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REVIEW

Current status of hyphenated mass spectrometry in studies of the metabolism of drugs of abuse, including doping agents

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Abstract This paper reviews scientific contributions on the identification and/or quantification of metabolites of drugs of abuse in in vitro assays or various body samples using hyphenated mass spectrometry. Gas chromatography–mass spectrometry (GC-MS) as well as liquid chromatography–mass spectrometry (LC-MS) approaches are considered and discussed if they have been reported in the last five years and are relevant to clinical and forensic toxicology or doping control. Workup and artifact formation are discussed, and typical examples of studies of the metabolism of designer drugs, doping agents, herbal drugs, and synthetic cannabinoids are provided. Procedures for quantifying metabolites in body samples for pharmacokinetic studies or in enzyme incubations for enzyme kinetic studies are also reviewed. In conclusion, the reviewed papers showed that both GC-MS and LC-MS still have important roles to play in research into the metabolism of drugs of abuse, including doping agents.

Keywords Drugs of abuse · Doping · Mass spectrometry · Liquid chromatography · Gas chromatography · Metabolism

Abbreviations

2C-I	4-Iodo-2,5-dimethoxy-beta-phenethylamine
2C-T-2	2,5-Dimethoxy-4-ethylthio-beta-phenethylamine
AC	Acetylation
AEME	Anhydroecgonine methyl ester
APCI	Atmospheric pressure chemical ionization
BDB	Benzodioxolyl-butanamine
CID	Collision-induced dissociation
CYP	Cytochrome P450
DOA	Drugs of abuse
DOB	4-Bromo-2,5-dimethoxyamphetamine
DOC	4-Chloro-2,5-dimethoxyamphetamine
DOI	4-Iodo-2,5-dimethoxyamphetamine
EDDP	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EI	Electron ionization
EMDP	2-Ethyl-5-methyl-3,3-diphenylpyrroline
ESI	Electrospray ionization
GC	Gas chromatography
GC-MS	Gas chromatography–mass spectrometry
GHB	Gamma-hydroxybutyric acid
HFB	Heptafluorobutyrilation
HRMS	High-resolution mass spectrometry
LC	Liquid chromatography
LC-MS	Liquid chromatography–mass spectrometry
LIT	Linear ion trap
LLE	Liquid–liquid extraction
MBDB	Methylbenzodioxolylbutanamine
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-Methylenedioxyethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MDOB	4-Bromo-2,5-dimethoxymethamphetamine
MDPV	Methylenedioxypyrovalerone
MPBP	4-Methylpyrrolidinobutyrophenone
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NICI	Negative-ion chemical ionization
ODT	*O*-demethyl tramadol
PCEEA	*N*-(1-phenylcyclohexyl)-2-ethoxyethanamine
PCEPA	*N*-(1-phenylcyclohexyl)-3-ethoxypropanamine

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PCMEA	*N*-(1-phenylcyclohexyl)-2-methoxyethanamine
PCMPA	*N*-(1-phenylcyclohexyl)-3-methoxypropanamine
PICI	Positive-ion chemical ionization
PMMA	*P*-methoxymethamphetamine
PP	Protein precipitation
Q	Quadrupole
QTOF	Quadrupole–time-of-flight hybrid
QTRAP	Quadrupole–linear ion trap hybrid
RI	Retention index
S-HFBPCl	*S*-heptafluorobutyrylprolyl chloride
SPE	Solid-phase extraction
TFA	Trifluoroacetylation
TMA-2	2,4,5-Trimethoxyamphetamine
TMS	Trimethylsilylation
TOF	Time of flight
UGT	Uridine-5′-diphosphoglucuronosyltransferases

Introduction

In the context of preclinical drug discovery, metabolism studies are mandatory, as metabolites can be pharmacologically active in addition to or instead of the parent drug, or they can show toxic effects. Also, the extent to which the drug is metabolized by the various phase I and II isoenzymes may markedly influence its duration of action and its elimination. Thus, metabolism studies must be presented when a new drug is submitted for approval by drug administrations. In contrast, new drugs of abuse (DOAs: chemically synthesized or herbal drugs) are distributed on the black market without any safety (pharmacology/toxicology) testing. Therefore, clinical and forensic toxicological institutions or doping control organizations need to study the pharmaco/toxicokinetics of DOAs, especially their metabolism.

