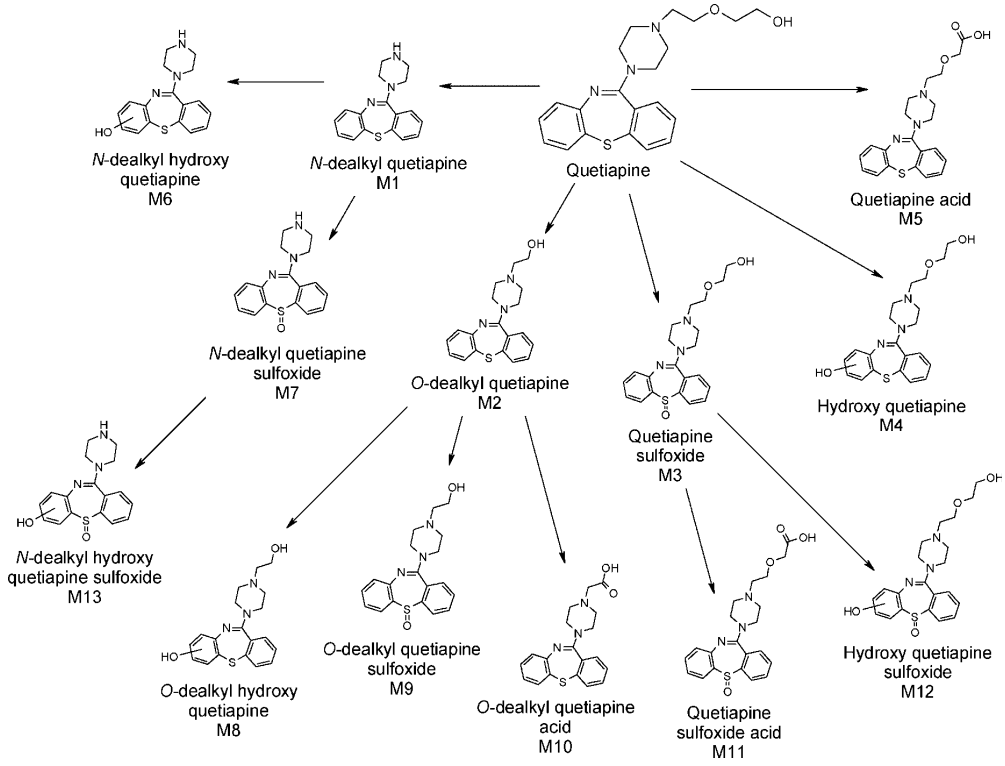

Figure 1. Structures of the 13 metabolites detected and identified in ten autopsy urine samples containing quetiapine. The order of the metabolic reactions may differ from those presented.

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been described.[16–19] However, detailed structures of these metabolites have not been included in the papers nor in the manufacturers' prescription information.[20]

Compared to the manual reasoning of potential metabolic routes and products, Meteor served as a valuable tool, providing molecular structures and exact masses automatically. The predictions made by Meteor were logical, and the categorical distinction between plausible and probable metabolic steps made the interpretation of the results unambiguous. However, the inability of the software to predict one of the main metabolic routes can be considered as a major drawback, and this suggests that predicting metabolism without any previous information would be much more challenging.

Detection of metabolites by MetaboliteDetect software

The predicted and detected metabolites of quetiapine in ten autopsy urine samples are indicated in Table 1, and the corresponding structures are illustrated in Fig. 1. The use of MetaboliteDetect software in this study differed from the procedure suggested by the manufacturer, because authentic

Table 2. The molecular formulae, average mass accuracies and SigmaFit values of the observed fragments

Compound	Molecular formula	$[M+H]^+$	RT	Fragment no	Fragment molecular formula protonated	$[M+H]^+$ theoretical	$[M+H]^+$ Measured	Mass error average ppm	Mass error average mDa	Sigma average
Quetiapine	C21H25N3O2S	384.1740	11.29	1	$[C17H15N2S]^+$	279.0950	279.0951	3.91	1.10	0.006
				2	$[C15H13N2S]^+$	253.0794	253.0793	3.13	0.57	0.008
				3	$[C13H8NS]^+$	210.0372	210.0370	2.60	0.55	0.019
M1	C17H17N3S	296.1216	10.95	1	$[C17H15N2S]^+$	279.0950	279.0947	1.79	0.77	0.009
				2	$[C15H13N2S]^+$	253.0794	253.0783	2.31	0.56	0.012
				3	$[C13H8NS]^+$	210.0372	210.0369	2.05	0.47	0.027
M2	C19H21N3OS	340.1478	11.07	1	$[C17H15N2S]^+$	279.0950	279.0945	2.76	0.50	0.008
				2	$[C15H13N2S]^+$	253.0794	253.0790	2.22	0.58	0.007
				3	$[C13H8NS]^+$	210.0372	210.0369	2.22	0.43	0.024
M3 peak 1	C21H25N3O3S	400.1689	8.68	1	$[C21H26N3O2]^+$	352.2020	352.2015	2.21	0.78	0.009
				2	$[C15H13N2OS]^+$	269.0743	269.0738	2.29	0.62	0.005
				3	$[C17H18N3]^+$	264.1495	264.1491	2.45	0.68	0.007
				4	$[C15H13N2]^+$	221.1073	221.1071	2.18	0.48	0.006
M3 peak 2			8.87	1	$[C21H26N3O2]^+$	352.2020	352.2013	3.12	1.11	0.007
				2	$[C15H13N2OS]^+$	269.0743	269.0739	2.45	0.66	0.004
				3	$[C17H18N3]^+$	264.1495	264.1486	3.70	0.97	0.029
				4	$[C15H13N2]^+$	221.1073	221.1071	2.28	0.51	0.004
M4	C21H25N3O3S	400.1689	6.73	1	$[C17H15N2OS]^+$	295.0900	295.0894	1.97	0.58	0.007
				2	$[C15H13N2OS]^+$	269.0743	269.0738	1.90	0.51	0.008
				3	$[C8H16NO2]^+$	158.1176	158.1167	3.86	0.62	0.035
M5	C21H23N3O3S	398.1533	11.80	1	$[C17H18N3S]^+$	296.1216	296.1211	3.06	0.91	0.019
				2	$[C17H15N2S]^+$	279.0950	279.0946	2.76	0.77	0.007
				3	$[C15H13N2]^+$	253.0794	253.0790	2.31	0.58	0.003
M6	C17H17N3OS	312.1165	4.64	1	$[C15H13N2OS]^+$	269.0743	269.0739	1.72	0.47	0.005
				2	$[C13H8NOS]^+$	226.0321	226.0319	1.39	0.32	0.004
M7 peak 1	C17H17N3OS	312.1165	8.42	1	$[C17H18N3]^+$	264.1495	264.1492	2.29	0.61	0.008
				2	$[C15H13N2]^+$	221.1073	221.1072	2.21	0.48	0.006
				3	$[C13H11N2]^+$	195.0917	195.0911	3.34	0.65	0.004
M7 peak 2			8.60	1	$[C17H18N3]^+$	264.1495	264.1492	2.29	0.61	0.008
				2	$[C15H13N2]^+$	221.1073	221.1071	1.93	0.43	0.006
				3	$[C13H11N2]^+$	195.0917	195.0911	3.21	0.63	0.005
M8	C19H21N3O2S	356.1427	5.88	1	$[C17H18N3O2S]^+$	328.1114	328.1110	1.83	0.60	0.006
				2	$[C15H13N2OS]^+$	269.0743	269.0741	1.71	0.46	0.005
M9 peak 1	C19H21N3O2S	356.1427	8.40	1	$[C19H22N3O]^+$	308.1757	308.1751	2.27	0.70	0.006
				2	$[C17H18N3]^+$	264.1495	264.1492	2.35	0.62	0.007
				3	$[C13H11N2]^+$	195.0917	195.0910	3.31	0.65	0.004
M9 peak 2			8.61	1	$[C19H22N3O]^+$	308.1757	308.1751	2.40	0.74	0.007
				2	$[C17H18N3]^+$	264.1495	264.1491	2.34	0.61	0.008
				3	$[C13H11N2]^+$	195.0917	195.0911	3.18	0.62	0.005
M10	C19H19N3O2S	354.1271	7.76	No identified fragments						
M11 peak 1	C21H2N3O4S	414.1482	9.31	1	$[C21H24N3O3]^+$	366.1812	366.1810	2.07	0.75	0.023
				2	$[C15H13N2OS]^+$	269.0743	269.0734	3.26	0.88	0.005
				3	$[C15H13N2]^+$	221.1073	221.1069	2.23	0.49	0.003
M11 peak 2			9.49	1	$[C21H24N3O3]^+$	366.1812	366.1812	2.09	0.77	0.026
				2	$[C15H13N2OS]^+$	269.0743	269.0738	2.21	0.59	0.006
				3	$[C15H13N2]^+$	221.1073	221.1069	2.57	0.57	0.010
M12 peak 1	C21H25N3O4S	416.1639	6.03	1	$[C21H26N3O3]^+$	368.1969	368.1969	1.08	0.40	0.005
				2	$[C15H13N2O2S]^+$	285.0692	285.0688	1.82	0.52	0.015
M12 peak 2			6.50	1	$[C21H26N3O3]^+$	368.1969	368.1969	1.28	0.47	0.004
				2	$[C15H13N2O2S]^+$	285.0692	285.0693	1.89	0.54	0.034
M13	C17H17N3O2S	328.1114	5.17	1	$[C17H18N3O]^+$	280.1444	280.1438	2.24	0.63	0.008

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blank reference urine samples are not available in post-mortem investigation. A pooled pseudo-reference was used instead, resulting in a chromatogram that did not differ much from the sample chromatogram. This resulted in complicated lists of unexpected metabolites, thus falling outside the scope of this study. However, most of the metabolites predicted by Meteor and the additional hydroxy metabolites (see above) were detected by MetaboliteDetect. The software provided information on retention time, mass accuracy, and isotopic pattern match (SigmaFit) automatically. The use of the pooled pseudo-reference sample was necessary for producing the chromatographic and mass spectral information on a routine basis. In general, the EICs obtained were unambiguous. As hydroxy and sulfoxy metabolites have identical molecular formulae, they could not be differentiated by accurate mass. Based on earlier experience with the chromatographic behaviour of metabolites, we hypothesised that the early eluting peak was the hydroxy metabolite. In addition, all of the sulfoxy metabolites gave a double peak, most likely due to sulfoxide stereochemistry.

Finding the most suitable settings in MetaboliteDetect for optimal results was laborious, and even then some of the metabolites readily detected by manual inspection were missed by the program (Table 1). This was due to the fixed nature of some of the parameters, in particular, the *Mass Spectrum Intensity Threshold* of the *Detect Masses* parameter. This threshold could only be selected as a relative value as a percentage of the base peak. In such instances, where a lot of compounds are co-eluting, even a relatively abundant ion could be rejected due to the relative cut-off. For instance, in the case of the *O*-dealkyl quetiapine sulfoxide (M9) metabolite, the *N*-dealkyl quetiapine sulfoxide (M7) co-eluted. The abundance of the *N*-dealkyl metabolite ions was always much higher, and consequently the relatively abundant *O*-dealkyl metabolite ions were not detected, because their abundances were lower than 30% of the former. This could have been fixed by lowering the value to 5–10%, but doing so resulted in very long and complex results lists; hence the selected 30% was a compromise between detectability and ease of interpretation. An option to select between absolute and relative threshold values would be a desirable addition to the software.

The detection capability of MetaboliteDetect varied from 40% to 100% in the ten urine samples, being better than 70% in 13 of the 18 metabolite peaks detected. Therefore, MetaboliteDetect worked well as a coarse search tool for

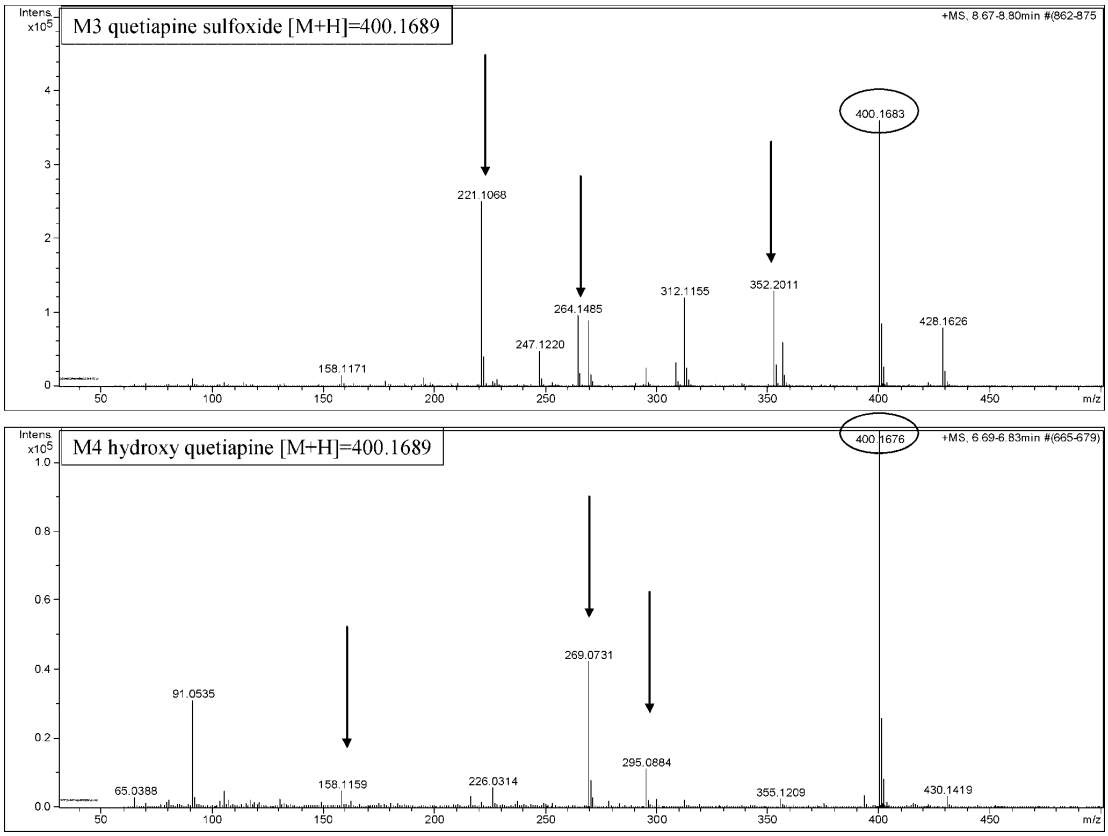

Figure 2. Mass spectra of the two quetiapine metabolites having identical molecular formula. The upper spectrum shows the characteristic fragment ions for the sulfoxide metabolite, corresponding to theoretical masses 352.2020, 264.1495 and 221.1073. The protonated molecule is circled.

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finding metabolites, but it cannot replace manual inspection if more detailed information is required. Hakala and co-workers had similar experience with Waters Metabolynx software.[21]

Identification of metabolites by accurate mass and prediction of fragmentation by ACD/MS Fragmenter software

The approach of substance identification by accurate mass measurement methods combined with isotopic pattern matching has been discussed earlier.[8] Table 1 shows that the previously established identification criteria, based on reproducible retention time (± 0.2 min), mass accuracy below 5 ppm, and isotopic pattern match (SigmaFit) below 0.03, were clearly achieved in this study. The mean standard deviation of retention time was 0.03 min, the mean mass accuracy was 1.68 ppm (0.62 mDa), and the mean SigmaFit value was 0.012.

The fragmentation pattern produced by ACD/MS Fragmenter, typically containing 150–200 suggested fragments, may be too complicated to interpret without experimental data. However, when the fragments generated in the ISCID experiment were selected from the fragment list provided by ACD/MS Fragmenter, the program generated concise information on the following: routes to the specific fragment, molecular structure and accurate mass of the protonated species, and information on neutral loss. The fragments detected and their suggested molecular formulae are listed in Table 2. Mean mass accuracy and SigmaFit values for the fragments were 2.40 ppm (0.62 mDa) and 0.010, respectively. The fragment structures provided by ACD/MS Fragmenter confirmed the metabolites predicted by Meteor. Overall, ACD/MS Fragmenter was found to be an easy-to-use tool for solving fragmentation patterns of quetiapine metabolites.

Metabolites M7 and M9 produced a mixed spectrum due to co-elution. These compounds were sulfoxy metabolites with two fragments in common, representing the cleavage of the sulfoxy (M7, M9) and *N*-alkyl group (M9) (*m/z* 264.1495) and further *N*-dealkylation (*m/z* 195.0917). This did not interfere with identification, as the molecular formulae of the precursor ions were different, but no conclusions could be made about the intensities of the fragments. Furthermore, ion *m/z* 195.0917 was not predicted by ACD/MS Fragmenter for M7, and therefore the source of the ion could not be confirmed. This example illustrates the limitations of the instrument, as only ISCID data were available, and tandem mass spectrometric (MS/MS) capabilities would be preferable for ideal interpretation of the data.

ACD/MS Fragmenter was found to be advantageous in differentiating compounds with identical molecular formulae. For the ions *m/z* 312.1165, 356.1427, and 400.1689, EICs with three separate peaks were obtained, representing the hydroxy/sulfoxy metabolites of quetiapine and their *N*- and *O*-dealkylated forms (metabolites M3, M4, M6, M7, M8, and M9, Fig. 1). The structural elucidation procedures for M3 and M4 are described in detail in the following. The average retention times (RT) obtained for *m/z* 400.1689 were 6.73, 8.68, and 8.87 min (Table 1). The two later eluting peaks had identical fragmentation, suggesting an identical structure (sulfoxy stereochemistry). The peak at RT 6.73 min had a distinctly different fragmentation pattern, as shown in Fig. 2. Analysed by ACD/MS Fragmenter, the fragments were readily identified and connected to specific structures, as shown in Fig. 3. Ion *m/z* 352.2020 resulted from cleavage of a sulfoxy group referring unambiguously to M3. Ion *m/z* 264.1495 resulted from cleavage of sulfoxy and *N*-dealkyl groups of M3. The fragments detected with the hydroxy metabolite were also possible for the sulfoxy metabolite, but they were of low abundance, as the sulfoxy cleavage appeared to dominate the fragmentation. No explicit conclusions about the site of hydroxylation could be made, except that it occurred at the tricyclic part of the molecule. According to the literature, hydroxylation of quetiapine takes place at position 7 of the aromatic ring (Fig. 3).[14] Hence, the peak with RT 6.73 min was assigned as M4, and the peaks with RTs 8.68 and 8.87 min as stereoisomers of M3. The fragmentation schemes of all identified compounds are available as Supporting Information in the online version of this article.

The presence of sulfone metabolites could be excluded, as both of the metabolites M12 and M13, predicted as sulfones by Meteor, had the characteristic fragment from sulfoxy cleavage. For most of the compounds, at least two characteristic fragments were observed repeatedly. For M10 no characteristic fragments could be found, as the peak was a minor one. For M13 only one fragment, resulting from sulfoxy cleavage, could be assigned. Two distinct fragments

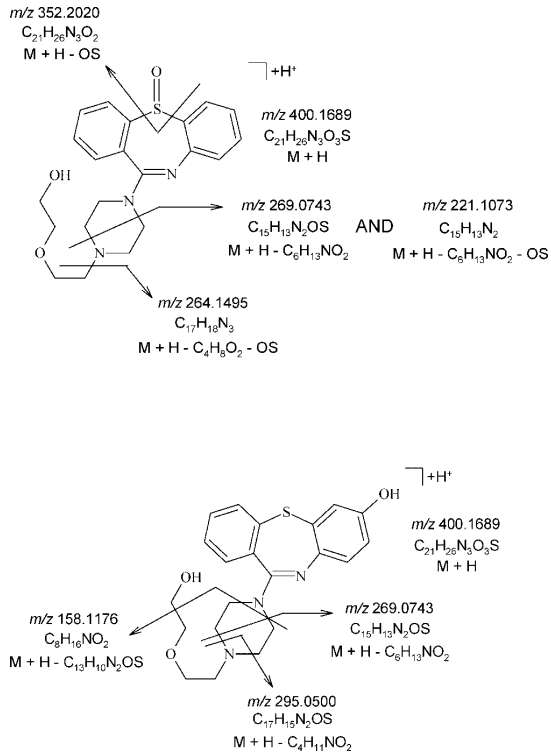

Figure 3. The fragmentation schemes of sulfoxy and hydroxy metabolites of quetiapine (M3 and M4 in the text and Tables 1 and 2) provided by the ACD/MS Fragmenter software.

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detected in experimental data for M3, M7 and M11 were not predicted by ACD/MS Fragmenter. These were combinations of sulfoxy and alkyl cleavages (ions *m/z* 221.1073 and 195.0917). The ions were abundant and the cleavages were logical, and they were thus evaluated as possible (see Figs. 2 and 3). Figure 4 shows a representative total ion chromatogram and a set of EICs with all 13 metabolites identified.

Accurate mass-based screening by LC/TOFMS in analytical toxicology

In analytical toxicology, there is a trend underway to move from triple quadrupole mass spectrometry towards accurate mass alternatives.[22] In addition to our own contributions, several other monitoring methods based on LC/TOFMS have been recently published.[23–25] In an interesting experimental approach, a very large target database (a subset of the PubChem Compound database containing approximately 50 500 compounds) was tested, but this application is still far from being routine.[26] Some authors applied CID or ISCID fragmentation to screen for pesticides[3] and drugs,[11] using corresponding target databases supplemented with fragment ions. These studies utilised reference standards to establish the fragment ions, but did not concentrate on structural elucidation of unknown compounds.

Within drug discovery, metabolic predictions are now an essential part of research.[27] Very recently, Tiller *et al.*,[28]

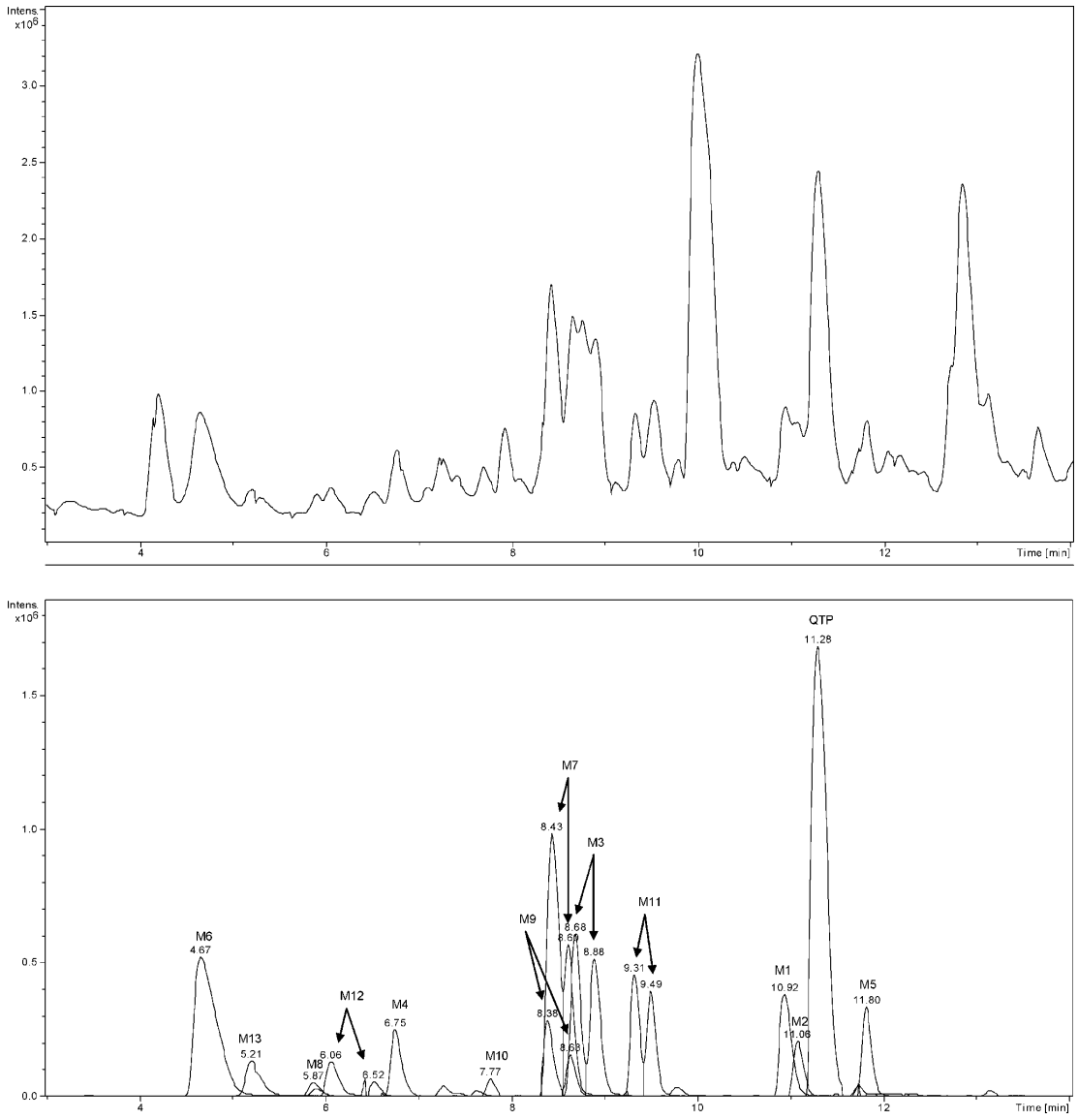

Figure 4. The total ion chromatogram of an autopsy urine sample containing quetiapine (upper), and the set of extracted ion chromatograms showing the 13 detected and identified metabolites (lower).

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utilising MetaboLynx software for predicting metabolism, highlighted the role of current accurate mass MS/MS technology as a 'first-line' high-throughput technique for the detection and characterisation of drug metabolites. Yet, in the field of analytical toxicology, prediction software has been unexplored as of to date. Our present study is pioneering in its use of *in silico* tools together with accurate mass measurement for predicting metabolism and MS fragmentation in the context of drug screening practice. ACD/MS Fragmenter software allowed a problem to be solved that has been much discussed in connection with LC/TOFMS:[29] how to differentiate drugs and metabolites with identical molecular formulae when the respective reference standards or reference spectra are unavailable.

CONCLUSIONS

The aim of the present study was to evaluate the feasibility of current *in silico* methods in predicting, detecting and identifying drug metabolites in the context of toxicological urine drug screening by LC/TOFMS when the respective reference standards are unavailable. The results showed that by applying readily available software to the antipsychotic drug quetiapine, it was possible to assign 13 metabolites in ten quetiapine-positive autopsy urine samples. In particular, the differentiation of metabolites with identical molecular formulae in ISCID experiments by ACD/MS Fragmenter software provided a new powerful instrument for substance identification without reference standards. For the experienced user, the whole procedure of predicting metabolites, assigning the corresponding ions in the LC/TOFMS acquisition data, and adding the spectral and retention time data into the target database, is a task that only takes a few days. Consequently, building up an extensive toxicology database containing exact monoisotopic masses of protonated molecules and verified retention times, with a comprehensive coverage of urinary drug metabolites, is a reasonable approach to be carried out in-house or on an interlaboratory basis.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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II

Research Article

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Differentiation of structural isomers in a target drug database by LC/Q-TOFMS using fragmentation prediction

Elli Tyrkkö,* Anna Pelander and Ilkka Ojanperä

Isomers cannot be differentiated from each other solely based on accurate mass measurement of the compound. A liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOFMS) method was used to systematically fragment a large group of different isomers. Two software programs were used to characterize *in silico* mass fragmentation of compounds in order to identify characteristic fragments. The software programs employed were ACD/MS Fragmenter (ACD Labs Toronto, Canada), which uses general fragmentation rules to generate fragments based on the structure of a compound, and SmartFormula3D (Bruker Daltonics), which assigns fragments from a mass spectra and calculates the molecular formulae for the ions using accurate mass data. From an in-house toxicology database of 874 drug substances, 48 isomer groups comprising 111 compounds, for which a reference standard was available, were found. The product ion spectra were processed with the two software programs and 1–3 fragments were identified for each compound. In 82% of the cases, the fragment could be identified with both software programs. Only 10 isomer pairs could not be differentiated from each other based on their fragments. These compounds were either diastereomers or position isomers undergoing identical fragmentation. Accurate mass data could be utilized with both software programs for structural elucidation of the fragments. Mean mass accuracy and isotopic pattern match values (SigmaFit; Bruker Daltonics Bremen, Germany) were 0.9 mDa and 24.6 mSigma, respectively. The study introduces a practical approach for preliminary compound identification in a large target database by LC/Q-TOFMS without necessarily possessing reference standards. **Copyright © 2010 John Wiley & Sons, Ltd.**

Keywords: structural isomers; drug; liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOFMS); mass fragmentation *in silico*; accurate mass

Introduction

Analytical techniques exploiting accurate mass measurement have become common in the pharmaceutical industry and drug metabolism studies,[1] as well as in analytical toxicology[2] and doping analysis[3] using large target databases. Current liquid chromatograpy/time-of-flight mass spectrometry (LC/TOFMS) instruments are fast, sensitive, and cost-effective in routine laboratory analysis.[4] They provide mass accuracy comparable to more expensive instruments together with moderately high mass resolution, which facilitates the determination of the elemental composition of small molecules.

An analytical challenge with accurate mass-based identification is the differentiation of isomers from each other, as these compounds cannot be differentiated solely based on accurate mass data, although in most cases they can be separated by means of LC. Further structural information can be produced with MS techniques by fragmenting the molecule and identifying the compound characteristic fragments.[5] Several large libraries of electron ionization (EI) reference mass spectra are available for use with gas chromatography-mass spectrometry (GC-MS),[6–8] which makes tentative identification of library compounds fast and convenient. Interpretation of the mass spectra acquired from electrospray ionization (ESI) LC/MS is more challenging, since less fragmentation occurs, and thus less structural information is achieved compared to EI with GC/MS.[9] ESI-MS fragment spectra tend to vary in ion intensities with different instruments,[5,9] and although reference mass spectral libraries for ESI-MS exist, it is not straightforward to exploit the data between different mass analyzers and laboratories without careful standardization of the conditions for compound identification.[10,11]

Both commercially available and in-house built software has been developed to predict *in silico* mass spectral fragmentation in MS analyses. In some programs, such as ACD/MS Fragmenter[12,13] or Mass Frontier,[14,15] the fragment prediction is mainly based on general rules of fragmentation reactions. Non-commercial software that simulates fragmentation and forms a reconstructed mass spectrum based on fragmentation rules includes MASSIS[16] and MASSIMO.[17] The following two software programs for fragment prediction do not rely on the general rules of mass fragmentation, but take into account optimal bond energies in order to predict the most stable fragments and estimate by a validated algorithm the probability of the predicted fragment. Fragment iDentificator (FiD)[18] uses scoring functions to rank competing fragmentation pathways of a molecule that can explain the mass peaks observed in the product ion (MS/MS) spectrum. The algorithm calculates the dissociation energies of the cleaved bonds and estimates the energetic favorability of the alternative fragments. Another recently published algorithm, Density Functional Theory (DFT),[19] calculates the thermodynamically most stable position for the

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protonation in a molecule. This information can be utilized in the prediction of the cleavage site of the molecule.

Mass fragmentation prediction with ACD/MS Fragmenter was successfully used in our previous study[20] for quetiapine metabolism and differentiation of the structurally isomeric metabolites. In that study, in-source collision-induced dissociation (ISCID) with LC/TOFMS was used to produce the fragments, and structural elucidation of the metabolites was done without reference standards. In ISCID analysis, sample background or other co-eluting analytes can interfere with the identification of compound characteristic ions. In the present study, a hybrid quadrupole TOFMS instrument (Q-TOFMS) is used for systematic, reference-standard-based analysis of a large number of different isomeric drugs with the purpose of producing compound characteristic fragments and differentiating the isomers from each other. Two software programs are used to specify mass fragmentation of the compounds *in silico*: one predicting the possible fragments based on the molecular structure of the compound (ACD/MS Fragmenter), and another assigning fragments from mass spectra acquired by MS/MS analysis and calculating the molecular formulae for the ions based on accurate mass measurement (SmartFormula3D).

Experimental Section

Materials

All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany), except the HPLC-grade methanol, which was purchased from Rathburn (Walkerburn, UK). Water was purified with a Millipore DirectQ-3 instrument (Bedford, MA, USA). The selected 111 standards were from several different suppliers.

Sample preparation

Isomeric compounds were searched from an LC/TOFMS in-house toxicology database of 874 substances, for which 462 reference standards were at hand, and 111 compounds were found from these standards. The compounds constituted 48 isomer groups with 2–4 compounds each, with *m/z* ranging from 150.1277 to 387.1559. Sixteen reference standard mixtures were prepared, containing 6–7 of the selected compounds of 1 μg/mL in 0.1% formic acid and methanol (9 : 1). Compounds in the same mixture were known to separate chromatographically.

Liquid chromatography/quadrupole time-of-flight mass spectrometry

The liquid chromatograph was an Agilent 1200 series instrument (Waldbronn, Germany) including a vacuum degasser, autosampler, binary pump, and column oven. Chromatographic separation was performed in gradient mode at 40 °C with Phenomenex Luna PFP(2) 100 × 2 mm (3 μm) column and a PFP 4 × 2.0 mm pre-column (Torrance, CA, USA). Mobile phase components were 2 mM ammonium acetate in 0.1% formic acid and methanol and the flow rate was 0.3 mL/min. The proportion of methanol was increased from 10% to 40% over 5 min, to 75% at 13.50 min, to 80% at 16 min and held at 80% for 4 min. The post-time was 8 min, comprising a total run time of 28 min per sample, and the injection volume was 10 μL.

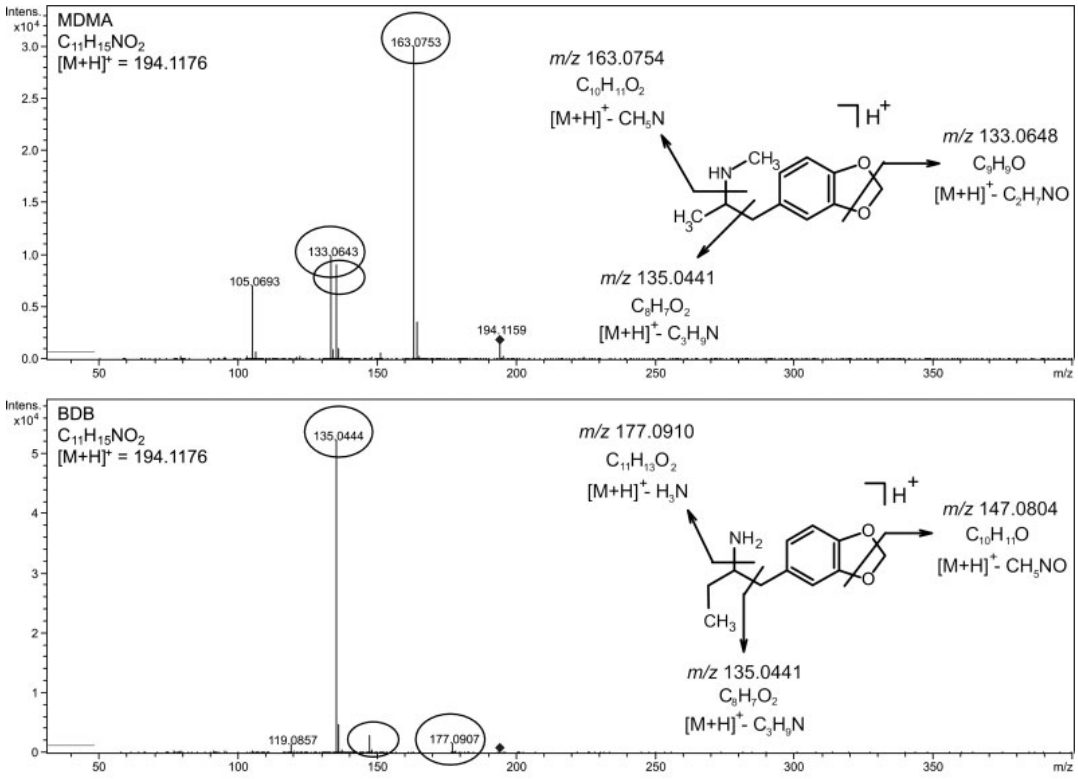
The mass analyzer was a Bruker Daltonics micrOTOF-Q mass spectrometer (Bremen, Germany) with an orthogonal electrospray ionization source and a six-port divert valve. The instrument was operated in positive ion mode with *m/z* range of 50–800. The nominal resolution of the instrument was 10 000 (FWHM). The nebulizer gas pressure was 1.6 bar and the drying gas flow 8.0 L/min. The drying temperature was 200 °C. The capillary voltage of the ion source was set at 4500 V and the end plate offset at −500 V. The quadrupole collision energy in MS mode was 6.0 eV and the collision cell radio-frequency 100.0 Vpp. The quadrupole transfer time was 60.0 μs and pre-pulse storage time 8.0 μs. The spectra rolling average was set at 2 and spectra time 0.6 s. Instrument calibration was performed externally with sodium formate solution, consisting of 10 mM sodium hydroxide in isopropanol and 0.2% formic acid (1 : 1, v/v). Ten sodium formate cluster ions, $(Na(NaCOOH)_{1-10})$ *m/z* values between 90.9766 and 702.8635, were selected for calibrating the instrument. Post-run internal mass scale calibration of individual samples was performed by injecting the calibrant at the beginning and at the end of each sample run. The calibrator ions in the post-run internal mass scale calibration were the same, excluding the ion *m/z* 702.8635, as used in the instrument calibration.

Mass fragmentation was performed in AutoMS(n) mode. When the intensity of the peak crosses the threshold level, the instrument measures every other spectrum in MS/MS mode and the alternate spectrum in MS mode. If several ions overlap, the instrument changes the ion for fragmentation after five spectra (3 s). The collision energy varies depending on the mass of the ion: light molecules are fragmented with less collision energy than heavier ones. Three different AutoMS(n) methods were created: general, high-collision energy and low-collision energy. In the general method, the collision energy for ions between 100 and 600 *m/z* varied linearly from 17 to 48 eV; in high-collision energy, from 22 to 56 eV; and in low-collision energy, from 12 to 36 eV. The absolute intensity threshold level in AutoMS(n) analysis was set at 30 000 cnts. All 16 mixtures were analyzed by the three methods to find out the optimal fragmentation energy for each compound.

Software

DataAnalysis 4.0 software by Bruker Daltonics (Bremen, Germany) was used for post-run internal mass spectrum calibration and further processing of the data acquired in the analyses. An automatic compound finding function of DataAnalysis, AutoMS(n), was used for fast identification of the compounds in the total ion current (TIC) chromatograms. Parameters for AutoMS(n) were determined: the intensity threshold was set at 2500 cnts and the maximum number of compounds to be identified was 250.

A mass spectra processing tool of DataAnalysis, SmartFormula3D, was used for calculating molecular formulae for possible fragments and precursor ions based on their accurate masses and isotope distribution matches, mSigma values. The elements included in the calculations were C, H, N, O, Cl and S. SigmaFit algorithm provides a numerical comparison of theoretical and measured isotopic patterns and can be utilized as an identification tool in addition to accurate mass determination. The calculation of SigmaFit value includes generation of the theoretical isotope pattern for the assumed protonated molecule,[21] and calculation of a match factor based on the deviations of the signal intensities.[22] The lower the mSigma value, the better the isotopic match. SmartFormula3D includes an algorithm that estimates whether a formula for a product ion is a subset of a formula for the precursor ion. It



Precursor MDMA	m/z calculated	error [mDa]	mSigma
C 11 H 16 N 1 O 2	194.117555	-0.56	13.7

Fragment	Intensity	m/z measured	m/z calculated	error [mDa]	mSigma	parity	Loss
C 10 H 11 O 2	29919	163.075328	163.075356	0	4.6	even	C 1 H 5 N 1
C 9 H 11 O 2	557	151.07462	151.075356	0.7	13.4	even	C 2 H 5 N 1
C 9 H 9 O 1	9785	133.064292	133.064791	0.5	5.4	even	C 2 H 7 N 1 O 1
C 8 H 9	7018	105.069337	105.069877	0.5	2	even	C 3 H 7 N 1 O 2

Precursor BDB	m/z calculated	error [mDa]	mSigma
C 11 H 16 N 1 O 2	194.117555	-0.78	11.4

Fragment	Intensity	m/z measured	m/z calculated	error [mDa]	mSigma	parity	Loss
C 11 H 13 O 2	23838	177.090918	177.091006	0.1	6.8	even	H 3 N 1
C 10 H 11 O 1	4941	147.081061	147.080441	-0.6	1.6	even	C 1 H 5 N 1 O 1
C 8 H 7 O 2	46629	135.043977	135.044056	0.1	0.7	even	C 3 H 9 N 1
C 9 H 11	536	119.085435	119.085527	0.1	25.4	even	C 2 H 5 N 1 O 2

Figure 1. Mass spectra and fragmentation schemes of MDMA and BDB ($[M+H]^+ = 194.1176$, $C_{11}H_{15}NO_2$). Results of SmartFormula3D identification are presented in tables automatically formed by the software. The identified characteristic fragments, corresponding to theoretical masses of *m/z* 163.0754, 135.0441 and 133.0648 for MDMA; and *m/z* 177.0910, 147.0804 and 135.0441 for BDB, are circled in the spectra. Possible structures for fragments provided by ACD/MS Fragmenter and SmartFormula3D are represented with arrows.

calculates a formula for the neutral or radical loss and determines if it fits with the observed mass difference for precursor and product ions. Product ions that cannot be related to the precursor ion are omitted; conversely, precursor ions that cannot be composed of any of the product ions are excluded. The precursor and product ion spectra of each compound were processed with the SmartFormula3D program. The mass tolerance for the precursor ion was set at 4 mDa and the isotopic pattern match value at 50 mSigma, and for product ions, 5 mDa and 100 mSigma, respectively. Electron configuration was set even for precursor ions and both even and odd for product ions. SmartFormula3D gives the sum formula, mass error, isotopic pattern match and electron configuration of the precursor and product ions in a chart (Figure 1), which can automatically be transferred to a spreadsheet.

ACD/MS Fragmenter 11.01 from Advanced Chemistry Development (Toronto, Canada) is a rule-based fragmentation prediction software. The program generates a fragmentation scheme for the drawn molecular structure using fragmentation rules of mass spectrometry known in the literature, as well as the selected ionization mode and the number of fragmentation steps. ACD/MS Fragmenter predicts both even and odd electron fragments, and forms a tree-model of all the possible fragments. The software provides information about the routes of fragmentation and all possible structures for a specific mass as well as the exact masses of the fragments. Experimental spectra of each compound were compared to the predicted fragment schemes, and the detected fragments were selected from the tree. The program parameters used in this study were API positive mode ionization, and the number of fragmentation steps was five. The fragmentation reactions were selected to include hetero and homolytic cleavage, neutral losses and hydrogen rearrangements. Other parameters of ACD/MS Fragmenter were left at their default values.

Results and Discussion

The results of SmartFormula3D and ACD/MS Fragmenter for each compound were compared and compound characteristic fragments were identified based on the information achieved from the programs. The most abundant and isomer specific fragment ions in a mass spectrum were selected for each parent compound. Table 1 shows all of the 111 compounds studied, belonging to 48 isomer groups, and the fragmentation data. For each compound, 1–3 fragments were identified, adding up to 305 fragments. In 80% of the cases the total number of identified fragments was three. For six compounds, only one fragment could be identified, due to poor fragmentation (e.g. ropivacaine and metolazone) or because neither of the programs predicted the observed fragments (e.g. chlorcyclizine). Ten isomer pairs could not be differentiated from each other based on fragmentation; however, eight of these pairs could be differentiated with proper chromatographic separation. The compounds, which had similar fragmentation, were either diastereomers (e.g. ephedrine and pseudoephedrine, $[M+H]^+ = 166.1226$; Table 1, isomer group 2), or position isomers (e.g. 2C-T-4 and 2C-T-7, $[M+H]^+ = 256.1366$; Table 1, isomer group 21), where the position of the fragmenting side chain or substituent did not affect the fragments formed in the MSn analysis. The differences in spectra intensities were not used as an identification parameter in this research, because neither of the software predicted the ion abundances. Two isomer pairs, protriptyline and nortriptyline ($[M+H]^+ = 264.1747$; Table 1, isomer group 23), as well as *cis*-3-methylfentanyl and *trans*-3-methylfentanyl ($[M+H]^+ = 351.2431$; Table 1, isomer group 46), could be differentiated from each other neither by chromatography nor by their fragmentation.

From the 305 identified fragments, ACD/MS Fragmenter predicted 89% and SmartFormula3D 93%, while in 82% of cases the identified fragment was predicted by both programs. Only 7% of the fragments were identified solely by ACD/MS Fragmenter and 11% by SmartFormula3D. Of the identified fragments, 89% were formed by even electron neutral losses and 11% by odd electron radical losses. The structure of the identified fragment could not be determined based on SmartFormula3D results alone, because the program does not give structural information, only the sum formula. The validity of the fragment identification based on SmartFormula3D evaluation was ensured with mass accuracy and isotopic pattern match. The reason why ACD/MS Fragmenter and SmartFormula3D did not predict the same fragments in all cases remained unclear. The aim of this study was not to identify all fragments formed in the analysis, but to find the characteristic fragments in order to differentiate isomers from each other.