In recent years, a series of new drug classes have appeared on the illicit drugs market [1–3]. Although designer drugs have the reputation of being safe, several experimental studies in rats and humans and epidemiological studies have indicated risks to humans, including a life-threatening serotonin syndrome, hepatotoxicity, neurotoxicity, psychopathology, and abuse potential [1, 4–11]. As metabolites are suspected to contribute to some of these toxic effects [5, 12, 13], and knowledge of them is important when developing screening approaches, the main steps in the metabolism of these new drugs must be elucidated. Furthermore, variations in the formation of pharmacologically active metabolites, the formation of toxic metabolites, or interactions with other medicaments may affect the analytical results obtained in clinical or forensic toxicology as well as in doping control. Additionally, there is good evidence that the metabolism of a drug by genetically variable cytochrome P450 (CYP) isoforms can influence the risk of drug dependence, the amount of drug consumed by dependent individuals, and some of the toxicities associated with drug-taking behavior [2].

In metabolism studies, one major step is the elucidation of the chemical structures of the phase I and II metabolites, while the other step is the quantification of the metabolites in either in vitro assays for enzyme kinetics or body samples for pharmacokinetics. Today, different hyphenated mass spectral technologies are available, such as gas chromatography (GC) or liquid chromatography (LC) coupled to single-stage or tandem mass spectrometry (MS or MS/MS) with quadrupole (Q), linear ion trap (LIT), or time-of-flight (TOF) analyzers [14]. GC-MS is still used for the identification and quantification of phase I metabolites of smaller molecules, while LC-MS is preferable for larger phase I metabolites, and particularly for phase II metabolites such as glucuronides and sulfates [2]. In certain cases, however, GC-MS could benefit the analysis of compounds with higher masses, such as steroids or cannabinoids [15, 16]. High-resolution MS (HRMS) provides better selectivity and thereby sensitivity, but it also markedly improves the identification power by providing the elemental compositions of the fragments. Thus, isobaric fragments with different elemental compositions from different parts of the molecule can be differentiated. Another review article to be published in a special issue of this journal on HRMS will focus on the power of HRMS in drug metabolism studies (see Meyer and Maurer, Anal Bioanal Chem, 2012). Liang et al. [14] discussed recent developments in the field of MS techniques coupled to LC, including post-acquisition data processing and mining modes, and compared the suitabilities of various MS techniques coupled to LC for studies of drug metabolism.

In the following, recent papers that describe the application of hyphenated MS to the identification and quantification of metabolites of DOAs in in vitro assays and biosamples will be critically reviewed. Only papers written in English and published in the last five years were considered.

GC-MS for studies of the metabolism of DOAs, including doping agents

GC-MS has a high separation power, and provides—when electron ionization (EI) is used—fragment-rich mass spectra that are easily interpreted by following established fragmentation rules [17, 18]. While molecules with longer alkyl (amine) side chains do not form molecular ions after EI, such information can still be obtained using positive-ion chemical ionization (PICI) [19–28].

Sample workup

Sample workup is mandatory for GC-MS approaches, and may consist of cleaving conjugates, extraction, and derivati-

zation [29]. For metabolism studies, only gentle enzymatic cleavage of conjugates should be used, and the different selectivities of the various enzyme preparations must be considered, particularly for quantitative assays [30–33]. Liquid–liquid extraction (LLE) or (more selective) solid-phase extraction (SPE) procedures are used to isolate and enrich the metabolites, as shown in Tables 1 and 2. The use of common liquid–liquid extraction under alkaline or acidic conditions is not appropriate if the metabolites show amphoteric properties [34]. In contrast, mixed-mode SPE has been successfully applied to the extraction of amphoteric metabolites of pyrrolidinophenones [35–39]. In addition, the volatility of the free bases and the instability of the analytes under alkaline and high-temperature extraction conditions can cause difficulties with amphetamine-related compounds [40, 41]. Various reactions are currently used to derivatize mostly polar metabolites (Tables 1 and 2), such as methylation, acetylation (AC), trimethylsilylation (TMS), trifluoroacetylation (TFA), or heptafluorobutyrilation (HFB), which can be completed in a few minutes using microwave assistance [29]. Selective derivatization may help with the interpretation of mass spectral fragmentation, and thus with the identification of the metabolite structure. For example, methylation using diazomethane allowed differentiation between carboxylic acids (derivatized in minutes), phenolic groups (in hours), and aliphatic hydroxy groups (not at all) [26, 42]. However, as discussed below, the formation of artifacts during workup must be considered [29], as this can lead to incorrect metabolite identification and imprecise quantification.