Both programs exploit accurate mass data in their prediction, which was the key feature in the identification of the compound characteristic fragments. The mean mass accuracy was 0.9 mDa and the mean SigmaFit value 24.6, as calculated from the absolute values of the precursor and fragment ions. Several research articles about the relationship between mass accuracy and ion abundance with Q-TOFMS instrument have been published.[23,24] Both mass accuracy and isotopic pattern match values are dependent on the ion abundance and show reduced match values when ion abundance is very low (<1000) or high ($>1 \times 10^6$). The same feature was seen in the present study and was taken into account when identifying precursor ions and fragments with SmartFormula3D. In some occasions the identification parameters had to be extended as high as 250 mSigma (ketobemidone, hydrocodone and milnacipran) to enable the identification of the parent compound or an obvious fragment structure predicted by the ACD/MS Fragmenter. A poor SigmaFit value of fragments did not always arise from high or low ion abundance. An extensively fragmenting molecule can have fragments differing only 2 Da from each other, resulting in the overlap of the isotopes, which may cause errors in isotopic pattern match measurement. That is why a good mass accuracy could be achieved, although the SigmaFit value did not fulfill the identification criteria (Table 1), and thus SigmaFit values higher than 200 were left out of the calculations.

Differentiation of isomers is presented here in detail by three examples of different isomer groups: MDMA and BDB; histapyrrodine, imipramine and nortrimipramine; and cocaine and scopolamine (Figures 1–3).

Methylenedioxymethamphetamine (MDMA, Ecstacy) and 1,3-benzodioxolylbutanamine (BDB), sharing a molecular formula of $C_{11}H_{15}NO_2$ and $[M+H]^+$ 194.1176, are structurally very similar compounds (Figure 1, isomer group 8 in Table 1). The only difference in their structure is the position of one methyl group. MDMA and BDB are chromatographically well-separated (Rt 7.18 min and 8.38 min, respectively), and their individual mass spectra are visually dissimilar. Both molecules undergo fragmentation of the amine group, which leads to fragments *m/z* 163.0754 for MDMA ($[M+H]^+ - CH_5N$) and *m/z* 177.0910 for BDB ($[M+H]^+ - H_3N$). The fragments *m/z* 133.0648 for MDMA and *m/z* 147.0804 for BDB are formed as a summation of the cleavage of amino group and the breakage of the methylenedioxy ring. MDMA and BDB share one common fragment, *m/z* 135.0441, which forms in the cleavage of the aliphatic side chain ($[M+H]^+ - C_3H_9N$). SmartFormula3D did not identify the ion *m/z* 135.0441 with the selected software parameters to be cleaved from MDMA, although the mass accuracy and isotopic pattern match were within the identification criteria (Figure 1, Table 1) for that ion. The fragmentation reaction, from which the fragment *m/z* 135.0441 would be formed, is in congruence with the reactions of other compounds with similar structure, e.g. BDB, MDDMA, MDEA and MBDB, for which SmartFormula3D identified the fragment *m/z* 135.0441 correctly. All other fragments were predicted by both software programs and they were even electron neutral losses.

Table 1. Molecular formula, mass accuracy and isotopic pattern match for observed fragments of 111 compounds belonging to 48 isomer groups, listed by increasing molecular mass of precursor ions

	Mass $[M+H]^+$	Rt min	Molecular formula	Compound	Frag. 1 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 2 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 3 *m/z*	Error mDa	mSigma	Neutral or radical loss
1	150.1277	6.12	$C_{10}H_{15}N$	Methamphetamine	119.0855	−0.1	2.7	CH_5N	91.0542	−0.1	1.0	C_3H_9N				
	150.1277	7.27	$C_{10}H_{15}N$	Phentermine	133.1012	0.7	5.2	H_3N	91.0542	0.0	2.4	C_3H_9N				
2	166.1226	3.54	$C_{10}H_{15}NO$	Ephedrine[a]	148.1121	−0.4	1.0	H_2O	133.0886	1.1	4.3	CH_5O	117.0699	0.7	7.1	CH_7NO
	166.1226	3.97	$C_{10}H_{15}NO$	Pseudoephedrine[a]	148.1121	−0.6	1.3	H_2O	133.0886	0.7	3.4	CH_5O	117.0699	0.3	16.4	CH_7NO
	166.1226	7.08	$C_{10}H_{15}NO$	PMA	149.0961	−0.4	8.5	H_3N	121.0648	0.4	9.6	C_2H_7N				
3	178.1226	5.72	$C_{11}H_{15}NO$	Ethylcathinone	160.1121	−0.7	6.9	H_2O	132.0808	0.6	7.7	C_2H_6O				
	178.1226	6.08	$C_{11}H_{15}NO$	Phenmetrazine	160.1121	−0.2	1.9	H_2O	134.0964	0.7	6.9	C_2H_4O	117.0699	0.0	96.3	C_2H_7NO
	178.1226	7.66	$C_{11}H_{15}NO$	4-MMC	160.1121	0.1	4.6	H_2O	145.0886	−0.5	18.6	CH_5O	119.0855	0.5	1.3	C_2H_5NO
4	180.1019	6.66	$C_{10}H_{13}NO_2$	MDA	163.0754	0.1	7.1	H_3N	135.0441	0.8	3.5	C_2H_7N	133.0648	0.6	13.1	CH_5NO
	180.1019	12.08	$C_{10}H_{13}NO_2$	Phenacetin	152.0706	0.0	1.9	C_2H_4	138.0913	0.7	6.4	C_2H_2O	110.0600	0.1	2.6	C_4H_6O
5	180.1383	4.36	$C_{11}H_{17}NO$	Methylephedrine	162.1277	−0.3	5.4	H_2O	147.1043	−0.8	0.8	CH_5O	117.0699	0.1	9.1	C_2H_9NO
	180.1383	7.59	$C_{11}H_{17}NO$	PMMA[b]	149.0961	−0.4	4.2	CH_5N	121.0648	0.4	5.5	C_3H_9N				
	180.1383	8.40	$C_{11}H_{17}NO$	Methoxyphenamine[b]	149.0961	−0.8	1.5	CH_5N	121.0648	−0.2	2.8	C_3H_9N				
	180.1383	9.84	$C_{11}H_{17}NO$	Mexiletine	163.1117	−0.6	31.1	H_3N	121.0648	0.9	93.6	C_3H_9N	105.0699	0.9	30.0	C_3H_9NO
6	181.0720	5.63	$C_7H_8N_4O_2$	Theobromine	163.0614	1.0	7.2	H_2O	138.0662	0.1	10.5	CHNO				
	181.0720	7.51	$C_7H_8N_4O_2$	Theophylline	124.0505	0.3	4.9	C_2H_3NO								
7	182.1176	1.90	$C_{10}H_{15}NO_2$	Etilefrine	164.1070	0.0	6.1	H_2O	135.0679	0.2	>200	C_2H_7O				
	182.1176	1.97	$C_{10}H_{15}NO_2$	HHMA	151.0754	−0.5	0.2	CH_5N	133.0648	0.7	5.5	CH_7NO	123.0441	0.2	3.5	C_3H_9N
	182.1176	3.61	$C_{10}H_{15}NO_2$	HMA	165.0910	0.2	4.6	H_3N	137.0597	0.9	7.0	C_2H_7N	133.0648	0.4	3.8	CH_7NO
	182.1176	7.84	$C_{10}H_{15}NO_2$	2-CH	165.0910	0.0	2.0	H_3N	150.0675	−0.9	5.6	CH_6N	105.0699	0.7	5.1	$C_2H_7NO_2$
8	194.1176	7.18	$C_{11}H_{15}NO_2$	MDMA	163.0754	0.0	4.6	CH_5N	135.0441	0.4	8.5	C_3H_9N	133.0648	0.5	5.4	C_2H_7NO
	194.1176	8.38	$C_{11}H_{15}NO_2$	BDB	177.0910	0.1	6.8	H_3N	147.0804	−0.6	1.6	CH_5NO	135.0441	0.1	0.7	C_3H_9N
9	196.1332	4.01	$C_{11}H_{17}NO_2$	HMMA	165.0910	0.1	1.4	CH_5N	137.0597	0.8	1.0	C_3H_9N	133.0648	0.8	6.7	C_2H_9NO
	196.1332	7.04	$C_{11}H_{17}NO_2$	3,4-DMA[b]	179.1067	0.1	4.1	H_3N	164.0862	0.6	1.0	CH_6N	151.0754	−0.6	1.0	C_2H_7N
	196.1332	8.89	$C_{11}H_{17}NO_2$	2,5-DMA[b]	179.1067	0.0	4.1	H_3N	164.0862	−0.1	3.3	CH_6N	151.0754	−0.8	0.3	C_2H_7N
10	205.1335	5.34	$C_{12}H_{16}N_2O$	Psilocin	160.0757	0.2	1.8	C_2H_7N	132.0808	0.8	15.6	C_3H_7NO				
	205.1335	8.71	$C_{12}H_{16}N_2O$	5-MeO-AMT	188.1070	0.7	3.1	H_2N	173.0835	0.3	6.6	CH_6N	147.0679	−0.2	5.4	C_3H_8N
11	208.1332	7.36	$C_{12}H_{17}NO_2$	MDDMA[b]	163.0754	−0.3	2.0	C_2H_7N	135.0441	0.5	3.1	$C_4H_{11}N$	133.0648	0.5	3.9	C_3H_9NO
	208.1332	8.01	$C_{12}H_{17}NO_2$	MDEA[b]	163.0754	−0.4	2.4	C_2H_7N	135.0441	0.1	5.4	$C_4H_{11}N$	133.0648	0.1	6.1	C_3H_9NO
	208.1332	8.64	$C_{12}H_{17}NO_2$	MBDB	177.0910	0.2	9.1	CH_5N	147.0804	−0.2	7.0	C_2H_7NO	135.0441	−0.2	4.1	$C_4H_{11}N$
12	210.1489	10.80	$C_{12}H_{19}NO_2$	DOM	193.1223	0.1	6.5	H_3N	178.0988	−0.1	2.5	CH_6N	165.0910	−0.2	3.9	C_2H_7N
	210.1489	11.55	$C_{12}H_{19}NO_2$	2-CE	193.1223	−0.1	2.4	H_3N	178.0988	−0.2	3.6	CH_6N	135.0804	0.4	15.8	C_3H_9NO
13	226.1438	4.17	$C_{12}H_{19}NO_3$	Terbutaline	152.0706	−0.4	2.9	$C_4H_{10}O$	135.0441	−1.7	1.5	$C_4H_{13}NO$	125.0597	0.6	8.8	$C_5H_{11}NO$
	226.1438	7.75	$C_{12}H_{19}NO_3$	3,4,5-TMA	209.1172	0.7	1.5	H_3N	194.0937	0.5	1.8	CH_6N	181.0859	0.7	4.0	C_2H_7N

Table 1. (*Continued*)

	Mass $[M+H]^+$	Rt min	Molecular formula	Compound	Frag. 1 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 2 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 3 *m/z*	Error mDa	mSigma	Neutral or radical loss
14	236.1645	6.62	$C_{14}H_{21}NO_2$	*O*-desmethylnortramadol	218.1539	0.1	15.4	H_2O	187.1117	0.6	32.0	CH_7NO				
	236.1645	9.16	$C_{14}H_{21}NO_2$	Dinortramadol	218.1539	0.0	1.9	H_2O	189.1274	0.0	9.2	CH_5NO	121.0648	0.4	1.4	$C_6H_{13}NO$
15	237.1598	4.43	$C_{13}H_{20}N_2O_2$	Procaine	164.0706	0.1	3.0	$C_4H_{11}N$	120.0444	0.2	4.7	$C_6H_{15}NO$	100.1121	0.3	1.2	$C_7H_7NO_2$
	237.1598	4.81	$C_{13}H_{20}N_2O_2$	Dropropizine	175.1230	0.6	4.4	$C_2H_6O_2$	160.0995	0.2	16.6	$C_3H_9O_2$	132.0808	0.2	25.2	$C_4H_{11}NO_2$
16	245.2012	10.90	$C_{16}H_{24}N_2$	DPT	144.0808	−1.1	7.2	$C_6H_{15}N$	114.1277	−0.4	3.3	C_9H_9N				
	245.2012	13.24	$C_{16}H_{24}N_2$	Xylometazoline	230.1778	0.4	13.9	CH_3	175.1481	0.6	15.8	$C_3H_6N_2$	161.1325	0.6	1.8	$C_4H_8N_2$
17	247.1805	7.77	$C_{15}H_{22}N_2O$	Mepivacaine	98.0964	−0.1	1.8	$C_9H_{11}NO$								
	247.1805	9.39	$C_{15}H_{22}N_2O$	5-MeO-MIPT	174.0913	−0.9	4.9	$C_4H_{11}N$	159.0679	−0.1	3.6	$C_5H_{14}N$	86.0964	0.8	2.4	$C_{10}H_{11}NO$
	247.1805	10.51	$C_{15}H_{22}N_2O$	Milnacipran	230.1539	−0.9	7.5	H_3N	129.0699	0.3	>200	$C_5H_{14}N_2O$	100.0757	−0.1	2.0	$C_{10}H_{13}N$
18	248.1645	7.56	$C_{15}H_{21}NO_2$	Ketobemidone	230.1539	−0.6	9.5	H_2O	201.1148	−0.2	52.8	C_2H_7O	190.1226	0.3	>200	C_3H_6O
	248.1645	9.67	$C_{15}H_{21}NO_2$	Pethidine	220.1332	−1.0	7.4	C_2H_4	202.1226	0.4	4.3	C_2H_6O	174.1277	0.0	20.9	$C_3H_6O_2$
19	250.1802	6.72	$C_{15}H_{23}NO_2$	*O*-desmethyltramadol	232.1696	1.4	93.5	H_2O	187.1117	1.7	54.1	C_2H_9NO	58.0651	1.5	20.9	$C_{12}H_{16}O_2$
	250.1802	9.38	$C_{15}H_{23}NO_2$	Nortramadol	232.1696	3.6	19.6	H_2O	201.1274	1.0	36.2	CH_7NO	189.1274	1.4	5.6	C_2H_7NO
	250.1802	12.23	$C_{15}H_{23}NO_2$	Alprenolol	208.1332	0.5	5.6	C_3H_6	173.0961	0.3	6.1	$C_3H_{11}NO$	116.1070	0.5	38.3	$C_9H_{10}O$
20	253.1366	12.73	$C_{15}H_{12}N_2O_2$	Oxcarbazepine	236.0706	0.0	10.9	H_3N	208.0757	−0.2	157.1	CH_3NO	180.0808	0.6	183.0	$C_2H_3NO_2$
	253.1366	13.38	$C_{15}H_{12}N_2O_2$	Phenytoin	182.0964	−0.1	4.2	C_2HNO_2								
21	256.1366	12.32	$C_{13}H_{21}NO_2S$	2C-T-4[b]	239.1100	−0.7	8.5	H_3N	224.0866	−0.3	7.5	CH_6N	197.0631	−0.8	9.5	C_3H_9N
	256.1366	12.82	$C_{13}H_{21}NO_2S$	2C-T-7[b]	239.1100	−0.2	9.8	H_3N	224.0866	0.3	7.4	CH_6N	197.0631	0.3	10.9	C_3H_9N
22	256.1696	11.86	$C_{17}H_{21}NO$	Diphenhydramine	167.0855	−1.7	6.9	$C_4H_{11}NO$	152.0621	−0.5	0.7	$C_5H_{14}NO$				
	256.1696	12.94	$C_{17}H_{21}NO$	Nororphenadrine	181.1012	−0.4	8.6	C_3H_9NO	166.0777	−0.1	11.8	$C_4H_{12}NO$	153.0699	−0.2	2.4	$C_5H_{13}NO$
23	264.1747	14.11	$C_{19}H_{21}N$	Protriptyline[b]	233.1325	−0.7	10.3	CH_5N	191.0855	−0.6	14.5	$C_4H_{11}N$	155.0855	−0.5	2.2	$C_7H_{11}N$
	264.1747	14.24	$C_{19}H_{21}N$	Nortriptyline[b]	233.1325	−0.7	7.3	CH_5N	191.0855	−0.2	74.2	$C_4H_{11}N$	155.0855	−0.2	10.7	$C_7H_{11}N$
24	264.1958	8.26	$C_{16}H_{25}NO_2$	*O*-desmethylvenlafaxine	246.1852	0.1	4.5	H_2O	201.1274	0.9	1.8	C_2H_9NO	133.0648	0.8	4.4	$C_7H_{17}NO$
	264.1958	9.10	$C_{16}H_{25}NO_2$	Tramadol	246.1852	0.8	43.2	H_2O	201.1274	0.0	7.5	C_2H_9NO	58.0651	2.1	6.7	$C_{13}H_{18}O_2$
	264.1958	10.59	$C_{16}H_{25}NO_2$	Norvenlafaxine	246.1852	−0.4	21.8	H_2O	215.1430	−0.2	6.4	CH_7NO	121.0648	0.0	9.7	$C_8H_{17}NO$
25	266.1652	9.13	$C_{17}H_{19}N_3$	Mirtazapine	235.1230	−0.3	37.8	CH_5N	209.1073	−0.3	12.1	C_3H_7N	195.0917	−1.4	1.4	C_4H_9N
	266.1652	11.36	$C_{17}H_{19}N_3$	Antazoline	196.1121	−0.4	4.5	$C_3H_6N_2$	175.1104	0.7	3.0	C_7H_7	91.0542	−0.3	1.8	$C_{10}H_{13}N_3$
26	267.1703	4.12	$C_{14}H_{22}N_2O_3$	Atenolol	190.0863	0.5	3.5	$C_3H_{11}NO$	178.0863	0.7	191.9	$C_4H_{11}NO$	145.0648	0.0	8.5	$C_4H_{14}N_2O_2$
	267.1703	5.65	$C_{14}H_{22}N_2O_3$	Practolol	225.1234	0.2	2.6	C_3H_6	190.0863	0.1	2.0	$C_3H_{11}NO$	178.0863	0.1	4.7	$C_4H_{11}NO$
27	267.1856	12.25	$C_{18}H_{22}N_2$	Cyclizine	167.0855	−1.0	2.0	$C_5H_{12}N_2$	152.0621	−0.5	1.3	$C_6H_{15}N_2$				
	267.1856	14.00	$C_{18}H_{22}N_2$	Desipramine	236.1434	−0.3	4.8	CH_5N	208.1121	0.0	7.5	C_3H_9N	72.0808	1.8	0.6	$C_{14}H_{13}N$
28	268.1696	10.13	$C_{18}H_{21}NO$	Pipradrol	250.1590	−1.4	8.5	H_2O	172.1121	0.1	41.1	C_6H_8O	167.0855	0.1	3.9	$C_5H_{11}NO$
	268.1696	10.88	$C_{18}H_{21}NO$	Azacyclonol	250.1590	−0.2	0.4	H_2O	167.0855	0.5	1.0	$C_5H_{11}NO$	143.0855	0	21.8	$C_7H_{11}NO$

Table 1. (*Continued*)

	Mass $[M+H]^+$	Rt min	Molecular formula	Compound	Frag. 1 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 2 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 3 *m/z*	Error mDa	mSigma	Neutral or radical loss
29	275.2118	9.75	$C_{17}H_{26}N_2O$	Ropivacaine	126.1277	−0.7	2.1	$C_9H_{11}NO$								
	275.2118	10.71	$C_{17}H_{26}N_2O$	5-MeO-DIPT	174.0913	−0.8	3.8	$C_6H_{15}N$	159.0679	−0.1	1.8	$C_7H_{18}N$	114.1277	0.1	1.9	$C_{10}H_{11}NO$
30	278.1903	11.84	$C_{20}H_{23}N$	EDDP	249.1512	−0.7	6.6	C_2H_5	234.1277	−0.3	11.2	C_3H_8	186.1277	0.5	17.1	C_7H_8
	278.1903	13.67	$C_{20}H_{23}N$	Maprotiline	250.1590	−1.2	5.0	C_2H_4	219.1168	−0.2	8.4	C_3H_9N	191.0855	0.2	176.6	$C_5H_{13}N$
	278.1903	14.20	$C_{20}H_{23}N$	Amitriptyline	233.1325	−1.0	7.0	C_2H_7N	205.1012	−0.1	82.4	$C_4H_{11}N$	155.0855	−0.2	7.9	$C_8H_{13}N$
31	280.1696	10.75	$C_{19}H_{21}NO$	*E*-10-hydroxynortriptyline[a]	262.1590	−0.6	1.0	H_2O	231.1168	−0.9	8.9	CH_7NO	216.0934	−0.5	13.1	C_2H_9NO
	280.1696	11.97	$C_{19}H_{21}NO$	*Z*-10-hydroxynortriptyline[a]	262.1590	−0.9	8.0	H_2O	231.1168	−0.7	11.5	CH_7NO	216.0934	−0.6	16.5	C_2H_9NO
	280.1696	12.85	$C_{19}H_{21}NO$	Doxepin	235.1117	−0.5	8.9	C_2H_7N	217.1012	−0.3	41.9	C_2H_9NO	107.0491	−0.3	1.9	$C_{12}H_{15}N$
32	281.2012	12.81	$C_{19}H_{24}N_2$	Histapyrrodine	210.1277	−0.3	1.0	C_4H_9N	132.0808	1.0	18.4	$C_{10}H_{15}N$	98.0964	−0.3	1.3	$C_{13}H_{13}N$
	281.2012	13.99	$C_{19}H_{24}N_2$	Imipramine	236.1434	−0.4	1.5	C_2H_7N	208.1121	−0.2	4.0	$C_4H_{11}N$	86.0964	0.8	1.0	$C_{14}H_{13}N$
	281.2012	14.48	$C_{19}H_{24}N_2$	Nortrimipramine	250.1590	−0.2	3.4	CH_5N	208.1121	−0.4	0.7	$C_4H_{11}N$	196.1121	0.4	4.9	$C_5H_{11}N$
33	285.1420	13.20	$C_{17}H_{20}N_2S$	Promethazine	240.0841	−1.0	2.8	C_2H_7N	198.0372	−0.8	5.6	$C_5H_{13}N$	86.0964	0.7	1.2	$C_{12}H_9NS$
	285.1420	13.82	$C_{17}H_{20}N_2S$	Promazine	240.0841	−0.2	6.7	C_2H_7N	212.0528	−0.1	3.5	$C_4H_{11}N$	86.0964	0.8	1.9	$C_{12}H_9NS$
34	286.1438	3.02	$C_{17}H_{19}NO_3$	Hydromorphone	227.0703	0.9	183.0	C_3H_9N	185.0597	−0.1	10.2	$C_5H_{11}NO$				
	286.1438	1.97	$C_{17}H_{19}NO_3$	Morphine	229.0859	0.0	6.2	C_3H_7N	211.0754	0.1	25.9	C_3H_9NO	201.0910	0.7	11.8	C_4H_7NO
	286.1438	4.67	$C_{17}H_{19}NO_3$	Norcodeine	268.1332	−0.8	31.5	H_2O	225.0910	0.4	70.7	C_2H_7NO	215.1067	1.5	49.4	C_3H_5NO
35	287.0582	13.34	$C_{15}H_{11}N_2O_2Cl$	Demoxepam	269.0476	−0.9	>200	H_2O	241.0527	0.0	>200	CH_2O_2	179.9847	−0.5	15.6	C_7H_9N
	287.0582	14.66	$C_{15}H_{11}N_2O_2Cl$	Oxazepam	269.0476	−0.4	5.2	H_2O	241.0527	−0.5	32.8	CH_2O_3	231.0684	−0.1	26.1	C_2O_2
36	294.1852	10.78	$C_{20}H_{23}NO$	*E*-10-hydroxyamitriptyline[a]	276.1747	−1.6	1.6	H_2O	231.1168	−1.1	21.1	C_2H_9NO	216.0934	−0.7	11.6	$C_3H_{12}NO$
	294.1852	11.98	$C_{20}H_{23}NO$	*Z*-10-hydroxyamitriptyline[a]	276.1747	−1.4	1.2	H_2O	231.1168	−1.0	48.0	C_2H_9NO	216.0934	−0.5	14.7	$C_3H_{12}NO$
37	300.1594	5.19	$C_{18}H_{21}NO_3$	Codeine	243.1016	−0.3	2.8	C_3H_7N	225.0910	0.4	33.1	C_3H_9NO	215.1067	0.2	5.0	C_4H_7NO
	300.1594	6.68	$C_{18}H_{21}NO_3$	Hydrocodone	243.1016	−0.7	5.5	C_3H_7N	241.0859	0.9	>200	C_3H_9N	213.0910	−0.1	77.5	C_4H_9NO
38	301.0738	14.89	$C_{16}H_{13}N_2O_2Cl$	Clobazam	259.0633	−1.3	6.7	C_2H_2O	224.0944	0.0	5.8	C_2H_2ClO				
	301.0738	15.38	$C_{16}H_{13}N_2O_2Cl$	Temazepam	255.0684	−1.1	14.3	CH_2O_2	228.0575	−0.2	26.9	$C_2H_3NO_2$				
39	301.1466	14.13	$C_{18}H_{21}N_2Cl$	Chlorcyclizine	201.0466	−0.2	10.8	$C_5H_{12}N_2$								
	301.1466	15.53	$C_{18}H_{21}N_2Cl$	Norclomipramine	270.1044	−1.2	1.9	CH_5N	242.0731	−1.1	13.1	C_3H_9N	72.0808	1.5	2.1	$C_{14}H_{12}NCl$
40	302.1387	2.49	$C_{17}H_{19}NO_4$	Oxymorphone	284.1281	−1.1	5.6	H_2O	242.1176	0.0	1.7	$C_2H_4O_2$	227.0941	1.3	18.2	$C_3H_7O_2$
	302.1387	6.25	$C_{17}H_{19}NO_4$	Noroxycodone	284.1281	0.0	3.2	H_2O	227.0941	−0.1	70.3	$C_3H_7O_2$	187.0754	0.4	46.6	$C_5H_9NO_2$
41	302.1751	5.08	$C_{18}H_{23}NO_3$	Dihydrocodeine	245.1172	0.0	3.6	C_3H_7N	227.1067	0.0	17.3	C_3H_9NO	201.0910	0.2	15.9	$C_5H_{11}NO$
	302.1751	10.44	$C_{18}H_{23}NO_3$	Isoxsuprine	284.1645	−1.8	1.8	H_2O	150.0913	−0.7	34.8	$C_9H_{12}O_2$	135.0804	0.6	3.8	$C_9H_{13}NO_2$
42	304.1543	6.00	$C_{17}H_{21}NO_4$	Scopolamine	156.1019	−0.3	9.1	$C_9H_8O_2$	138.0913	−0.1	4.6	$C_9H_{10}O_3$	121.0648	0.4	14.3	$C_9H_{13}NO_3$
	304.1543	9.58	$C_{17}H_{21}NO_4$	Cocaine	182.1176	−0.5	2.3	$C_7H_6O_2$	150.0913	−0.5	11.1	$C_8H_{10}O_3$	105.0335	−0.6	9.0	$C_{10}H_{17}NO_3$

Table 1. (*Continued*)

	Mass $[M+H]^+$	Rt min	Molecular formula	Compound	Frag. 1 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 2 *m/z*	Error mDa	mSigma	Neutral or radical loss	Frag. 3 *m/z*	Error mDa	mSigma	Neutral or radical loss
43	314.1751	7.33	$C_{19}H_{23}NO_3$	Ethylmorphine	257.1172	0.0	4.4	C_3H_7N	239.1067	0.9	21.1	C_3H_9NO	229.1223	2.3	4.2	C_4H_7NO
	314.1751	12.57	$C_{19}H_{23}NO_3$	Reboxetine	176.1070	0.8	3.7	$C_8H_{10}O_2$	158.0964	0.7	11.1	$C_8H_{12}O_3$	91.0542	0.1	4.3	$C_{12}H_{17}NO_3$
44	325.1911	9.38	$C_{20}H_{24}N_2O_2$	Quinidine[a]	307.1805	−1.3	6.7	H_2O	253.1335	−0.1	16.8	C_4H_8O	160.0757	−0.1	37.8	$C_{10}H_{15}NO$
	325.1911	9.72	$C_{20}H_{24}N_2O_2$	Quinine[a]	307.1805	−1.2	3.5	H_2O	253.1335	−0.4	17.6	C_4H_8O	160.0757	−0.1	41.4	$C_{10}H_{15}NO$
45	328.1543	5.56	$C_{19}H_{21}NO_4$	Naloxone	310.1438	0.8	7.9	H_2O	268.1332	1.2	167.9	$C_2H_4O_2$	253.1097	0.1	33.3	$C_3H_7O_2$
	328.1543	6.75	$C_{19}H_{21}NO_4$	6-MAM	268.1332	−0.4	41.3	$C_2H_4O_2$	211.0754	0.3	6.0	$C_5H_{11}NO_2$	193.0648	−1.3	25.9	$C_5H_{13}NO_3$
46	351.2431	12.30	$C_{23}H_{30}N_2O$	*cis*-3-methylfentanyl[a]	230.1539	−0.4	5.3	$C_8H_{11}N$	202.1590	−1.3	10.3	$C_9H_{11}NO$	134.0964	0.4	0.3	$C_{14}H_{19}NO$
	351.2431	12.30	$C_{23}H_{30}N_2O$	*trans*-3-methylfentanyl[a]	230.1539	−0.1	8.4	$C_8H_{11}N$	202.1590	−2.2	33.4	$C_9H_{11}NO$	134.0964	0.7	2.1	$C_{14}H_{19}NO$
47	366.0674	12.93	$C_{16}H_{16}N_3O_3SCl$	Metolazone	258.9939	−1.5	20.3	C_7H_9N								
	366.0674	13.73	$C_{16}H_{16}N_3O_3SCl$	Indapamide	132.0808	0.2	6.4	$C_7H_7N_2O_3ClS$	117.0573	0.0	174.6	$C_8H_{10}N_2O_3ClS$	91.0542	0	6.4	$C_9H_{10}N_3O_3ClS$
48	387.1559	12.47	$C_{21}H_{26}N_2OS_2$	Thioridazine-5-sulfoxide	262.0355	−0.5	8.4	$C_8H_{15}N$	258.0406	−0.7	34.7	$C_7H_{15}NO$	126.1277	0.2	2.5	$C_{13}H_{11}NOS_2$
	387.1559	12.73	$C_{21}H_{26}N_2OS_2$	Mesoridazine	372.1325	−2.2	4.9	CH_3	274.0355	−1.0	9.8	$C_7H_{15}N$	126.1277	0.1	5.6	$C_{13}H_{11}NOS_2$

Abbreviations: PMA = paramethoxyamphetamine, 4-MMC = 4-methylmethcathinone, MDA = 3,4-methylenedioxyamphetamine, PMMA = paramethoxymethamphetamine, HHMA = 3,4-dihydroxymethamphetamine, HMA = 4-hydroxy-3-methoxyamphetamine, 2C-H = 2,5-dimethoxyphenethylamine, MDMA = methylenedioxymethamphetamine, BDB = 1,3-benzodioxolylbutanamine, HMMA = 4-hydroxy-3-methoxymethamphetamine, 3,4-DMA = 3,4-dimethoxyamphetamine, 2,5-DMA = 2,5-dimethoxyamphetamine, 5-MeO-AMT = 5-methoxy-α-methyltryptamine), MDDMA = 3,4-methylenedioxy-N,N-dimethylamphetamine, MDEA = 3,4-methylenedioxy-N-ethylamphetamine, MBDB = N-methyl-1,3-benzodioxolylbutanamine, DOM = 4-methyl-2,5-dimethoxyamphetamine, 2C-E = 2,5-dimethoxy-4-ethylphenethylamine, 3,4,5-TMA = 3,4,5-trimethoxyamphetamine, DPT = N,N-dibutyltryptamine, 5-MeO-MIPT = N-isopropyl-5-methoxy-N-methyltryptamine, 2C-T-4 = 4-isopropylthio-2,5-dimethoxyphenethylamine, 2C-T-7 = 4-propylthio-2,5-dimethoxyphenethylamine, 5-MeO-DIPT = N,N-diisopropyl-5-methoxytryptamine, EDDP = methadone metabolite, 6-MAM = 6-monoasethylmorphin.

[a] = diastereomers.

[b] = position isomers.

Histapyrrodine, imipramine and nortrimipramine share a molecular formula of $C_{19}H_{24}N_2$ and $[M+H]^+$ 281.2012 (Figure 2, isomer group 32 in Table 1). Imipramine and nortrimipramine are structural isomers with the same tricyclic molecule skeleton. Histapyrrodine is structurally different from these two compounds. All three compounds were chromatographically separated, and the retention times were 12.81 min for histapyrrodine, 13.99 min for imipramine, and 14.48 min for nortrimipramine. The mass spectrum of histapyrrodine was obviously different from the spectra of imipramine and nortrimipramine, which for their part were visually quite similar. The fragment *m/z* 210.1277 of histapyrrodine is formed by the pyrrolidine ring fragmentation ($[M+H]^+$ - C_4H_9N), the fragment *m/z* 132.0808 is formed after the benzene ring cleavage from the former ($[M+H]^+$ - $C_{10}H_{15}N$), and the fragment *m/z* 98.0964 is a methylaminobenzene residue ($C_6H_{12}N$). All the fragments detected for histapyrrodine were compound characteristic, so its differentiation from the other two structural isomers was undisputable. Imipramine and nortrimipramine could be differentiated from each other based on the fragment ions *m/z* 236.1434 of imipramine and *m/z* 250.1590 of nortrimipramine, which are formed in the cleavage of the amino group. The fragment ions *m/z* 208.1125, *m/z* 196.1121 and *m/z* 86.0964 are detected for both compounds; however, the ion *m/z* 196.1121 of imipramine is very low in intensity. The three fragment ions identified for imipramine and nortrimipramine were all built up in the fragmentation of the alkyl side chain. The identified fragments of histapyrrodine, imipramine and nortrimipramine were even electron neutral losses and were predicted by both ACD/MS Fragmenter and SmartFormula3D, except fragment *m/z* 250.1590 of nortrimipramine, which was only predicted by ACD/MS Fragmenter. The fragmentation reaction, which builds up the fragment *m/z* 250.1590, is logical and consistent with the known reactions for amines. The mass accuracy and isotopic pattern match for the ion were −0.2 mDa and 3.4 mSigma, respectively, and thus fulfill the identification criteria (Table 1).

Cocaine and scopolamine, which share molecular formula $C_{17}H_{21}NO_4$ and $[M+H]^+$ 304.1543, are plant alkaloids that include a tropane ring in their structure[18] (Figure 3, isomer group 42 in Table 1). The retention times were 9.58 min for cocaine and 6.00 min for scopolamine. The MSn spectra of cocaine and scopolamine are compound characteristic and can easily be differentiated from each other. The three fragments identified for cocaine and scopolamine are formed in the fragmentation of the ester bonds, or the carbon atom next to the ester bond. The fragments identified for cocaine were *m/z* 182.1176, *m/z* 150.0913 and *m/z* 105.0335, which were formed by fragmentation of $C_7H_6O_2$, $C_8H_{10}O_3$ and $C_{10}H_{17}NO_3$, respectively. The characteristic fragments of scopolamine were *m/z* 156.1019, *m/z* 138.0913 and *m/z* 121.0648. These fragments were formed in cleavage of $C_9H_8O_2$, $C_9H_{10}NO_3$ and $C_9H_{13}NO_3$, respectively. The fragments identified for cocaine and scopolamine were even electron neutral losses, predicted and identified by both ACD/MS Fragmenter and SmartFormula3D.

The number of fragments per compound predicted by ACD/MS Fragmenter ranged from 34 to 232, and SmartFormula3D suggested 1–4 different formulae for the precursor ions and 2–15 possible formulae as product ions relatable to the precursor ion, respectively. The long list of possible fragments of ACD/MS Fragmenter, with many potentially false positive predictions, might be difficult to use on its own for structural elucidation without comparison with experimental data. Also the fact that the software did not predict the same fragments in all cases shows some lack of robustness. That is why care should be taken when interpreting mass spectral data with these software programs. However, with accurate mass data and good chromatographic separation, the reliability of the software is superior compared to nominal mass data.

The programs used in this study give neither exact knowledge about the charge distribution and the location of the radical site nor approximations of the probability and abundance of the predicted fragment. These features have given rise to criticism,[18,19] and consequently software has been developed that take into account the thermodynamic and stability aspects as well as the probability rates of the predicted fragments. Identification and structural elucidation of all detected fragments might be crucial when studying drug metabolism where the structure of the metabolite is unknown and needs to be identified based on its fragments. In the present study, an adequate approach was to identify compound characteristic fragments in order to differentiate the structural isomers.

The present study was carried out using pure standards, and the compounds in the same mixture were known to be chromatographically separated. This arrangement does not quite correspond to authentic samples, where isomers with retention times close to each other can co-elute. Such a pair is for example etilefrine and HHMA, with a retention time difference of only 0.07 min (Table 1; isomer group 7). In this case, the MS/MS spectrum would be a combination of both of these compounds. However, etilefrine and HHMA have compound characteristic fragments, and hence, if fragment ions of HHMA are seen with the fragment ions of etilefrine in the same spectrum, it can be concluded that both compounds are in the sample.

The structural elucidation with tandem mass spectrometry has been used, for example, in drug metabolism research,[26] analysis of impurities in pharmaceuticals[27] and technical chemicals[28] as well as in detection of environmental toxins.[29] Several studies concerning differentiation of structural isomers with MS/MS techniques have been published, but these works deal with the differentiation of two or three compounds. For instance, studies have been published about differentiation of MDEA and MBDB; methamphetamine and phentermine;[30] hydromorphone, morphine and norcodeine;[31] clobazam and temazepam;[32] as well as metolazone and indapamide.[33] Tramadol and *O*-desmethyl venlafaxin sharing a molecular formula of $C_{16}H_{25}NO_2$ and $[M+H]^+$ 264.1958, have been reported to undergo similar fragmentation,[34] yielding only a single fragment of *m/z* 58. In our study, tramadol and *O*-desmethyl venlafaxine were differentiated based on *O*-desmethyl venlafaxine's characteristic fragment, *m/z* 133.0648. Similar results have been reported about isomers that could not be distinguished based on their fragments, including the compounds studied in the present work (MDEA and MDDMA,[35] and ephedrine and pseudoephedrine[36]).

To date, software for *in silico* fragment prediction has mostly been used for structural elucidation of drug metabolites[15,37,38] where the compound structures are unknown or just approximated and the identification and structural determination of all observed fragments in the mass spectra is crucial. There have been no publications on differentiation of structural isomers by MS/MS spectra and identification of the characteristic fragments with fragment prediction software, except our previous study of quetiapine.[20] The advantage of this method – combining accurate mass and fragment prediction in order to elucidate compound structure – over identification by spectral library comparison lies in the fragment structure determination it enables.

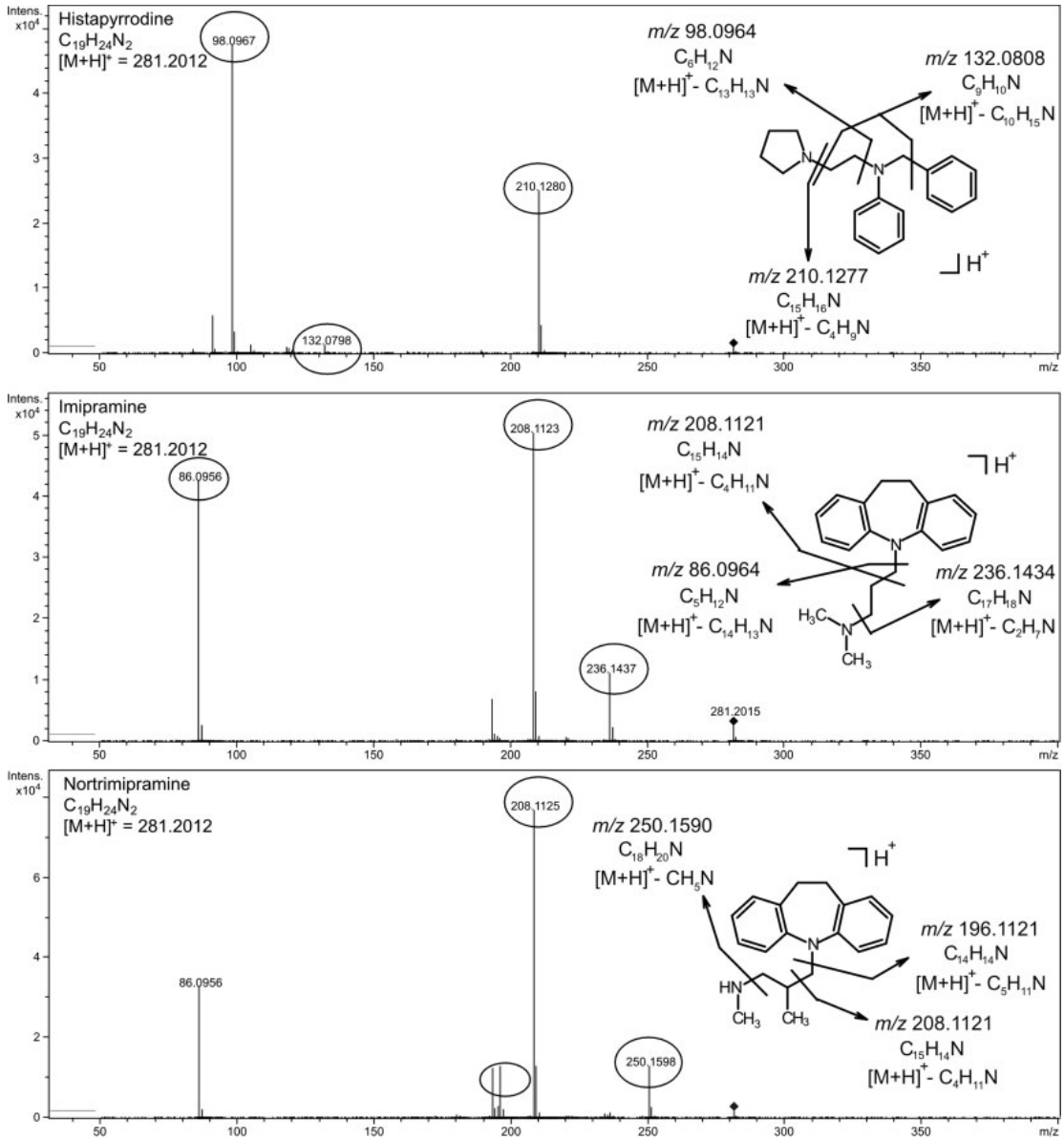


Figure 2. Mass spectra and fragmentation schemes of histapyrrodine, imipramine and nortrimipramine ($[M+H]^+ = 281.2012$, $C_{19}H_{24}N_2$).

Conclusion

Poor accessibility of reference standards has hindered substance identification within drug screening, especially for new drugs, designer drugs, and metabolites. Formula-based identification against a target database of exact monoisotopic masses is a partial remedy, but even this approach fails with isomeric compounds. The aim of the present study was to differentiate all isomers found in a comprehensive target database, based on LC/Q-TOFMS product ion spectra of the reference standards available, and to identify the compound characteristic fragments. The results from 48 isomer groups demonstrated an indisputable advantage of the predictive software in assigning relevant mass fragments to structural isomers and in defining the molecular formulae of the fragments. The two software programs proved to be valuable for interpretation of experimental accurate mass data. However, one should be aware of the differences in the performance of each software program and the possibility of false positive predictions. The use of fragmentation prediction allows a target database to be built up that contains the exact monoisotopic masses of both precursor and the most

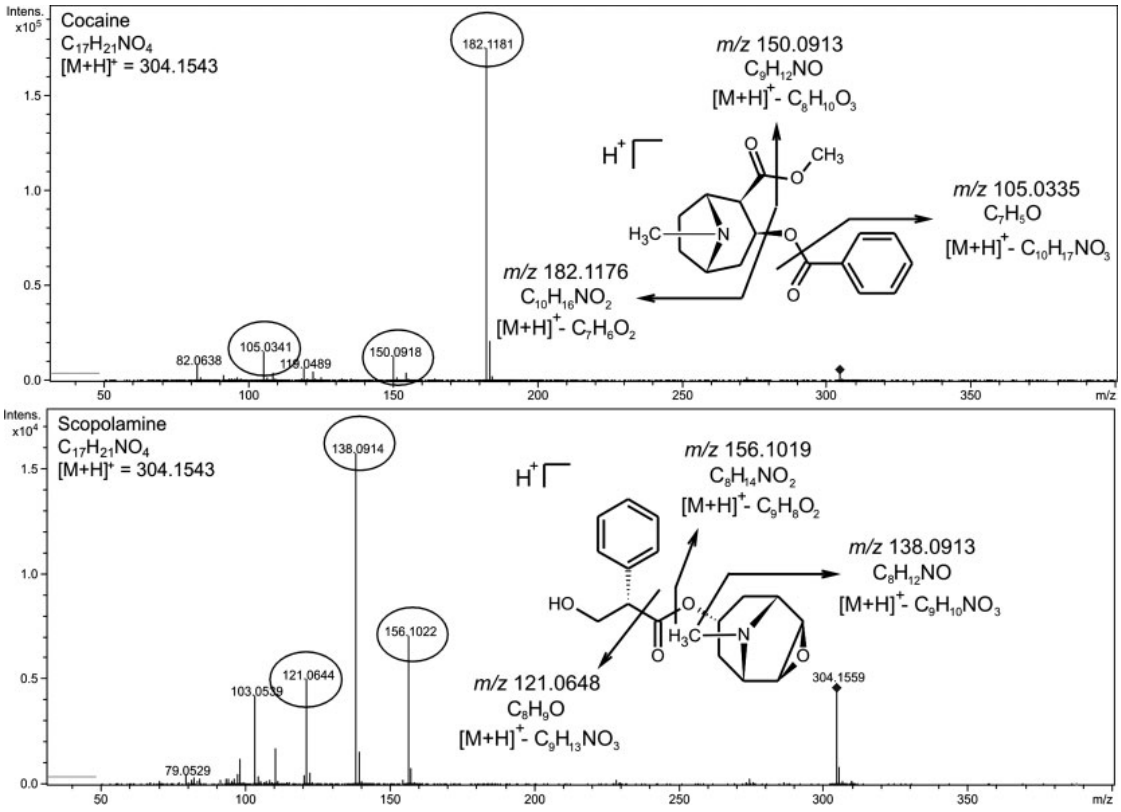


Figure 3. Mass spectra and fragmentation schemes of cocaine and scopolamine ($[M+H]^+ = 304.1543$, $C_{17}H_{21}NO_4$).

characteristic fragment ions, even for those compounds for which a reference standard cannot be readily obtained. Compound characterization in a biological sample can be carried out using these two fragment prediction software programs, as accurate mass data enables the elucidation of fragment structures. This, in turn, makes a rapid tentative identification of a range of compounds feasible in pharmaceutical, toxicology, and forensic contexts.