Pitfalls and artifact formation in GC-MS studies

Artifacts formed during sample preparation or passage through the hot injection port, the GC column, and/or the ion source can lead to false elucidations of the metabolite structure. Examples such as *N*- or *S*-oxidation, the hydrolysis of esters, ethers or amides, dehydration or hydration, formylation by methanol, Cope elimination of *N*-oxides, and decarboxylation are described in detail elsewhere [29]. Theobald et al. described for example the formation of methylene artifacts in methanolic solution upon studying the metabolism of the designer drugs 2,5-dimethoxy-4-ethylthio-beta-phenethylamine (2C-T-2) and 4-iodo-2,5-dimethoxy-beta-phenethylamine (2C-I) [43, 44]. They were able to prove the formation of these artifacts by using deuterated methanol as solvent. The resulting MS spectrum showed a shift of two mass units for the molecular ion compared to the mass spectrum of the presumed methylene artifact. Similar artifact formation was described for ephedrines, which were transferred to 3,4-dimethyl-5-phenyl-1,3-oxazolidine when performing an analysis in methanolic solution [45]. However, these formyl adducts can easily be avoided using derivatization, which is recommended anyway.

Metabolism of designer drugs of abuse

Most designer drugs of abuse are arylalkylamines with molecular masses of below 250 u. Metabolic changes in nitrogen-containing side chains such as in amphetamines are easier to elucidate with GC-MS than with any LC-MS method [46]. This is due to the formation of characteristic ions such as those at *m/z* 58, 72, 86, 100 as a result of radical-based alpha-cleavage. Such ions, when altered appropriately after derivatization (e.g., *m/z* 100 as acetylated 58), easily allow for example primary and secondary amines to be distinguished [18]. A series of metabolism studies on such designer drugs have been published by the authors' group over the last few years, based on the enzymatic cleavage of conjugates, LLE or SPE followed by acetylation, methylation, or silylation using EI and PICI techniques [2, 47]. The papers are summarized in Table 1. The limitation of GC-MS in these studies was evident: identification of the conjugates. Their existence was only postulated because the amount of phase I metabolites increased after conjugate cleavage. Therefore, more recent studies included LC-MS at the very least in order to identify the glucuronides and sulfates [27].

Theobald et al. studied designer drugs of the phenethylamine type [43, 44, 48–50], Ewald et al. investigated those of the 2,3-dimethoxyamphetamine type [19, 21, 51–53], Sauer et al. researched those of the phencyclidine-type [23–25, 54], and finally Sauer et al. [26] and Meyer et al. [27] studied new pyrrolidinophenones. The metabolism of phenethylamine-type drugs and piperazines was tested years ago and is reviewed elsewhere [47]. GC-MS after acetylation and silylation was applied to the studies of the distribution and metabolism of *para*-methoxymethamphetamine (PMMA) [55]. The rat and human phase I metabolites of cathinone derivatives—so-called beta-ketos, a new class of designer drugs—were also identified by GC-MS [28, 56, 57]. For example, Fig. 1 shows EI mass spectra, structures, and predominant fragmentation patterns of acetylated mephedrone and some of its metabolites. Correlation of the fragmentation pattern of the parent drug with those of the metabolites allowed their structures to be postulated. Kamata et al. [56] and Zaitsu et al. [57] further confirmed the phase I metabolite structure and identified the conjugates by LC-MS. This strategy was also applied to metabolism studies on methylenedioxypyrovalerone (MDPV), a DOA with some similarities in structure to the cathinones and the pyrrolidinophenones [27, 58].

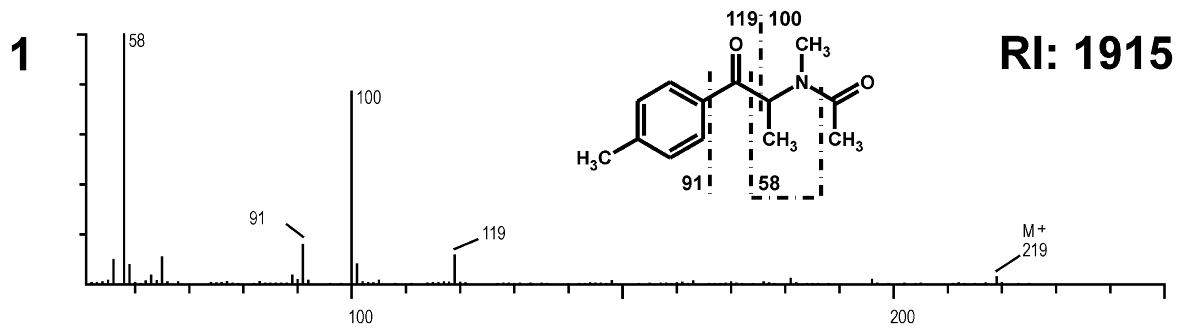
In contrast to all of these examples, GC-MS was only able to identify some of the major metabolites of larger drugs of abuse, such as synthetic cannabinoids [59–61] or