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Prediction of liquid chromatographic retention for differentiation of structural isomers

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ABSTRACT

A liquid chromatography (LC) retention time prediction software, ACD/ChromGenius, was employed to calculate retention times for structural isomers, which cannot be differentiated by accurate mass measurement techniques alone. For 486 drug compounds included in an in-house database for urine drug screening by liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOFMS), a retention time knowledge base was created with the software. ACD/ChromGenius calculated retention times for compounds based on the drawn molecular structure and given chromatographic parameters. The ability of the software for compound identification was evaluated by calculating the retention order of the 118 isomers, in 50 isomer groups of 2–5 compounds each, included in the database. ACD/ChromGenius predicted the correct elution order for 68% (34) of isomer groups. Of the 16 groups for which the isomer elution order was incorrectly calculated, two were diastereomer pairs and thus difficult to distinguish using the software. Correlation between the calculated and experimental retention times in the knowledge base tested was moderate, $r^2 = 0.8533$. The mean and median absolute errors were 1.12 min, and 0.84 min, respectively, and the standard deviation was 1.04 min. The information generated by ACD/ChromGenius, together with other *in silico* methods employing accurate mass data, makes the identification of substances more reliable. This study demonstrates an approach for tentatively identifying compounds in a large target database without a need for primary reference standards.

1. Introduction

Liquid chromatography (LC) is a separation method that is increasingly important and widely used in the pharmaceutical industry as well as in doping analysis and clinical and forensic toxicology. LC techniques have developed and improved significantly during the last decade: New ultra-high performance instruments provide efficiency, sensitivity, and higher resolution. Reversed-phase LC, especially coupled with mass spectrometry (MS), is a fast, robust, and reliable analytical technique in routine analysis [1–3].

A wide variety of *in silico* tools are available for the analyst to facilitate and speed up the analytical processes and compound identification. For example, computer software may aid in the study of drug metabolism [4] and toxicity [5], in metabolite identification [6], in mass fragmentation prediction [7], and in optimization of sample preparation [8] or chromatographic separation [9]. Commercial software for chromatographic method development, optimization, and validation [10–13] include DryLab (Molnár-Institute for Applied Chromatography, Berlin, Germany) [14] and ChromSword (ChromSword, Riga, Latvia) [15]. The DryLab software is based on experimental data and simple calculations, like the linear solvent strength model. ChromSword, a more advanced software, combines the chemical structure of compounds with chromatographic data. These software, however, have predicted retention times for compounds only in simple isocratic or linear gradient separations with a small number of analytes [9,10].

More extensive information about retention phenomena can be obtained from quantitative structure–retention relationship (QSRR) models [16–18]. The aim of these models is to discover the relation between the molecular descriptors – calculated from the chemical structure – and retention. QSRR models describe chromatographic retention in single chromatographic systems. QSRR analyses can identify the most useful structural descriptors in a molecule, detect the molecular mechanism of retention of a given compound, compare the separation mechanisms of various chromatography columns, calculate the physicochemical properties of the analytes, and estimate biological activity of xenobiotics. QSRR models have been used for predicting the retention times of drug compounds in order to determine their retention behaviors [19–21]; however, for identification purposes they have only been applied to peptides [22–25].

Despite the advantages of QSRR models, they have not yet become part of routine LC method development or of compound

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identification. A crucial challenge is to select the most informative molecular descriptors from a large number of possibilities [16]; Dragon software (Talete, Milano, Italy) [26], for example, calculates almost 5000 molecular descriptors. The evaluation of prediction performance is an important and critical phase in model validation [18]. The use of QSRR also requires personnel conversant with computational modeling.

The lack of reference standards for new drugs, metabolites, and designer drugs forces the analyst to find alternative methods for tentative compound identification. Our in-house toxicology database for urine drug screening by liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOFMS) includes the molecular formulae of almost 900 substances (legal and illegal drug compounds and their metabolites), while retention time data obtained by reference standards is available for 486.

High resolution mass spectrometry (HRMS) instruments for systematic toxicological analysis in clinical and forensic toxicology laboratories have become more common [3,27] since accurate mass data enables the determination of elemental analyte composition. Differentiation of structural isomers is not possible by accurate mass measurement alone, which is why good chromatographic separation is necessary.

Here we used an *in silico* method to determine the retention order of 118 isomers comprising 50 isomer groups of 2–5 compounds. We created a retention time knowledge base of the 486 compounds included in our LC/Q-TOFMS in-house toxicology database with ACD/ChromGenius software (ACD/Labs, Toronto, Canada) [28]. This software uses a self-created knowledge base of structures and experimental retention times as a basis to predict, using built-in physicochemical prediction algorithms, retention times and chromatograms for new compounds. Another knowledge base of 458 compounds that were analyzed under different chromatographic conditions was created to test if the knowledge bases would yield similar prediction accuracies. The two knowledge bases included 454 compounds in common. The aim of the study was to evaluate the ability of the software to predict retention times for structural isomers as an additional tool for identifying compounds in LC/Q-TOFMS urine drug screening.

2. Materials and methods

2.1. Chemicals and reagents

All the solvents and reagents were of analytical grade (Merck, Darmstadt, Germany), except high performance liquid chromatography (HPLC)-grade methanol (T.J. Baker, Deventer, The Netherlands) and acetonitrile (Rathburn, Walkerburn, UK). Water was purified with a Millipore DirectQ-3 instrument (Bedford, MA, USA). The standards were from several different suppliers.

2.2. Liquid chromatography/quadrupole time-of-flight mass spectrometry

The liquid chromatograph was an Agilent 1200 series instrument (Waldbronn, Germany) with a vacuum degasser, binary pump, autosampler and column oven. Reference standard mixtures (1 μg mL^{-1} in 0.1% formic acid and methanol or acetonitrile, 9:1) of the compounds in the in-house toxicology databases were analyzed by two chromatographic methods. In the first method, a Luna PFP(2) (pentafluorophenyl) 100 mm × 2 mm (3 μm) column and a PFP 4 mm × 2 mm pre-column (Phenomenex, Torrance, CA, USA) were used in gradient mode at 40 °C. The mobile phase components were 2 mM ammonium acetate in 0.1% formic acid, and methanol. The injection volume was 5 μL, and the flow rate 0.3 mL min^{-1}. The proportion of methanol was increased from 10% to 40% at 5 min, to 75% at 13.50 min, to 80% at 16 min and held 4 min at 80%. The hold-up time of the PFP column was 0.75 min. The analysis time was 20 min, and the post-time 8 min. In the other method, the separation was performed in gradient mode at 40 °C with a Luna C-18(2) 100 mm × 2 mm (3 μm) column and a 4 mm × 2 mm pre-column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5 mM ammonium acetate in 0.1% formic acid and acetonitrile. The flow rate was 0.3 mL min^{-1}, and the injection volume was 10 μL. The amount of the organic phase was increased from 10% to 40% at 10 min, to 75% at 13.50 min, to 80% at 16 min and held for 5 min, for a total analysis time of 21 min, and the post-time was 6 min. The C-18 column hold-up time was 0.70 min.

The mass analyzer was a Bruker Daltonics micrOTOF-Q mass spectrometer (Bremen, Germany) with an orthogonal electrospray ionization source. The nominal resolution of the instrument was 10,000 (FWHM). The instrument was operated in positive ion mode with an *m/z* range of 50–800. The nebulizer gas pressure was 1.6 bar, the drying gas flow 8.0 L min^{-1}, and the drying gas temperature was 200 °C. The capillary voltage was 4500 V, and the end plate offset was set at −500 V. The quadrupole ion energy was 3.0 eV, and the collision cell radio frequency was 100.00 Vpp. The quadrupole transfer time was 60.0 μs and pre-pulse storage time 8.0 μs. The spectra rate was 1.7 Hz, and the spectra rolling average were set at 2. External instrument calibration was performed with sodium formate solution, comprised of 10 mM sodium hydroxide in isopropanol and 0.2% formic acid (1:1, v/v). The calibration was completed with ten sodium formate cluster ions, with exact masses between 90.9766 and 702.8635. Post-run internal mass calibration for each sample was performed by injecting the calibrant at the beginning and at the end of the run.

2.3. Software

Post-run internal mass spectrum calibration and processing of the sample data was performed with DataAnalysis 4.0 software (Bruker Daltonics, Bremen, Germany). The retention times of the compounds were recorded in the in-house toxicology database. Two retention time knowledge bases were created from the in-house databases: PFP (including 486 compounds) and C18 (458 compounds, respectively), by ACD/ChromGenius 12.00 software from Advanced Chemistry Development (Toronto, Canada). The chemical structure of each compound was drawn with ACD/ChemSketch, and the structure was added to the knowledge base with the experimental retention time (min) and peak width at half maximum (min), as well as the chromatographic conditions. ACD/ChromGenius software predicted retention times for compounds using chemical structures and calculated physicochemical properties. It calculated the retention time for each compound by comparing the structure to the most similar compounds in the knowledge base, which were selected by a specified algorithm. It looked for the chemically most similar structures, and searched for the physical properties of these structures that most closely correlated with the retention time. A prediction equation for each compound, relating retention time to physical properties, was generated: The calculated retention time was a sum of the most correlated properties, for which a specific coefficient was determined based on experimental results. The software estimated the prediction accuracy of the knowledge base by performing a leave-one-out study for each compound. The software parameters used for calculating physicochemical parameters included log *D*, log *P*, polar surface area, molecule volume and weight, molar refractivity, as well as the number of hydrogen donors and acceptors. The retention time calculations for the compounds in the knowledge base were performed using 30 of the most similar compounds obtained by Dice coefficient similarity search and linear regression. Reversed phase mode was selected as the chromatographic system.

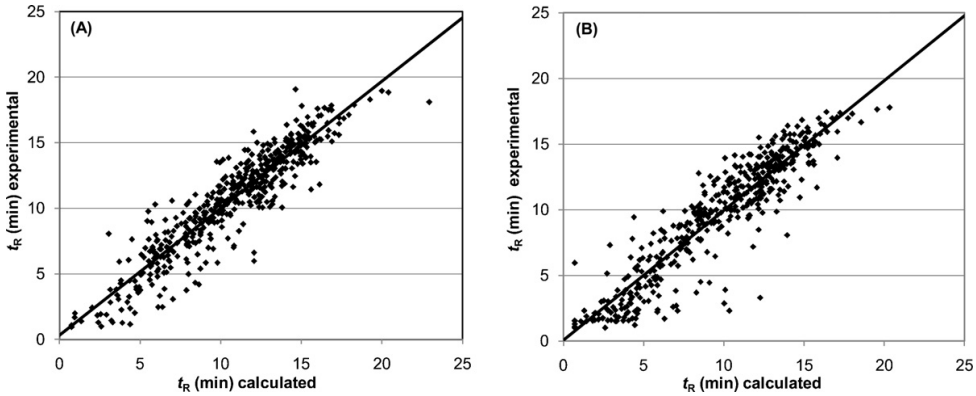

Fig. 1. Correlation between experimental and calculated retention times (t_R) of (A) 486 compounds in the PFP knowledge base (r^2 = 0.8533) and (B) 454 compounds in the C18 knowledge base (r^2 = 0.8497).

The parameters of ACD/ChromGenius also included the software default values. The calculated retention times of the compounds in the PFP and C18 knowledge bases were compared to the true experimental values. ACD/ChromGenius also calculated the retention factor (k) for the compounds. This would have been the parameter of choice, if the method was transferred to another column system, however, to clarify the comparison between experimental and calculated retention times, the absolute values were chosen for this study. The predicted retention order of the 118 isomers in the PFP knowledge base was compared to experimentally determined data. The C18 knowledge base was employed for comparing the prediction accuracies between the two chromatographic methods, not for isomer retention order determination.

3. Results and discussion

The correlation between the calculated and experimental retention times of the 486 compounds in the PFP knowledge base is shown in Fig. 1A. The prediction accuracy of the C18 knowledge base of 458 compounds (Fig. 1B) was virtually equal to the PFP knowledge base. A statistical comparison between PFP and C18 knowledge bases is presented in Table 1. The C18 knowledge base was created to test if the ACD/ChromGenius software was able to predict retention times with equal accuracy in two common chromatographic systems. The prediction accuracy of the PFP knowledge base turned out to be slightly more precise, which might result from the 6% (28 compounds) size difference of the two knowledge bases, as the prediction accuracy increases with knowledge base size and diversity [28].

As can be observed from the distribution of retention time errors in the PFP knowledge base (Fig. 2), most of the compounds fall in a range of error of ±1.00 min. The absolute error between the calculated and experimental retention time was less than 0.50 min for 33% (162), less than 1.00 min for 57% (279), and less than 2.00 min for 84% (407) of compounds. The calculated retention time value was higher than the experimental in 48% (232 compounds); thus, no tendency for the predicted values being higher or lower than the experimental retention times was observed. The minimum and maximum errors were −6.10 min, and 5.00 min, respectively.

Guidelines for compound identification criteria by LC retention time are available. The technical document for identification criteria for qualitative assays in doping analysis by the World Anti-Doping Agency (WADA) states that the HPLC retention time of the compound and the reference standard should not differ by more than 2% or ±0.1 min in the same analysis [29]. According to the Society of Toxicological and Forensic Chemistry guidelines, a tolerance for relative retention time repeatability of 5% is acceptable as one criterion for positive compound identification [30]. Here, the retention times calculated by ACD/ChromGenius differed by ±2% for 17% (82), by ±5% for 35% (171) and by ±10% for 58% (280) of the compounds in the PFP knowledge base. Since the absolute errors between the calculated and experimental retention times were relatively large, the results of ACD/ChromGenius alone are invalid for compound identification.

Table 1
Comparison of the PFP and C18 knowledge bases.

	PFP knowledge base	C18 knowledge base
r^2	0.8533	0.8497
Mean absolute error[a]	1.12	1.26
Median absolute error[a]	0.84	0.95
Standard deviation[a]	1.04	1.19
RMSE[a,b]	1.53	1.74
Minimum absolute error[a]	0.01	0.00
Maximum absolute error[a]	6.10	8.97

[a] Minutes.
[b] Root mean square error.

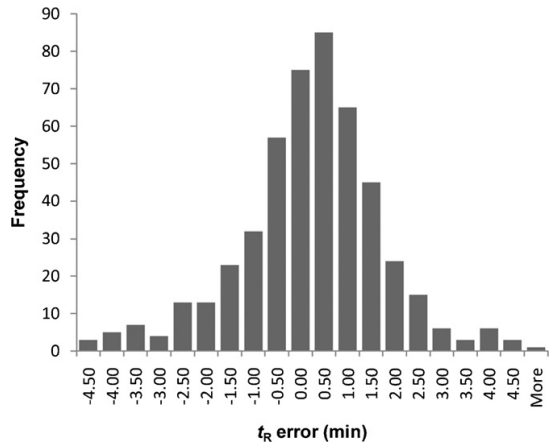

Fig. 2. Distribution of retention time (t_R) errors for 486 compounds in the PFP knowledge base.

The prediction accuracy was somewhat poorer for early eluting (experimental retention time, $t_R \leq 9.00$ min; 153 compounds), more polar analytes, for which the average absolute retention time error in the PFP knowledge base was 1.29 min. For substances eluting after 9.00 min (333 compounds), the error was 1.04 min. The error between the experimental and calculated retention time was large for compounds that share very few similar physicochemical properties with other compounds, such as hydroxychloroquine (Δt_R 6.10 min), minoxidil (5.00 min), amiodarone (4.83 min), and clonidine (4.10 min). Variation in prediction accuracy was also observed between different drug compound categories. The average absolute error was 0.78 min (median 0.64 min) for the 50 phenethylamines, and 0.88 min (median 0.62 min) for the 47 tri- and tetracyclic central nervous system drugs, while the error was 1.01 min (median 0.73 min) for the 45 opioids. This may due to the greater variety in chemical structure and retention behavior between opiates and synthetic opioids. The prediction accuracy in the C18 knowledge base varied similarly.

Why the software yielded such poor results for some of the compounds remains unclear. Increasing the size of the knowledge base might improve prediction accuracy as well as the precision of the calculated retention times, since the number of structurally similar compounds would rise. The rather complex chromatographic conditions used in this study, including non-linear gradient and PFP column, represent a real-life analytical separation. Our approach of employing a retention time prediction software for determination of compound elution order is novel, as commercial retention time prediction software are generally employed for HPLC method development, not for compound identification. Comparison to previous published studies on retention time predictions [9,10,19–21] is disparate, since the chromatographic conditions and the diversity of the database in this study are widely different from those in previous studies.

ACD/Labs does not provide the full algorithm for retention time calculation, and consequently the present evaluation of the prediction performance of ACD/ChromGenius software relies on examination of retention time errors. A critical study of the algorithm and how it relates physicochemical properties to the calculated retention time would possibly help analyzing the errors in the individual calculated retention times. Unfortunately, commercial software, developed by non-academic communities, are rarely transparent by their algorithms. This is why the results obtained by these software should be regarded with a critical aspect.

Despite the rather large absolute errors between the experimental and calculated retention times, the ACD/ChromGenius software proved to be useful in predicting the compound elution order. It calculated the right isomer elution order for 68% (34) of groups. The results of the 50 isomer groups with experimental and calculated retention times, as well as the absolute retention time errors, are presented in Table 2. Of those 16 groups for which the software was unable to calculate the isomer elution order correctly, two were diastereomer pairs: ephedrine and pseudoephedrine (Table 2; group 4; $[M+H]^+$ 166.12264; $C_{10}H_{15}NO$), as well as quinine and quinidine (group 46; $[M+H]^+$ 325.19105; $C_{20}H_{24}N_2O_2$), which differ only in their three-dimensional orientation. Apparently, the differentiation of stereoisomers was extremely challenging for the software. Two diastereomer pairs, *E*- and *Z*-10-hydroxyamitriptyline, as well as *E*- and *Z*-10-hydroxynortriptyline, were left out of the isomer differentiation because the ACD/ChromGenius software could not distinguish between them.

In two groups, anabasine and nicotine (group 3; $[M+H]^+$ 163.12298; $C_{10}H_{14}N_2$), and the 2-, 3-, and 4-fluoromethamphetamines (group 5; $[M+H]^+$ 168.11830; $C_{10}H_{14}NF$), the experimental retention time difference between the isomers was less than 0.20 min, which also made the differentiation demanding for the software. The calculated retention times for the three fluoromethamphetamines were within 0.06 min, which would have been inadequate for chromatographic separation of the isomers, although the retention order was correct.

In six cases of the groups with a falsely predicted retention order, the error did not extend to all of the isomers of the group. For example, in group 20 ($[M+H]^+$ 247.18049; $C_{15}H_{22}N_2O$), the retention order was predicted correctly for 3-methylnorfentanyl, 5-MeO-MIPT and mepivacaine, but the large retention time error of milnacipran, 4.35 min, made the isomer group unresolved. Milnacipran is structurally different from other compounds in the PFP knowledge base, which led to poor retention time calculation. The same was observed in group 34 ($[M+H]^+$ 278.19033; $C_{20}H_{23}N$), where the poor prediction accuracy of EDDP, Δt_R 2.87 min, induced an incorrect calculation of the retention order, though the order was correctly predicted for amitriptyline and maprotiline. Thirteen of the 50 isomer groups included one or more opioids, for which the prediction accuracy was quite poor in the PFP knowledge base. The presence of a single compound for which retention time calculation is difficult can obscure the exact retention order in the isomer group. Thus a critical perspective should be adopted when interpreting the results and using the predicted retention times generated by ACD/ChromGenius for compound identification.

The retention order of the isomers was more likely to be correctly calculated if the prediction accuracy of a compound was adequate. Of the nine isomer groups that included tri- and tetracyclic central nervous system drugs (23, 26, 28, 30, 34, 35, 36, 41, and 50), the retention order was correctly predicted in eight cases. The retention order was also correct in groups where a tri- or tetracyclic compound was an isomer with a structurally different compound, such as mirtazapine with antazoline (group 28; $[M+H]^+$ 266.16517; $C_{17}H_{19}N_3$), as well as in groups where two tri- or tetracyclic compounds form an isomer pair, *e.g.* chlorcyclizine and norclomipramine (41; $[M+H]^+$ 301.14660; $C_{18}H_{21}N_2Cl$). The incorrect order in one group (34) was due to the poor prediction accuracy of EDDP, as is explained in more detail in the previous paragraph.

Correct prediction of the substance elution order gives valuable information for the separation of isomeric compounds. New designer drugs are often different phenethylamine derivates [31], which vary by substituent and substituent position. These compounds are potential structural isomers to each other, as in group 10 (Table 2): 2-CH, DMPEA, etilefrine, HHMA and HMA ($[M+H]^+$ 182.11756; $C_{10}H_{15}NO_2$). The separation of these five isomers by predicting the right elution order demonstrates the value of ACD/ChromGenius software. A similar situation in a biological sample would be very unlikely, but the software predictions provide an indication of the compound retention time compared to other possible substances.

In our previous study [7] we employed a software for mass fragmentation prediction in order to differentiate isomers. Two isomer pairs, also included in this study, 2,5-DMA and 3,4-DMA (group 12; $[M+H]^+$ 196.13321; $C_{11}H_{17}NO_2$) as well as 2C-T-4 and 2C-T-7 (group 24; $[M+H]^+$ 256.13658; $C_{13}H_{21}NO_2S$), could not then be separated by fragmentation prediction alone, since they had identical fragments. In the present study, however, these isomer pairs were successfully separated by retention order prediction. In a situation where an unidentified compound is known to be a phenethylamine analog, retention time prediction offers significant information. The retention time data generated by ACD/ChromGenius combined with data produced by other prediction software, together with accurate mass data, can complete the identification of a substance.

It has been shown elsewhere that HRMS enables non-targeted screening or screening against very large databases. To attain the correct chemical structure, all possible structures for a determined molecular formula are first filtered by different heuristic rules [32].

Table 2
Molecular formulae, experimental and calculated retention times, and absolute retention time errors of 118 structural isomers in the PFP knowledge base listed by increasing molecular mass of precursor ions. Isomer groups with correctly calculated retention order are italicized.

	Mass $[M+H]^+$	Molecular formula	Compound	Retention time (exp[a])	Retention time (calc[b])	Retention time error (abs[c])
1	150.12773	$C_{10}H_{15}N$	*Metamphetamine*	5.96	5.54	0.42
	150.12773	$C_{10}H_{15}N$	*Phentermine*	7.04	6.64	0.40
2	161.10733	$C_{10}H_{12}N_2$	*Anatabine*	1.28	3.30	2.02
	161.10733	$C_{10}H_{12}N_2$	*Tryptamine*	5.94	6.82	0.88
3	163.12298	$C_{10}H_{14}N_2$	Anabasine	1.43	3.22	1.79
	163.12298	$C_{10}H_{14}N_2$	Nicotine	1.25	3.90	2.65
4	166.12264	$C_{10}H_{15}NO$	Ephedrine[d]	3.37	3.58	0.21
	166.12264	$C_{10}H_{15}NO$	Paramethoxyamphetamine (PMA)	6.87	6.29	0.58
	166.12264	$C_{10}H_{15}NO$	Pseudoephedrine[d]	3.82	3.53	0.29
5	168.11830	$C_{10}H_{14}NF$	2-Fluoromethamphetamine	6.66	5.83	0.83
	168.11830	$C_{10}H_{14}NF$	3-Fluoromethamphetamine	6.85	5.80	1.05
	168.11830	$C_{10}H_{14}NF$	4-Fluoromethamphetamine	7.04	5.78	1.26
6	178.12264	$C_{11}H_{15}NO$	4-Methylmethcathinone (4-MMC)	7.48	6.03	1.45
	178.12264	$C_{11}H_{15}NO$	Ethylcathinone	5.56	6.50	0.94
	178.12264	$C_{11}H_{15}NO$	Phenmetrazine	5.81	5.77	0.04
7	180.10191	$C_{10}H_{13}NO_2$	*3,4-Methylenedioxyamphetamine (MDA)*	6.40	6.72	0.32
	180.10191	$C_{10}H_{13}NO_2$	*Phenacetin*	11.75	11.77	0.02
8	180.13829	$C_{11}H_{17}NO$	Methoxyphenamine	8.19	6.73	1.46
	180.13829	$C_{11}H_{17}NO$	Methylephedrine	4.16	5.74	1.58
	180.13829	$C_{11}H_{17}NO$	Mexiletine	9.59	8.48	1.11
	180.13829	$C_{11}H_{17}NO$	Paramethoxymethamphetamine (PMMA)	7.39	6.83	0.56
9	181.07200	$C_7H_8N_4O_2$	*Theobromine*	5.47	6.90	1.43
	181.07200	$C_7H_8N_4O_2$	*Theophylline*	7.27	7.90	0.63
10	182.11756	$C_{10}H_{15}NO_2$	*2,5-Dimethoxyphenethylamine (2C-H)*	7.63	5.54	2.09
	182.11756	$C_{10}H_{15}NO_2$	*3,4-Dimethoxyphenethylamine (DMPEA)*	5.08	5.18	0.10
	182.11756	$C_{10}H_{15}NO_2$	*Etilefrine*	1.82	2.35	0.53
	182.11756	$C_{10}H_{15}NO_2$	*3,4-Dihydroxymethamphetamine (HHMA)*	1.87	2.91	1.04
	182.11756	$C_{10}H_{15}NO_2$	*4-Hydroxy-3-methoxyamphetamine (HMA)*	3.34	3.61	0.27
11	194.11756	$C_{11}H_{15}NO_2$	*4-Methoxymethcathinone (4-MeOMC)*	6.89	6.21	0.68
	194.11756	$C_{11}H_{15}NO_2$	*1,3-Benzodioxolylbutanamine (BDB)*	8.18	8.76	0.58
	194.11756	$C_{11}H_{15}NO2$	*Methylenedioxymethamphetamine (MDMA)*	6.96	6.75	0.21
12	196.13321	$C_{11}H_{17}NO_2$	*2,5-Dimethoxyamphetamine (2,5-DMA)*	8.63	7.28	1.35
	196.13321	$C_{11}H_{17}NO_2$	*3,4-Dimethoxyamphetamine (3,4-DMA)*	6.81	6.95	0.14
	196.13321	$C_{11}H_{17}NO_2$	*4-Hydroxy-3-methoxymethamphetamine (HMMA)*	3.75	5.02	1.27
13	205.13354	$C_{12}H_{16}N_2O$	*5-Methoxy-α-methyltryptamine (5-MeO-AMT)*	8.38	8.01	0.37
	205.13354	$C_{12}H_{16}N_2O$	*Psilocin*	5.07	6.49	1.42
14	208.13321	$C_{12}H_{17}NO_2$	N-Methyl-1,3-benzodioxolylbutanamine (MBDB)	8.45	8.47	0.02
	208.13321	$C_{12}H_{17}NO_2$	3,4-Methylenedioxy-N,N-dimethylamphetamine (MDDMA)	7.17	7.90	0.73
	208.13321	$C_{12}H_{17}NO_2$	3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	7.85	7.89	0.04
15	210.14886	$C_{12}H_{19}NO_2$	*2,5-Dimethoxy-4-ethylphenethylamine (2C-E)*	11.19	10.03	1.17
	210.14886	$C_{12}H_{19}NO_2$	*4-Methyl-2,5-dimethoxyamphetamine (DOM)*	10.50	9.16	1.34
16	226.14377	$C_{12}H_{19}NO_3$	*3,4,5-Trimethoxyamphetamine*	7.56	6.58	0.98
	226.14377	$C_{12}H_{19}NO_3$	*Terbutaline*	3.82	4.77	0.95
17	236.16451	$C_{14}H_{21}NO_2$	*Dinortramadol*	8.91	7.82	1.09
	236.16451	$C_{14}H_{21}NO_2$	*O-desmethylnortramadol*	6.66	7.07	0.41
18	237.15975	$C_{13}H_{20}N_2O_2$	Procaine	4.18	8.65	4.47
	237.15975	$C_{13}H_{20}N_2O_2$	Dropropizine	4.53	5.61	1.08
19	245.20123	$C_{16}H_{24}N_2$	N,N-Dibutyltryptamine (DPT)	10.62	12.36	1.74
	245.20123	$C_{16}H_{24}N_2$	Xylometazoline	12.83	12.23	0.60
20	247.18049	$C_{15}H_{22}N_2O$	3-Methylnorfentanyl	9.03	8.32	0.71
	247.18049	$C_{15}H_{22}N_2O$	N-isopropyl-5-methoxy-N-methyltryptamine (5-MeO-MIPT)	9.16	9.08	0.08
	247.18049	$C_{15}H_{22}N_2O$	Mepivacaine	7.65	7.79	0.14
	247.18049	$C_{15}H_{22}N_2O$	Milnacipran	10.29	5.94	4.35

Table 2 (*Continued*)

	Mass $[M+H]^+$	Molecular formula	Compound	Retention time (exp[a])	Retention time (calc[b])	Retention time error (abs[c])
21	248.16451	$C_{15}H_{21}NO_2$	*Ketobemidone*	7.39	8.00	0.61
	248.16451	$C_{15}H_{21}NO_2$	*Pethidine*	9.49	9.01	0.48
22	250.18016	$C_{15}H_{23}NO_2$	*Alprenolol*	11.85	11.79	0.06
	250.18016	$C_{15}H_{23}NO_2$	*Nortramadol*	9.17	8.98	0.19
	250.18016	$C_{15}H_{23}NO_2$	*O-desmethyltramadol*	6.48	8.56	2.08
23	253.13658	$C_{15}H_{12}N_2O_2$	*Oxcarbazepine*	12.48	11.91	0.58
	253.13658	$C_{15}H_{12}N_2O_2$	*Phenytoin*	13.08	13.05	0.03
24	256.13658	$C_{13}H_{21}NO_2S$	*4-Isopropylthio-2,5-dimethoxyphenethylamine (2C-T-4)*	11.91	11.95	0.04
	256.13658	$C_{13}H_{21}NO_2S$	*4-Propylthio-2,5-dimethoxyphenethylamine (2C-T-7)*	12.35	13.41	1.06
25	256.16959	$C_{17}H_{21}NO$	*Diphenhydramine*	11.57	11.12	0.45
	256.16959	$C_{17}H_{21}NO$	*Nororphenadrine*	12.57	11.55	1.02
26	264.17468	$C_{19}H_{21}N$	*Nortriptyline*	13.81	13.56	0.25
	264.17468	$C_{19}H_{21}N$	*Protriptyline*	13.67	13.22	0.45
27	264.19581	$C_{16}H_{25}NO_2$	Norvenlafaxine	10.30	9.74	0.56
	264.19581	$C_{16}H_{25}NO_2$	O-desmethylvenlafaxine	8.00	9.34	1.34
	264.19581	$C_{16}H_{25}NO_2$	Tramadol	8.94	9.80	0.86
28	266.16517	$C_{17}H_{19}N_3$	*Antazoline*	11.02	12.36	1.34
	266.16517	$C_{17}H_{19}N_3$	*Mirtazapine*	9.07	10.07	1.00
29	267.17032	$C_{14}H_{22}N_2O_3$	*Atenolol*	3.99	4.77	0.78
	267.17032	$C_{14}H_{22}N_2O_3$	*Practolol*	5.53	5.64	0.11
30	267.18558	$C_{18}H_{22}N_2$	*Cyclizine*	11.91	10.38	1.53
	267.18558	$C_{18}H_{22}N_2$	*Desipramine*	13.57	12.92	0.66
31	268.16959	$C_{18}H_{21}NO$	Azacyclonol	10.54	10.46	0.08
	268.16959	$C_{18}H_{21}NO$	Pipradrol	9.92	10.85	0.93
32	275.21179	$C_{17}H_{26}N_2O$	*N,N-diisopropyl-5-methoxytryptamine (5-MeO-DIPT)*	10.45	10.90	0.45
	275.21179	$C_{17}H_{26}N_2O$	*Ropivacaine*	9.58	10.58	1.00
33	276.15942	$C_{16}H_{21}NO_3$	*Homatropine*	6.11	7.87	1.76
	276.15942	$C_{16}H_{21}NO_3$	*Methylenedioxypyrovalerone (MDPV)*	9.73	9.57	0.16
34	278.19033	$C_{20}H_{23}N$	Amitriptyline	13.75	13.30	0.45
	278.19033	$C_{20}H_{23}N$	EDDP (methadone metabolite)	11.62	14.49	2.87
	278.19033	$C_{20}H_{23}N$	Maprotiline	13.29	12.82	0.47
35	281.20123	$C_{19}H_{24}N_2$	*Histapyrrodine*	12.45	12.43	0.02
	281.20123	$C_{19}H_{24}N_2$	*Imipramine*	13.51	13.68	0.17
	281.20123	$C_{19}H_{24}N_2$	*Nortrimipramine*	14.04	13.73	0.31
36	285.14200	$C_{17}H_{20}N_2S$	*Promazine*	13.39	12.77	0.62
	285.14200	$C_{17}H_{20}N_2S$	*Promethazine*	12.82	12.73	0.09
37	286.14377	$C_{17}H_{19}NO_3$	Hydromorphone	2.83	4.20	1.37
	286.14377	$C_{17}H_{19}NO_3$	Morphine	1.92	2.56	0.64
	286.14377	$C_{17}H_{19}NO_3$	Norcodeine	4.48	3.82	0.66
38	287.05818	$C_{15}H_{11}N_2O_2Cl$	*Demoxepam*	13.13	11.73	1.40
	287.05818	$C_{15}H_{11}N_2O_2Cl$	*Oxazepam*	14.43	13.55	0.88
39	300.15942	$C_{18}H_{21}NO_3$	*Codeine*	5.02	4.94	0.08
	300.15942	$C_{18}H_{21}NO_3$	*Hydrocodone*	6.51	6.02	0.49
40	301.07383	$C_{16}H_{13}N_2O_2Cl$	*Clobazam*	14.69	13.85	0.84
	301.07383	$C_{16}H_{13}N_2O_2Cl$	*Temazepam*	15.21	14.36	0.85
41	301.14660	$C_{18}H_{21}N_2Cl$	*Chlorcyclizine*	13.63	11.73	1.91
	301.14660	$C_{18}H_{21}N_2Cl$	*Norclomipramine*	15.00	13.36	1.64
42	302.13868	$C_{17}H_{19}NO_4$	Oxymorphone	2.36	4.38	2.02
	302.13868	$C_{17}H_{19}NO_4$	Noroxycodone	6.05	4.29	1.76
43	302.17507	$C_{18}H_{23}NO_3$	*Dihydrocodeine*	4.95	5.63	0.68
	302.17507	$C_{18}H_{23}NO_3$	*Isoxsuprine*	10.12	10.02	0.10

Table 2 (*Continued*)

	Mass $[M+H]^+$	Molecular formula	Compound	Retention time (exp[a])	Retention time (calc[b])	Retention time error (abs[c])
44	304.15434	$C_{17}H_{21}NO_4$	*Cocaine*	9.45	10.61	1.16
	304.15434	$C_{17}H_{21}NO_4$	*Scopolamine*	5.79	6.06	0.27
45	314.17507	$C_{19}H_{23}NO_3$	*Ethylmorphine*	7.21	6.32	0.89
	314.17507	$C_{19}H_{23}NO_3$	*Reboxetine*	12.22	11.44	0.78
46	325.19105	$C_{20}H_{24}N_2O_2$	Quinidine[d]	9.38	8.45	0.93
	325.19105	$C_{20}H_{24}N_2O_2$	Quinine[d]	9.68	8.42	1.26
47	328.15433	$C_{19}H_{21}NO_4$	6-Monoacethylmorphine (6-MAM)	6.63	5.91	0.72
	328.15433	$C_{19}H_{21}NO_4$	Naloxone	5.36	6.14	0.78
48	366.06737	$C_{16}H_{16}N_3O_3SCl$	*Indapamide*	13.40	15.09	1.69
	366.06737	$C_{16}H_{16}N_3O_3SCl$	*Metolazone*	12.64	13.74	1.10
49	377.20710	$C_{20}H_{28}N_2O_5$	*Enalapril*	12.25	11.86	0.39
	377.20710	$C_{20}H_{28}N_2O_5$	*Remifentanil*	9.83	10.16	0.33
50	387.15593	$C_{21}H_{26}N_2OS_2$	*Mesoridazone*	12.15	13.24	1.09
	387.15593	$C_{21}H_{26}N_2OS_2$	*Thioridazine-5-sulfoxide*	12.45	14.61	2.16

[a] Experimental, min.
[b] Calculated, min.
[c] Absolute value, min.
[d] Diastereomers.

The predicted retention time can be used as a powerful orthogonal filter to cut down the number of possible chemical structures [33–36]. Hence, retention time prediction plays an important role also in data mining procedures, such as in the metabolomics context [37].

4. Conclusion

We have demonstrated a novel approach for differentiation of structural isomers in a large target database by liquid chromatography retention order prediction. For the first time, retention time prediction software has been employed to help identify for small molecules. The results showed that, despite the rather large absolute errors between the calculated and experimental retention times, the software turned out to be a feasible predictor of the correct retention order of most compounds. The predictions were particularly adequate for structurally similar compounds, such as phenethylamine derivates. While insufficient for compound identification on its own, ACD/ChromGenius does nevertheless bring valuable information by determining retention orders, and thus making the identification of unknown compounds more reliable, even when primary reference standards are unavailable. Molecular formula determination by accurate mass measurement is an outstanding method for reliable compound identification; however, it requires further assistance in order to distinguish isomers. The software used in the present study provided additional data that can be utilized in the context of clinical and forensic toxicology drug screenings in order to tentatively characterize compounds in biological samples.

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RESEARCH PAPER

In silico and in vitro metabolism studies support identification of designer drugs in human urine by liquid chromatography/quadrupole-time-of-flight mass spectrometry

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Abstract Human phase I metabolism of four designer drugs, 2-desoxypipradrol (2-DPMP), 3,4-dimethylmethcathinone (3,4-DMMC), α-pyrrolidinovalerophenone (α-PVP), and methiopropamine (MPA), was studied using in silico and in vitro metabolite prediction. The metabolites were identified in drug abusers' urine samples using liquid chromatography/quadrupole-time-of-flight mass spectrometry (LC/Q-TOF/MS). The aim of the study was to evaluate the ability of the in silico and in vitro methods to generate the main urinary metabolites found in vivo. Meteor 14.0.0 software (Lhasa Limited) was used for in silico metabolite prediction, and in vitro metabolites were produced in human liver microsomes (HLMs). 2-DPMP was metabolized by hydroxylation, dehydrogenation, and oxidation, resulting in six phase I metabolites. Six metabolites were identified for 3,4-DMMC formed via *N*-demethylation, reduction, hydroxylation, and oxidation reactions. α-PVP was found to undergo reduction, hydroxylation, dehydrogenation, and oxidation reactions, as well as degradation of the pyrrolidine ring, and seven phase I metabolites were identified. For MPA, the nor-MPA metabolite was detected. Meteor software predicted the main human urinary phase I metabolites of 3,4-DMMC, α-PVP, and MPA and two of the four main metabolites of 2-DPMP. It assisted in the identification of the previously unreported metabolic reactions for α-PVP. Eight of the 12 most abundant in vivo phase I metabolites were detected in the in vitro HLM experiments. In vitro tests serve as material for exploitation of in silico data when an authentic urine sample is not available. In silico and in vitro designer drug metabolism studies with LC/Q-TOF/MS produced sufficient metabolic information to support identification of the parent compound in vivo.

Keywords 2-Desoxypipradrol · 3,4-Dimethylmethcathinone · α-Pyrrolidinovalerophenone · Methiopropamine · Metabolite prediction · Liquid chromatography/quadrupole-time-of-flight mass spectrometry

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Introduction

An increasing number of new designer drugs arrive annually on the illicit drug market. Between 2005 and 2012, 237 new psychoactive compounds were reported through the early warning system in Europe, and a record number of 73 new substances were notified in 2012 [1, 2]. Analytical laboratories in the fields of forensic and clinical toxicology, as well as customs and criminal investigation, need to tackle this challenge and develop methods for compound identification, even if a certified reference standard is not always available.

In terms of toxicological risk assessment and analytical method development aspect, the knowledge about designer drug metabolism is highly substantial [3–5]. The metabolism of designer drugs is commonly studied in animals, especially rats [3, 5]. In vitro experiments in metabolism studies have become popular among forensic and clinical toxicology research groups [6] and in doping control laboratories [7–10] during recent years. Drug incubation with human liver microsomes (HLMs), S9 fraction, or hepatocytes is an established ethical and cost-effective method in studies of the metabolism of new designer drugs and psychoactive compounds [6]. Their main application to date has been in enzyme kinetics.

High-resolution mass spectrometry (HRMS) applications are today widely employed in drug metabolism studies [11, 12]. Modern quadrupole-time-of-flight (Q-TOF) instruments

provide high mass resolution and accuracy on a routine basis. Q-TOF instruments operate at high data acquisition speed, and their full-range data acquisition enables retrospective data mining. Q-TOF mass analyzers are frequently used in drug metabolism research in substance identification and in confirmation of nominal mass MS data [11, 13]. Software solutions play a key role in systematic drug metabolism studies by HRMS, and most of the MS manufacturers provide software tools such as MetaboLynx (Waters, Milford, MA, USA) and MetWorks (Thermo Scientific, Waltham, MA, USA) to detect products of common metabolic pathways [14]. Mass defect filtering (e.g., MassLynx, Waters) [15] and MS/MS spectra comparison (e.g., SmileMS, GeneBio, Geneva, Switzerland) [16] software enable nontargeted metabolite screening and have served in the identification of drugs of abuse metabolites. Several software tools for metabolite prediction of xenobiotics are commercially available, three being MetaSite (Molecular Discovery Ltd, Middlesex, UK), MetaDrug (Thomson Reuters, NY, USA), and Meteor (Lhasa Limited, Leeds, UK). Drug metabolite prediction in silico is widely used to study the biotransformation of pharmaceuticals [17–21]. Prediction using commercial or self-coded software has not become common in studies of designer drugs or other toxicologically interesting compounds, however.

Here, we studied the metabolism of four structurally different designer drugs: 2-desoxypipradrol (2-DPMP, Daisy), 3,4-dimethylmethcathinone (3,4-DMMC), α-pyrrolidinovalerophenone (α-PVP), and methiopropamine (MPA). At the moment, only 2-DPMP is classified as a controlled drug of abuse in Finland, which makes the other compounds attractive among drug users, as the penalties are less severe than those for drug offenses. Methiopropamine and α-PVP are classified as pharmaceutical compounds, whereas 3,4-DMMC is not yet subject to any legislation and is categorized as a research chemical. 2-DPMP is a phencyclidine-derived compound, and its metabolism has not previously been reported. For the pyrrolidinophenone analog α-PVP, 12 phase I metabolites in rats have been reported [22]. These include hydroxylation, dehydrogenation, ring opening and oxidation reactions, and degradation of the pyrrolidine ring to the corresponding primary amine. These metabolites have not been confirmed from authentic human urine samples. The main human urinary phase I metabolites of the β-keto-structured cathinone 3,4-DMMC have recently been reported by Shima et al. [23]. They identified three metabolites, formed after *N*-demethylation and reduction of the β-ketone, using synthesized standards. In a recent metabolism study of the thiophene derivative of methamphetamine MPA, a nor-metabolite was detected in human urine [24].

We identified the main human urinary phase I metabolites of the designer drugs studied using in silico and in vitro methods for metabolite prediction. The aim was to produce sufficient metabolic information for urine drug screening to support the identification of the parent compound. In our previous study, the metabolite prediction software Meteor was found to be useful in assigning metabolites to quetiapine [20]. Here, the ability of the Meteor software to predict the metabolism of designer drugs was evaluated by comparing the results between in silico predictions and our own predictions based on known metabolic reactions of structural analogs. The in vitro metabolism of 2-DPMP, 3,4-DMMC, α-PVP, and MPA was studied using HLMs to support the in vivo metabolite findings and to test whether the HLM experiments produced the main phase I metabolites. Several authentic human urine samples, which had tested positive for the designer drugs studied, were analyzed using liquid chromatography (LC)/Q-TOF/MS, and the metabolites were characterized from their MS/MS spectra without reference standards.