Table 1 Qualitative or quantitative GC-MS procedures used in metabolism studies of various drugs, along with information on workup and the MS device used

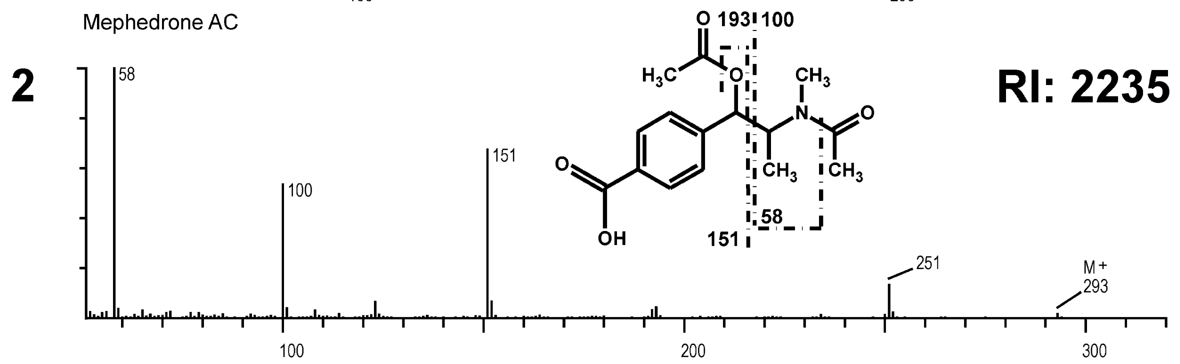
Compounds	Sample, workup	MS device	Quantitative/ qualitative	References
Designer drugs of the phenethylamine type	Urine, LLE, AC	GC-Q (EI, PICI)	Qualitative	[43, 44, 48–50]
Designer drugs of the 2,3-dimethoxyamphetamine type	Urine, LLE, AC	GC-Q (EI, PICI)	Qualitative	[19, 21, 51–53]
Designer drugs of the phencyclidine type	Urine, LLE, AC	GC-Q (EI, PICI)	Qualitative	[23–25, 54]
Designer drugs of the pyrrolidinophenone type	Urine, LLE, AC	GC-Q (EI, PICI)	Qualitative	[26, 27]
Designer drugs of the amphetamine type (PMMA)	Urine, LLE, AC, TMS plasma, tissue, SPE, AC	GC-Q (EI)	Qualitative/ quantitative	[55]
Designer drugs of the cathinone type (butylone, methylone, mephedrone)	Urine, SPE, AC	GC-Q (EI, PICI)	Qualitative	[28]
Designer drugs of the cathinone type (butylone, ethylone)	Urine, PP/LLE, TFA	GC-Q (EI), LC-Q LC-QQQ (ESI+)	Qualitative/ quantitative	[57]
Designer drugs of the cathinone type (methylone)	Urine, PP/LLE, TFA	GC-Q (EI), LC-Q (ESI+)	Qualitative/ quantitative	[56]
Designer drugs of the cathinone type (MDPV)	Urine, SPE/PP	GC-Q (EI) LC-HRMS (Orbitrap, ESI+)	Qualitative	[27]
Designer drugs of the cathinone type (MDPV)	Urine, LLE	GC-Q (EI) LC-QTOF (ESI+)	Qualitative	[58]
Cocaine, cocaethylene, benzoylecgonine	Plasma/urine, LLE	GC-MS (no details given)	Quantitative	[148]
Synthetic cannabinoids (JWH-018 and JWH-073)	Urine, LLE/SPE	GC-Q (EI) LC-QTOF (ESI+)	Qualitative	[59]
Synthetic cannabinoids (JWH-018)	Urine, LLE	GC-QQQ LC-QQQ (ESI+/−)	Qualitative	[61]
Anabolic steroids	Horse liver homogenate, LLE, TMS	GC-Q (EI)	Qualitative	[68]
Tramadol and metabolites	Camel blood, urine, SPE	GC-Q (EI)	Qualitative/ quantitative	[69]
Doping agent S107	Urine, LLE, TMS	GC-Q, GC-QQQ, GC-HRMS (EI)	Qualitative/ quantitative	[70]
Benfluorex and metabolites	Urine, LLE	GC-Q (EI) LC-HRMS (Orbitrap) LC-QQQ	Qualitative/ quantitative	[71]
Cannabinoids and metabolites	Plasma, SPE, TMS	GC-Q (EI)	Quantitative	[72, 74, 77, 81]
Cannabinoids and metabolites	Urine, PP/SPE, TMS,	GC-Q (EI)	Quantitative	[80]
Cannabinoids and metabolites	Oral fluid, SPE, TMS/TFA	GC-Q (EI/NICI)	Quantitative	[73, 75, 149]
Amphetamines (methamphetamine, MDMA) and metabolites	Mouse plasma/brain SPE, HFB	GC-Q (EI)	Quantitative	[82]
MDMA and metabolites	Plasma SPE, HFB	GC-GC-Q (EI)	Quantitative	[78, 83]
MDMA and metabolites	Urine, SPE, HFB	GC-Q (EI)	Quantitative	[76]
Methadone, heroin, cocaine, and metabolites	Sweat, SPE, TMS,	GC-Q (EI)	Quantitative	[79]
MDMA and metabolites	Urine, SPE/LLE	GC-Q (NICI) LC-HRMS (Orbitrap)	Quantitative	[32]
MDMA, MDEA, MBDB, BDB, MDA and metabolites	CYP incubate, LLE, *S*-HFBPCl	GC-Q (NICI)	Quantitative	[85–89]
Dextromethorphan and metabolites	Plasma, urine, CYP incubate, LLE	GC-Q (EI)	Quantitative	[90]
GHB	Liver cytosol, LLE, TMS	GC-IT (EI)	Quantitative	[91]