Experimental

Materials

Methanol (LC-MS grade) and isopropanol (analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents (analytical grade) were from Merck (Darmstadt, Germany). Water was purified with a MilliQ Integral5 instrument (Millipore, Billerica, MA, USA). α-PVP (HCl) was obtained from Toronto Research Chemicals (Toronto, Canada), tramadol (HCl) from Nycomed Christiaens (Brussels, Belgium), and dibenzepin (HCl) of pharmaceutical purity from Sandoz (Holzkirchen, Germany). 2-DPMP, 3,4-DMMC, and MPA were obtained from material seized by the Finnish National Bureau of Investigation or from the Finnish Customs. The purity of the substances was determined by LC-chemiluminescence nitrogen detection [25] with a linear gradient separation. The purity of 2-DPMP was 50.7 %, 3,4-DMMC 85.0 %, and MPA 97.6 %, as their hydrochlorides (measurement uncertainty 6.5 %, unpublished data). The unpurified seized drug material (0.1 mM in 0.1 % formic acid + methanol) was analyzed by the LC/Q-TOF/MS method before the in vitro incubations to exclude possible contaminating active ingredients. No such substances were found, which would interfere with the metabolic reactions. Thus, the impurity was presumed to be from inactive excipients. HLMs (BD UltraPool™ HLM 150) and NADPH regenerating system solutions A (26 mM $NADP^+$, 66 mM glucose-6-phosphate and 66 mM $MgCl_2$ in H_2O) and B (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) were supplied by BD Biosciences (Woburn, MA, USA). β-glucuronidase was obtained from Roche (Mannheim, Germany). Mixed-mode solid phase extraction (SPE) cartridges with strong cation exchange and hydrophobic interactions (Isolute HCX-5, 130 mg, 10 mL) were provided by Biotage (Uppsala, Sweden).

Urine samples were either collected at autopsy or they were clinical toxicology cases investigated at our laboratory. The urine samples tested positive for one or more of the designer drugs studied in our routine drug screening by LC-TOF/MS [26]. The positive findings were confirmed and quantified by gas chromatography-MS [27]. Ten 2-DPMP cases (concentrations in urine between 0.29 and 42 mg/L), two 3,4-DMMC cases (0.53 and 1.4 mg/L), eight α-PVP cases (0.08–13 mg/L), and three MPA cases (0.52–19 mg/L) were studied. The urine samples contained several other toxicological findings, including prescription drugs and illicit drugs and their metabolites, as well as alcohol, caffeine, and nicotine. The maximum number of positive findings was 22, and the average and median were 10 and 9, respectively. The predicted metabolites were checked against the in-house toxicology database to exclude potential false-positive findings resulting from other compounds.

Urine sample preparation

Urine samples (1 mL) were hydrolyzed overnight (15 h) at 37 °C, with β-glucuronidase. Solid phase extraction [26] was used for sample preparation. The samples were reconstituted with 150 μL methanol/0.1 % formic acid (1:9 *v*/*v*). The methanol used for washing the SPE cartridge between neutral and basic extractions was evaporated and reconstituted likewise and analyzed by LC/Q-TOF/MS method to find out if any of the most polar metabolites were lost in sample preparation.

In vitro metabolism studies

Phase I metabolism in vitro of the four designer drugs, 2-DPMP, 3,4-DMMC, α-PVP, and MPA, was studied using HLMs. The reaction mixture consisted of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride, in 100 mM phosphate buffer at pH 7.4. The final reaction mixture volume was 100 μL, where the drug concentration was 100 μM and the protein concentration was 2.0 mg/mL. The reaction was initiated by addition of the microsomes, and the incubation time was 4 h at 37 °C. The reaction was terminated with 100 μL of ice-cold methanol. The samples were centrifuged (16,000 rpm, 25,000 g-units, 10 min), and the supernatant was stored at −80 °C until analysis. Test samples were prepared in triplicate. In addition to the test samples, a blank sample without the drug, biological control samples without either HLMs or NADPH regenerating solutions, and a chemical control sample without both HLMs and NADPH were prepared to determine any interference resulting from matrix compounds and spontaneously formed metabolites. A standard control sample with a compound of known in vitro metabolism, tramadol, was also included in the test set. The presence of the main in vitro phase I metabolites of tramadol [28] confirmed the incubation conditions.

In silico metabolite prediction

The metabolism of the designer drugs was predicted using Meteor 14.0.0 software (Lhasa Limited, Leeds, UK). Meteor predicts the metabolism of xenobiotics using a rule-based expert system [29]. First, substructures of a query compound labile towards a biotransformation reaction are checked in the knowledge base. Second, an absolute reasoning algorithm evaluates the possibility of that reaction taking place on five likelihood levels: probable, plausible, equivocal, doubted, and improbable [30]. Last, relative reasoning ranks concomitant metabolic reactions to remove the more improbable transformations. The following prediction parameters were used here: metabolism was outlined to include phase I reactions, the maximum number of metabolic steps was set at four, and the maximum number of predicted metabolites was 400. Absolute reasoning likelihood included probable and plausible levels for 3,4-DMMC and α-PVP and was expanded to cover equivocal level for 2-DPMP and MPA. Relative reasoning level was set at one, and mammals were selected as species. Absolute reasoning was set at a higher likelihood level for 3,4-DMMC and α-PVP because at the equivocal level, Meteor predicted over 200 metabolites for these compounds, which was thought to include an excessive number of false-positive predictions. After processing, duplicate metabolites and metabolites with a molecular mass lower than 100 were removed from the results, as they were thought most likely to be false-positive predictions. A database with the molecular formulae of both the origin compound and the predicted metabolites for each designer drug was created in Excel 2003 (Microsoft, Redmond, WA, USA).

Metabolite prediction based on published analogous reactions

The metabolism of the compounds studied was also predicted from the published metabolic reactions of their structural analogs in order to test the ability of the Meteor software to predict designer drug metabolism. The metabolism of 2-DPMP was deduced from the metabolism of phencyclidine-derived designer drugs [3]. Phencyclidines are known to undergo *N*-dealkylation, which in this case was thought to result in opening of the piperidine ring, and further oxidation of the primary alcohol. Those metabolites predicted after β-keto-structured cathinones, especially 4-methylmethcathinone (4-MMC, mephedrone) [3, 31], were added to the list of published metabolites of 3,4-DMMC [23]. The metabolites of α-PVP were taken from the study of Sauer et al. [22] in rats, supplemented with the metabolism of other pyrrolidinophenone-derived designer drugs [3, 5]. The MPA metabolites were based both on the published normetabolite [24] and as predicted by methamphetamine [32], as well as thiophene-structured compounds [33]. Metabolic

reactions were thought to be overlapping, even though this resulted in unreported metabolites. An Excel-based metabolite database of each designer drug was created as above.

Liquid chromatography/quadrupole-time-of-flight-mass spectrometry

The liquid chromatograph was a Waters Acquity Ultra Performance instrument (Milford, MA, USA) including a high-pressure mixing binary-gradient pump with a six-channel solvent degasser, a sample manager, and a thermostated column manager. A Luna PFP(2) column (pentafluorophenyl, 100×2 mm, 3 μm) and a PFP pre-column (4×2 mm, from Phenomenex, Torrance, CA, USA) were used for separation in a gradient mode at 40 °C. The mobile phase components were 2 mM ammonium acetate in 0.1 % formic acid and methanol. The flow rate was 0.3 mL/min, and the pre-run time was 5 min. The gradient started at 5 % of methanol and was increased to 40 % in 5 min, to 75 % in 13.50 min, to 80 % in 16 min, and held at 80 % for 2 min. A partial loop with needle overfill mode was used for injection, and the volume was 3 μL for the urine samples and 7.5 μL for the HLM incubation samples.

The mass spectrometer was a Bruker Daltonics micrOTOF-Q instrument (Bremen, Germany) with an orthogonal electrospray ionization source. The nominal resolution of the instrument was 10,000 FWHM. It was operated in a positive ion mode over a *m/z* range of 50–800. The drying gas flow was 8.0 mL/min, temperature was 200 °C, and the nebulizer gas pressure was 1.6 bar. The capillary voltage of the ion source was set at 4,500 V and the end plate offset at −500 V. In MS mode, the quadrupole ion energy was 5.0 eV, the collision cell radiofrequency 150.0 Vpp, the quadrupole transfer time 60.0 μs, and pre-pulse storage time 8.0 μs. The spectra time was 0.6 s and the spectra rolling average was set at 2. An external instrument calibration with sodium formate solution (10 mM NaOH in isopropanol and 0.2 % formic acid, 1:1 *v/v*) was performed using ten cluster ions ($Na(NaCOOH)_{1–10}$), *m/z* values from 90.9766 to 702.8635. The same calibrant was injected at the beginning and end of each sample run for post-run internal mass scale calibration.

AutoMS(n) methods were built for each designer drug for mass fragmentation. In the AutoMS(n) system, the instrument alternates MS and MS/MS modes in each spectrum. The selection of the precursor ion was based on a preselected mass list and an intensity threshold of 500 counts. If none of the ions from the list were detected, the most abundant ion was fragmented. A precursor ion list of the metabolites detected in the screening mode was constructed for each drug. A smart exclusion technique was used to reduce background noise, and an active exclusion mode allowed the rejection of the precursor ion after three spectra. The voltages for fragmentation were optimized by flow injection of a mixture of the compounds studied (1 μg/mL in methanol) and using an auto-optimization function. The collision energy was 20–40 eV for ions between *m/z* 100 and 500, and the energy varied from 80 to 120 % of the set value using collision sweeping. Ion source and transfer parameters were as in the screening mode. Spectra time was shortened to 0.5 s.

Data analysis

DataAnalysis 4.1 (Bruker Daltonics, Bremen, Germany) was employed to process the analysis data. An automatic database search function was created for each designer drug in TargetAnalysis 1.2 (Bruker Daltonics, Bremen, Germany) to find the predicted metabolites. This reverse database search reported hits from the LC/Q-TOF/MS acquisition data within the selected identification criteria: peak area counts of 2,000, a mass tolerance of ±3 mDa, and an isotopic pattern match, mSigma, threshold of 200. The script has been described in more detail by Ojanperä et al. [34].

Rule-based fragmentation prediction software, MS/Fragmenter 12.01 (Advanced Chemistry Development, ACD/Labs, Toronto, Canada), was used to identify and aid in structural determination of the designer drugs and their metabolites. This software was used in our previous studies [20, 35] and is described in more detail in these papers. The function "atmospheric pressure positive ion protonation" was selected as the ionization type, and the number of fragmentation steps was set at five. Other fragmentation parameters outlined aromatic bond cleavage, ring formation, resonance reaction, hydride shift, heterolytic and homolytic cleavages, hydrogen rearrangements, and neutral losses. For some product ions unidentified by the MS/Fragmenter, a DataAnalysis built-in tool for MS spectra interpretation, SmartFormula3D [36], was used to provide additional information in structural determination of the metabolites.

Results and discussion

Table 1 shows the reactions used by the Meteor software for metabolite prediction, the pathways collected from the published analogous reactions, as well as the total number of predicted metabolites, with prediction likelihood levels for each compound.

The identified human urinary metabolites of the designer drugs studied are listed in Table 2 and described below in detail. The structures of the metabolites detected in the human urine samples and in vitro experiments were confirmed by comparing the mass spectra of the metabolites with the product ions identified for the parent compounds in MS/MS spectra using MS/Fragmenter and SmartFormula3D. Fragment prediction for each metabolite was also performed, to determine the site of the metabolic reaction in the molecule and also to differentiate between possible structural isomers. In the analysis

Table 1 List of biotransformation reactions (in alphabetical order) applied to designer drug metabolite prediction based on Meteor software and the analogous reactions found in the literature

2-DPMP	3,4-DMMC	α-PVP	MPA
Meteor (n=42) pro: n=5; pla: n=14; equ: n=23	Meteor (n=69) pro: n=9; pla: n=60	Meteor (n=15) pro: n=1; pla: n=14	Meteor (n=21) pla: n=14; equ: n=7
β-Oxidation of carboxylic acids (pla)	5-Hydroxyl. of 1,2,4-subst. benzenes (pla)	**Hydroxylation of alkyl methylene (pla)**	Benzylic hydroxylation (pla)
Decarboxylation (equ)	**Hydroxylation of aromatic methyl (pro)**	Hydroxylation of terminal methyl (pla)	Decarboxylation (equ)
Hydrolysis of cyclic carboxyamides (equ)	**Hydroxylation of terminal methyl (pla)**	**Lactams from aza-alicyclic comp. (pla)**	Hydroxylation of terminal methyl (pla)
Hydroxylation of aromatic methine (equ)	**Oxidation of primary alcohols (pla)**	**Oxidation of primary alcohols (pla)**	*N*-hydroxylation of secondary amines (equ)
Lactams from aza-alicyclic comp. (pla)	Oxidation of secondary alcohols (pla)	Oxidation of secondary alcohols (pla)	Oxidation of primary alcohols (pla)
N-hydroxyl. of secondary amines (equ)	**Oxidative *N*-demethylation (pro)**	**Oxidative *N*-dealkylation (pro)**	Oxidation of secondary alcohols (pla)
Oxidation of primary alcohols (pro)	**Reduction of aliphatic ketones (pla)**	**Reduction of aliphatic ketones (pla)**	Oxidative deamination (equ)
Oxidation of secondary alcohols (pro)			**Oxidative *N*-demethylation (pla)**
Oxidative deamination (equ)			Reduction of aliphatic ketones (pla)
Oxidative *N*-dealkylation (pla)			
Para-hydroxylation of benzenes (pla)			
Reduction of aliphatic ketones (pla)			
Published analogous reactions (n=14)	Published analogous reactions (n=11)	Published analogous reactions (n=23)	Published analogous reactions (n=13)
Dehydrogenation	**Hydroxylation**	**Degradation of pyrrolidine ring**	Hydroxylation
Hydroxylation	***N*-demethylation**	**Dehydrogenation**	***N*-demethylation**
***N*-dealkylation → ring opening**	**Oxidation**	**Hydroxylation**	Sulfoxidation
Oxidation	**Reduction**	**Ring opening + oxidation**	

Total number of predicted metabolites and their likelihood levels are in brackets. True identified metabolic reactions are presented in bold
pro probable likelihood level, *pla* plausible likelihood level, *equ* equivocal likelihood level

of the wash methanol (see "Urine sample preparation"), no designer drug metabolites other than those seen in the urine samples were detected.

2-DPMP

2-DPMP (at *m/z* 252.1747) was found to undergo extensive oxidative metabolism, including aromatic hydroxylation, hydroxylation at the piperidine ring, and oxidation after ring opening. Dehydrogenated hydroxy metabolites were also found, resulting in six identified phase I metabolites in total. The proposed phase I metabolism of 2-DPMP is presented in Fig. 1a. Several characteristic product ions could be identified with the MS/Fragmenter for 2-DPMP and these are presented in detail in Fig. 2a. Product ions at *m/z* 193.1012, 181.1012, 167.0855, and 91.0542, or their modifications formed in metabolic reactions, could be detected from the MS/MS spectra of the metabolites, making the identification reliable.

Six peaks fitting the exact mass of *m/z* 268.1696, corresponding to a hydroxylated metabolite, were observed in the total ion chromatograms of the urine samples. The site of the hydroxylation reaction was concluded to take place at both aromatic (M1) and piperidine (M2) rings, which could be differentiated by their characteristic product ion spectra. Product ions (exact masses) at *m/z* 197.0961 ($C_{14}H_{13}O$) and 183.0804 ($C_{13}H_{11}O$), formed by the addition of a hydroxyl group to the corresponding 2-DPMP product ions (Fig. 2a), indicate

Table 2 Phase I metabolites identified for 2-DPMP, 3,4-DMMC, α-PVP, and MPA in authentic human urine samples. Identification criteria were ±3 mDa for mass error, mSigma threshold of 200, and peak area counts of 2,000

Metabolite found in human urine in vivo	Metabolic reaction	Formula	Rt (min)	$[M+H]^+$ (*m/z*)	Found in vitro	Found in silico	Deduced from analogous reactions
2-DPMP		$C_{18}H_{21}NO$	7.10	252.1747			
M1[a]	Hydroxylation (aromatic)	$C_{18}H_{21}NO$	5.06	268.1696		X	X
			6.30				
M2[a]	Hydroxylation (aliphatic, i.e., piperidine ring)	$C_{18}H_{21}NO$	5.86	268.1696			X
			6.09				
			6.50		X		
			8.68		X		
M3[a]	Hydroxylation (aliphatic) + dehydrogenation	$C_{18}H_{19}NO$	12.11	266.1539	X	X	X
M4[a]	2 × hydroxylation (aliphatic) + 1 × dehydrogenation	$C_{18}H_{19}NO_2$	10.46	282.1489			X
			10.83				
M5	3 × hydroxylation (aliphatic and aromatic) + 1 × dehydrogenation	$C_{18}H_{19}NO_3$	8.34	298.1438			X
M6	2 × hydroxylation (aromatic) + ring opening + oxidation	$C_{18}H_{21}NO_4$	9.97	316.1543			X
3,4-DMMC		$C_{12}H_{17}NO$	5.89	192.1383			
M1[a]	*N*-demethylation	$C_{11}H_{15}NO$	5.51	178.1226	X	X	X
M2[a]	Reduction	$C_{12}H_{19}NO$	5.45	194.1539	X	X	X
M3[a]	Reduction + *N*-demethylation	$C_{11}H_{17}NO$	4.88	180.1383		X	X
			5.06				
M4[a]	Hydroxylation	$C_{12}H_{17}NO_2$	3.18	208.1332	X	X	X
			3.67		X		
M5	*N*-demethylation + hydroxylation	$C_{11}H_{15}NO_2$	5.88	194.1176		X	X
M6	Reduction + hydroxylation + oxidation	$C_{12}H_{17}NO_3$	4.68	224.1281	X	X	X
α-PVP		$C_{15}H_{21}NO$	5.70	232.1696			
M1[a]	Reduction	$C_{15}H_{23}NO$	5.84	234.1852	X	X	
M2	Hydroxylation	$C_{15}H_{21}NO_2$	5.35	248.1645	X	X	X
M3[a]	Hydroxylation + dehydrogenation	$C_{15}H_{19}NO_2$	12.02	246.1489	X	X	X
M4[a]	Reduction + hydroxylation + dehydrogenation	$C_{15}H_{21}NO_2$	8.90	248.1645		X	
			9.95		X		
M5	Degradation of pyrrolidine ring	$C_{11}H_{15}NO$	4.95	178.1226	X		X
M6	Hydroxylation + dehydrogenation + ring opening + oxidation	$C_{15}H_{21}NO_3$	5.97	264.1594	X		X
			6.67				
M7	Hydroxylation + oxidation	$C_{15}H_{19}NO_3$	10.42	262.1438		X	
MPA		$C_8H_{13}NS$	3.13	156.0841			
M1[a]	*N*-demethylation	$C_7H_{11}NS$	2.79	142.0685	X	X	X

[a] Abundant metabolites in human urine

aromatic hydroxylation. The product ion spectra from the M1 peaks were identical; thus, differentiation of the compounds was not possible, nor could the exact site of the hydroxylation be determined. Metabolite M1 was detected in HLM experiments only at trace levels.

In addition to the corresponding product ions of 2-DPMP, the loss of water was detected in the spectra of metabolites M2 hydroxylated at the piperidine ring. Metabolites M2 were present in relatively high abundance in the urine samples, signifying that hydroxylation at the piperidine ring is the main phase I metabolism route of 2-DPMP. These peaks are possibly a mixture of regioisomers and conformational isomers, forming sum spectra, as the peaks were not fully separated at their baseline. The metabolite M2 eluting at

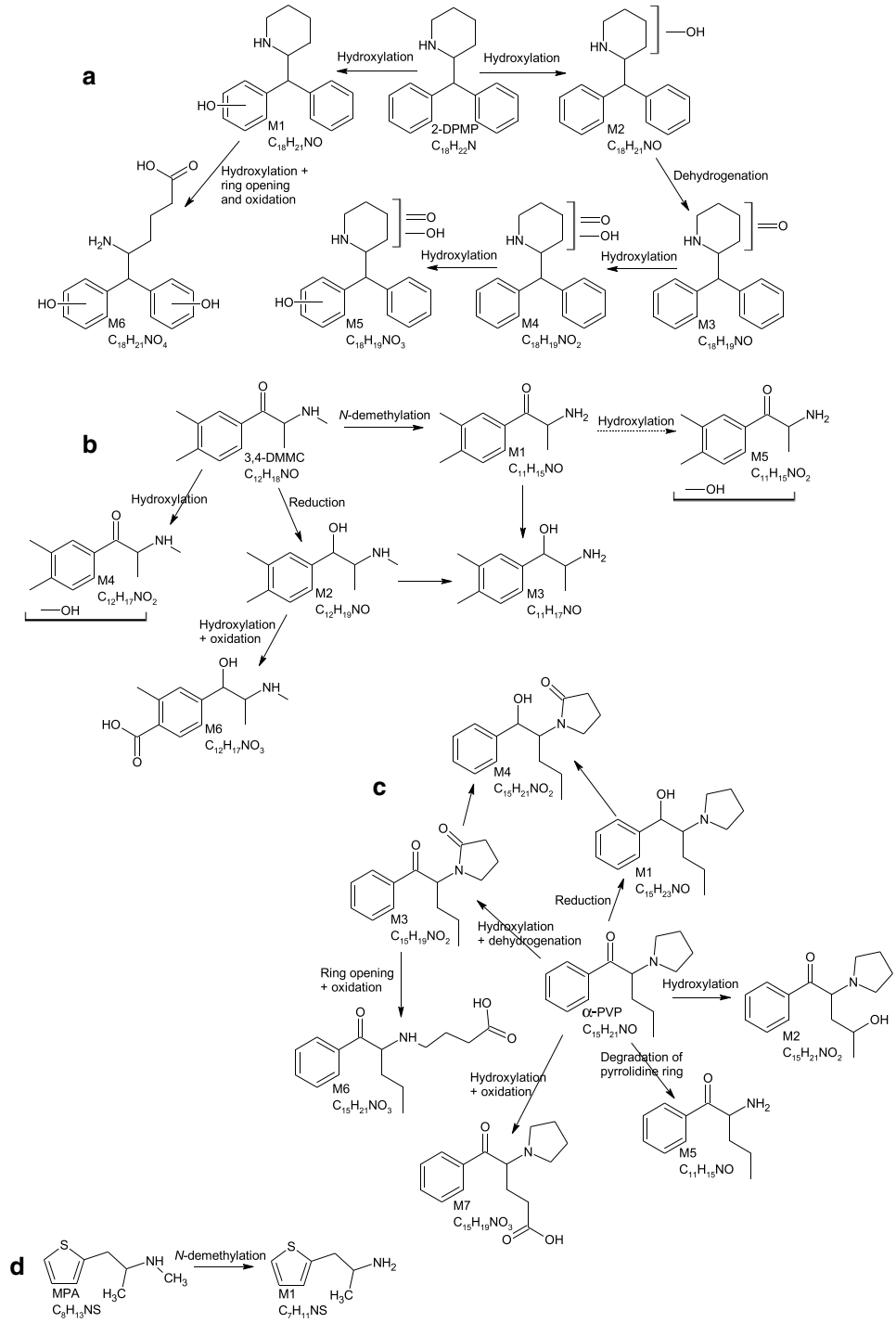


Fig. 1 Proposed phase I metabolism of 2-DPMP, 3,4-DMMC, α-PVP, and MPA in humans. Metabolite M5 of 3,4-DMMC (*dashed arrow*) was identified solely by exact mass and isotopic pattern comparison

8.68 min was a minor urinary metabolite, but it was present in the HLM samples in rather high abundance. The Meteor software did not predict hydroxylation at carbon atoms in the piperidine ring under the chosen reasoning constraints. However, the α-carbinolamine structure is expressed as a reaction intermediate in the lactam (metabolite M3) formation. In the absence of an intermolecular hydrogen bonding stabilization, this structure is generally not observed in vivo. Because of the relative reasoning filter applied, biotransformations expressing hydroxylation at the alicyclic carbon atoms are discarded. With a lower filter, these metabolites would also have been observed. *N*-hydroxylation at the piperidine nitrogen was predicted for 2-DPMP by Meteor (Table 1) at equivocal likelihood level. *N*-hydroxylated secondary aliphatic amines may react further to produce more complex compounds [37], which means that these metabolites could not be detected in human urine. The presence of this metabolite is therefore rather doubtful.

2-DPMP M2 metabolites were found to undergo dehydrogenation (M3 at *m/z* 266.1539) to a corresponding lactam or ketone. In the HLM samples, three peaks showing $[M+H]^+$ at *m/z* 266.1539 at 11.00, 11.47, and 12.11 min were observed. Traces of these two first eluting compounds were also present in the urine samples, and thus, reliably interpretable MS/MS spectra could not be produced to confirm the structures.

The 2-DPMP metabolite M4 (at *m/z* 282.1489) was detected in rather high abundance. It was formed via two hydroxylation reactions at the piperidine ring, one of the hydroxyl groups subsequently undergoing dehydrogenation. The metabolic reactions of M4 were found to take place only at the piperidine ring, as the product ion at *m/z* 167.0855 indicated the aromatic rings were not hydroxylated. The loss of H_2O (at *m/z* 264.1383), followed by the loss of CO (at *m/z* 236.1434) and NH_3 (at *m/z* 219.1168), confirmed this presumption. A minor peak of M4, detected at 10.46 min, indicates possible isomerism of this metabolite. Metabolite M5 (at *m/z* 298.1438) was formed via aromatic hydroxylation of M4. Characteristic product ions for aromatic hydroxylated compounds (see M1 above) were detected for metabolite M5. In addition, product ions formed via loss of H_2O, CO, and NH_3 could be seen, similar to M4, at 15.9949 atomic mass units and higher *m/z* values. This shows a difference of one hydroxyl group between M4 and M5, located outside the piperidine ring system. M4 and M5 were not detected in in vitro experiments nor were they predicted by the Meteor software.

2-DPMP was found to undergo oxidative *N*-dealkylation resulting in ring opening of the piperidine structure and oxidation of the primary alcohol to a carboxylic acid. Metabolite M6 (at *m/z* 316.1543) was found to comprise the previous structure and in addition was twice hydroxylated at the aromatic rings. Product ions $[M+H–NH_3]^+$ at *m/z* 299.1278 and $[M+H–NH_3–CO_2H_2]^+$ at *m/z* 253.1223, and those identical to other aromatic hydroxy metabolites, specified this structure. Meteor suggested an oxidative *N*-dealkylation reaction, followed by oxidation of the primary alcohol. Nevertheless, the formation of M6 was not predicted by the software, as the likelihood of sequential hydroxylation reactions in the two aromatic rings is assigned as doubted.

Fig. 2 Proposed fragmentation schemes identified using fragment prediction for 2-DPMP, 3,4-DMMC, α-PVP, and MPA and the corresponding MS/MS spectra. Product ions denoted with an *asterisk* (*) were detected in metabolite MS/MS spectra

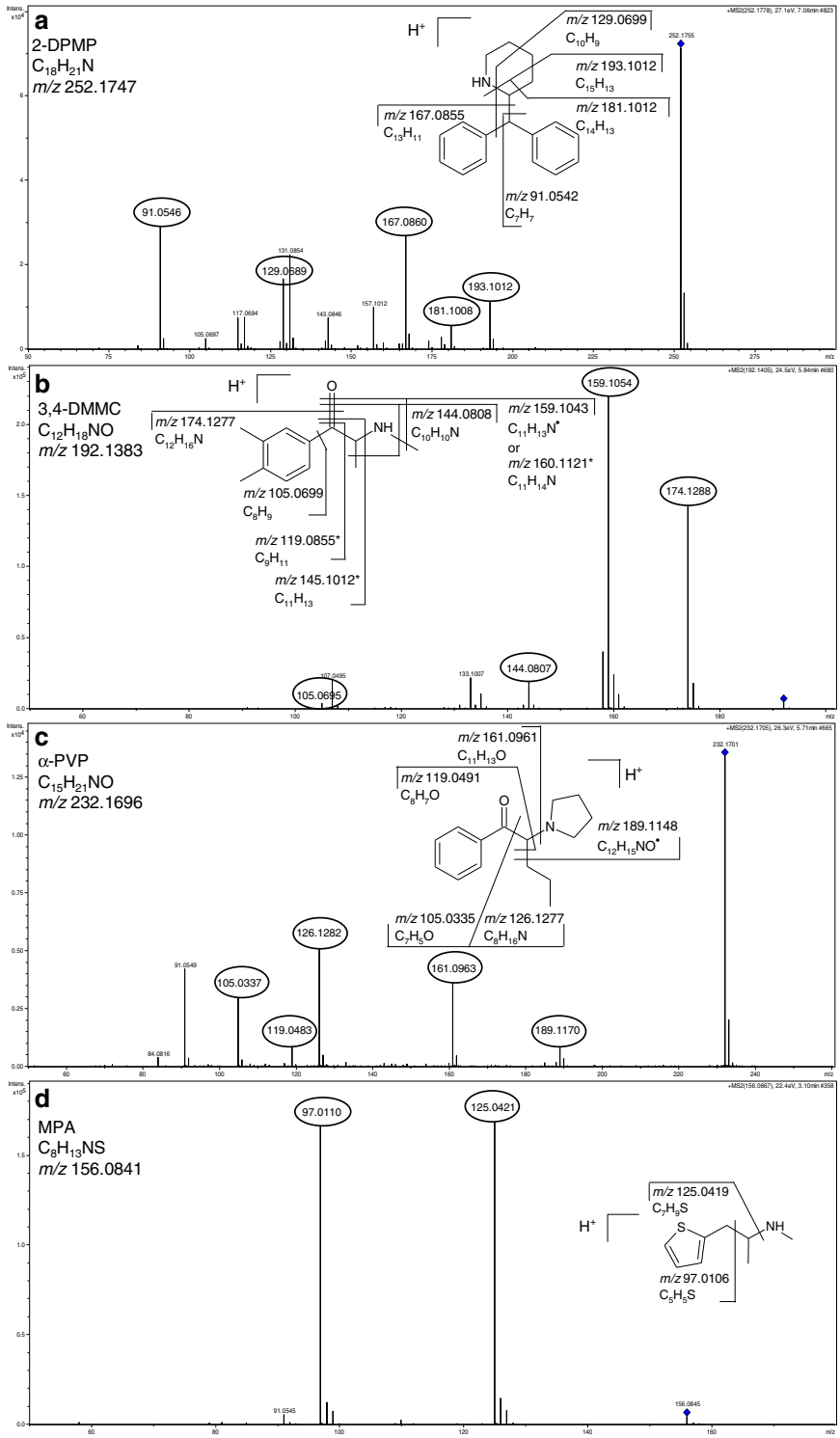
Great individual variation in metabolite abundances was noticed between the 2-DPMP cases studied, which makes the determination of the main metabolites difficult. Metabolites M1 and M2 were identified in nine cases out of ten, indicating hydroxy metabolites would be the primary phase I human metabolites. Metabolites M3–M6 were thought to be minor 2-DPMP metabolites, as the intensities of the compounds were in some cases quite low. The number of other toxicological findings in the urine cases studied was up to 15. Possible drug–drug interactions may have an influence on the metabolic ratios. The urine concentrations of 2-DPMP itself varied greatly as well, and thus, care should be taken when concluding the quantity of the metabolites.

3,4-DMMC

Six metabolites could be identified for 3,4-DMMC in human autopsy urine cases (Table 2). They included the recently reported [23] *N*-demethylated (M1) and reduced metabolites (M2) and the combination of the reactions (M3). Hydroxylated (M4 and M5) and further oxidated (M6) metabolites were detected and identified here as well. Compounds M4 and M6 have previously been reported as putative metabolites, as the identification was based on nominal mass MS/MS data and relative retention times [23]. All metabolites identified here were predicted by the Meteor software. The proposed metabolism of 3,4-DMMC is presented in Fig. 1b.

In the MS/MS spectrum of 3,4-DMMC (at *m/z* 192.1383), two main peaks formed after fragmentation of the ketone group (at *m/z* 174.1277), together with further loss of a methyl radical (at *m/z* 159.1043), were detected (Fig. 2b). Fragmentation of the metabolites mainly followed the path of 3,4-DMMC. An additional loss of water was detected in the MS/MS spectra of hydroxylated and oxidated metabolites. Other characteristic product ions, identified for the metabolites using the MS/Fragmenter, are presented with an asterisk (*) in Fig. 2b.

Two peaks were identified for metabolite M3 (at *m/z* 180.1383), formed via *N*-demethylation and reduction reactions (Table 2). The product ion spectra of these compounds were identical, indicating the formation of diastereomers [23].


a
2-DPMP
$C_{18}H_{21}N$
m/z 252.1747
m/z 129.0699
$C_{10}H_9$
m/z 193.1012
$C_{15}H_{13}$
m/z 181.1012
$C_{14}H_{13}$
m/z 167.0855
$C_{13}H_{11}$
m/z 91.0542
C_7H_7
91.0546
167.0860
129.0589
193.1012
181.1008
b
3,4-DMMC
$C_{12}H_{18}NO$
m/z 192.1383
m/z 174.1277
$C_{12}H_{16}N$
m/z 144.0808
$C_{10}H_{10}N$
m/z 159.1043
$C_{11}H_{13}N^{\bullet}$
or
m/z 160.1121*
$C_{11}H_{14}N$
m/z 105.0699
C_8H_9
m/z 119.0855*
C_9H_{11}
m/z 145.1012*
$C_{11}H_{13}$
159.1054
174.1288
144.0807
105.0695
c
α-PVP
$C_{15}H_{21}NO$
m/z 232.1696
m/z 161.0961
$C_{11}H_{13}O$
m/z 119.0491
C_8H_7O
m/z 189.1148
$C_{12}H_{15}NO^{\bullet}$
m/z 105.0335
C_7H_5O
m/z 126.1277
$C_8H_{16}N$
126.1282
105.0337
161.0963
119.0483
189.1170
d
MPA
$C_8H_{13}NS$
m/z 156.0841
97.0110
125.0421
m/z 125.0419
C_7H_9S
m/z 97.0106
C_5H_5S

Racemization of the reduced metabolite M2 could not be shown in the analysis of the urine and in vitro samples. However, confirmation of the possible isomerism of M2 would require further analysis under different separation conditions, e.g., with a chiral column.

Two peaks for the hydroxylated metabolite M4 (at *m/z* 208.1332) were detected in urine and HLM samples (Table 2). Previous studies [23, 29] indicate that the hydroxylation takes place at the aromatic methyl group. In addition to this, Meteor predicted hydroxylations at terminal methyl groups of the side chain (Table 1). The MS/MS spectra of the hydroxy metabolites were identical in both ion quality and abundance, possibly indicating that the hydroxylation reaction takes place at either of the aromatic methyl groups. The prediction likelihood also applies to the aromatic methyl hydroxylation (Table 1). However, hydroxylation of the aliphatic methyl group could not be excluded.

A previously unreported metabolite of 3,4-DMMC, metabolite M5, resulted from *N*-demethylation and hydroxylation (at *m/z* 194.1176). It eluted at the same time as 3,4-DMMC (at *m/z* 192.1383); thus, a characteristic MS/MS spectrum of M5 could not be produced, as the separation mass window in the quadrupole had to be wide enough to utilize the isotopic pattern match comparison in the compound identification in MS/MS analysis. The identification of M5 is based on accurate mass measurement only, and further studies should be carried out to confirm its existence.

Metabolite M6 (at *m/z* 244.1281) was produced via reduction of the ketone group, followed by hydroxylation and oxidation of the aromatic methyl group. The most abundant product ion in its spectrum at *m/z* 174.0913 corresponded to the structure $C_{11}H_{12}NO$ ($[M+H–H_4O_2–CH_2]^+$). Detection of an ion at *m/z* 137.0597 (product ion at *m/z* 105.0699+O_2, Fig. 2b.) showed that the position of the carboxylic acid group was in the xylyl methyl. Metabolite M6 was confirmed in only one of the urine samples and was detected in vitro only as trace levels, which would indicate it is a minor metabolite of 3,4-DMMC.

α-PVP

α-PVP (at *m/z* 232.1696) was metabolized extensively, as seven phase I metabolites were detected in the human urine samples (Table 2). The proposed metabolism is presented in Fig. 1c. Characteristic product ions were identified using fragment prediction (Fig. 2c). α-PVP was found to metabolize by hydroxylation at the propyl side chain (M2 at *m/z* 248.1645), hydroxylation followed by dehydrogenation at the pyrrolidine ring to form a lactam structure (M3 at *m/z* 246.1489), and degradation of the pyrrolidine ring to a primary amine (M5 at *m/z* 178.1226). These metabolites were previously identified in rat urine [22] and were found here in the HLM samples as well. For the hydroxy-α-PVP (M2), the product ion at *m/z* 189.1148 ($[M+H–C_3H_7O]^{+\cdot}$) showed that the hydroxylation takes place at the propyl side chain. Metabolite M3 showed a characteristic product ion of a γ-lactam structure at *m/z* 86.0600 (C_4H_8NO). For metabolite M5, product ions corresponding to the loss of water (at *m/z* 160.1121) followed by the loss of a propyl side chain (at *m/z* 118.0651) were identified.

Metabolite M1 (at *m/z* 234.1852), formed by reduction of the ketone structure to a corresponding alcohol, was the most abundant metabolite of α-PVP in both human urine and in vitro experiments. Loss of water, followed by pyrrolidine ring loss, or propyl side chain loss as a radical cation was identified as an indication of the hydroxylated structure. This metabolic reaction has not previously been reported for α-PVP [22]. Here, it was predicted by the Meteor software. Metabolite M4 (at *m/z* 248.1645) was found to be derived through a combination of reduction (M1) and lactam formation (M3) reactions. Metabolite M4, which has two chiral atoms, was expressed as potential diastereomers [38, 39], at 8.90 and 9.95 min, with identical MS/MS spectra. The structure of M4 was verified by identification of product ions at *m/z* 230.1539 from the loss of water and the loss of the lactam ring at *m/z* 145.1012.

Metabolite M6 (at *m/z* 264.1594) was formed from metabolite M3 by oxidation after pyrrolidine ring opening. Product ions from the loss of water, the loss of acetic acid, and the loss of aminobutyric acid were identified, demonstrating the proposed structure. Two peaks of M6 with identical MS/MS spectra and ion abundances were detected (Table 2), indicating the possible formation of enantiomers. In the HLM incubations, only one form of this metabolite was produced, as only the first eluting compound was observed.

Hydroxylation followed by oxidation at the propyl side chain produced metabolite M7 (at *m/z* 262.1438). This proposed structure was based on the detection of benzaldehyde and a loss of propanoic acid from the parent compound. Oxidation of the propyl side chain was not identified for α-PVP in rat urine [22] nor was it reported for other pyrrolidinophenone derivatives [38–44]. Metabolite M7 was predicted by Meteor, but not seen in the in vitro tests.

An additional metabolite of α-PVP, formed via reduction and hydroxylation (at *m/z* 250.1802), was detected in HLM incubations, but was not present in human urine samples, however.

Four of the seven metabolites identified here for α-PVP were new metabolites, and two of them formed via an unreported metabolic reaction, i.e., reduction of the β-ketone and oxidation of the propyl side chain. Three out of the ten phase I metabolites of α-PVP detected in rat urine [22] were identified here. For the pyrrolidinophenone derivatives studied in rats, reduced metabolites have only been reported as minor metabolites for α-pyrrolidinopropiophenone, PPP [38], 4′-methyl-α-pyrrolidinohexanophenone, MPHP [39], and 4′-

methyl-α-pyrrolidinobutyrophenone, MPBP [43]. Here, the reduced metabolite M1 was found to be as the most abundant metabolite in all eight human urine samples studied. The fact that reduction of the ketone group was not detected in rats may be because of differences between species in the dominant metabolic reactions [45, 46]. Metabolites formed via reduction of the oxo group in humans have been reported for β-keto-structured cathinones [31, 47, 48], however. Based on the findings in this study, reduction of the ketone should be taken into consideration in the identification of human urinary metabolites of pyrrolidinophenone derivatives.

Sauer et al. [22] proposed that the urine screening procedure used for α-PVP, which is metabolized to a great extent in rats, should be based on its metabolites. Although α-PVP also undergoes significant metabolism in humans, the drug itself was the most abundant finding in all urine samples analyzed. Thus, identification of the metabolites in conjunction with α-PVP in human urine makes identification of this compound substantially more reliable.

MPA

In the analysis of three urine cases that tested positive for MPA (at *m/z* 156.0841), the *N*-demethylated metabolite nor-MPA (M1 at *m/z* 142.0685) was identified (Fig. 1d and Table 2). The product ions identified were identical for MPA and nor-MPA (Fig. 2d). Traces of MPA hydroxy metabolites could also be seen in the in vitro experiments; however, they could not be identified in human urine. The results are in concordance with the recent paper by Welter et al. [24], who stated that MPA metabolized to only a minor extent, as only one phase I metabolite, nor-MPA, was detected in human urine.

Feasibility of in silico and in vitro experiments in identification of metabolites in vivo

The likelihood level of a metabolic reaction calculated by Meteor was found to be a reasonable indicator of the probability of the prediction. All the reactions identified were either at a probable or plausible level. Four of the five probable reactions, oxidation of primary alcohol, hydroxylation of aromatic methyl, and oxidative *N*-demethylation or *N*-dealkylation (Table 1), were found to occur. The predictions proved to be most successful for the phenethylamine-structured designer drugs: Meteor predicted all the identified metabolites of 3,4-DMMC and MPA, as well as five of the seven metabolites of α-PVP. It improved the identification of the previously unreported metabolic reactions for α-PVP: reduction of the ketone group and oxidation of the propyl side chain, resulting in detection of three new metabolites. The Meteor prediction results were less successful for 2-DPMP, as only two of the six metabolites identified had been proposed. One of the most abundant metabolites of 2-DPMP, M2, was predicted only as an intermediate in the formation of a lactam structure. It is likely that Meteor finds only a few metabolic reactions from its knowledge base for compounds structurally similar to 2-DPMP, as most of the predictions were created at the equivocal likelihood level. This would explain the low prediction sensitivity [17]. The unpredicted metabolites would probably be achievable by widening the Meteor processing settings. This would, however, also produce a great number of false-positive predictions. The Meteor software has shown a tendency towards overprediction [17], as also discovered in this study. Thus, the relevance of metabolites predicted at a likelihood level lower than "plausible" is at least questionable when the aim is to identify the main urinary metabolites.

In silico metabolite prediction has not previously been applied to designer drugs, and one of the goals in this study was to evaluate the applicability of the Meteor software in this area. Based on the findings in this study, Meteor is a suitable tool for predicting the main human phase I metabolites for amphetamine analogs and phenethylamine analogs. For phencyclidine compounds, or structurally completely novel designer drugs, the prediction results need to be viewed more critically. It is necessary to evaluate the suitability of in silico prediction for each designer drug class individually. The software predictions should always be compared with biological material, in vivo or in vitro samples, to screen for the true-positive metabolites.

Drug metabolism prediction based on published analogous reactions is time consuming and, in the case of structurally new compounds, sometimes uncertain. Although metabolite prediction in silico did not prove here to be better than our own judgment based on the metabolic reactions of structural analogs, it definitely speeds up the creation of the list of possible metabolites to be used in an automated database search for Q-TOF/MS data.

Eight of the 12 most abundant phase I metabolites of the four designer drugs studied in human urine could be detected in the in vitro HLM experiments. A few designer metabolites detected in the HLM samples in minor abundance were not seen in human urine. Thus, the difference between in vitro and in vivo metabolism [6] should be taken into consideration when extrapolating the metabolic data from in vitro experiments to identification of metabolites in vivo. Although in vitro tests do not absolutely predict human in vivo metabolism [6], they definitely serve as material for exploitation of in silico data when an authentic urine sample is not available. The results can be extrapolated for qualitative drug screening analysis in toxicology to support a positive finding of a parent compound. However, to perform in vitro metabolite experiments on designer drugs requires the availability of reference standards for the parent drugs, which limits the usage of the procedure for rare compounds.

The metabolites proposed for the designer drugs studied here do not necessarily cover all the possible phase I metabolites of the compounds. Drug–drug interactions may also

have an influence on the metabolite ratios. A comprehensive designer drug metabolism study on forensic cases is demanding, as information about the possible intake of the drug, or the time since intake, is rarely available. The benefits of in silico predictions are in qualitative metabolite identification which was the main objective of this study.

Conclusion

Applying in silico and in vitro experiments to support identification of designer drug metabolites in drug abusers' urine samples by LC/Q-TOF/MS was both effective and straightforward. The LC/Q-TOF/MS instrumentation used provided sufficient sensitivity for identification of designer drug metabolites in a complex biological matrix that also contained a great number of prescription drugs and street drugs. Compound identification with the automated reverse database search method was feasible, even though the metabolite peaks were partly overlapping. Structural characterization of fragments by accurate mass data, assisted by the MS/MS data interpretation tool MS/Fragmenter, served well in the differentiation between structural isomers. Eleven previously unreported metabolites for the four designer drugs studied, 2-DPMP, 3,4-DMMC, α-PVP, and MPA, were identified here. Six metabolites, including hydroxylated and further dehydrogenated and oxidated compounds, were detected for 2-DPMP, for which the metabolism has not been published earlier. The hydroxy-*N*-desmethyl metabolite was a new product found in this study for 3,4-DMMC. Four of the α-PVP metabolites found here, formed via reduction of the β-ketone and oxidation reactions, were not detected in the earlier metabolism studies in rats. The in silico metabolite prediction method proved to be a rapid way to create a list of possible metabolites for a novel designer drug, which can further be screened from in vitro incubation samples to identify the true-positive metabolites. These tentatively identified metabolite formulas could then be added to the database used for routine urine drug screening, in order to facilitate designer drug identification in authentic urine samples.

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