Table 2 Qualitative or quantitative LC-MS procedures used in metabolism studies of various drugs, along with information on workup and the MS device used

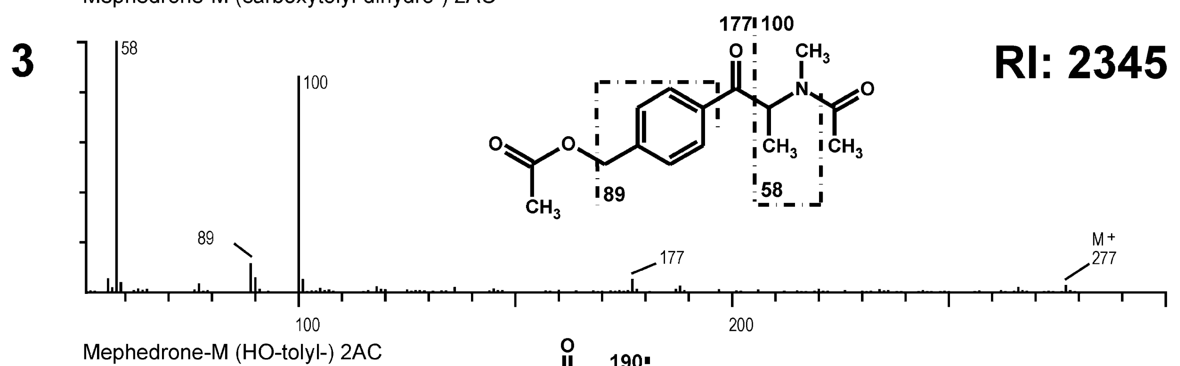
Compounds	Sample, workup	MS device	Quantitative/ qualitative	References
Alkaloids of the herbal DOA Kratom and metabolites	Urine, SPE	LC-LIT, LC-Orbitrap (ESI+)	Qualitative	[42, 96–99]
JWH-018, JWH-073 and metabolites	Urine, LLE/SPE	GC-Q (EI) LC-QTOF (ESI+)	Qualitative	[59]
JWH-018 and metabolites	Urine, LLE	GC-QQQ LC-QQQ (ESI+/−)	Qualitative	[61]
JWH-018 and metabolites	Urine, LLE	LC-QTRAP (ESI+)	Qualitative	[60]
Methoxyphenamine and metabolites	Urine, direct injection	LC-QQQ	Qualitative	[150]
Stanazolone and metabolites	Urine, LLE	LC-QTOF	Qualitative	[101]
Steroid metabolism	Urine (chimeric mice), LLE	LC-QQQ	Qualitative	[102]
Steroid metabolism	Humanized livers (chimeric mice), LLE	LC-QQQ	Qualitative	[104]
Doping agent S107	Liver microsomes	LC-QTRAP, LC-Orbitrap	Qualitative/ quantitative	[103]
Buprenorphine, norbuprenorphine, and metabolites	Urine, SPE	LC-QQQ (ESI+)	Quantitative	[129]
Buprenorphine, methadone, cocaine, opiates and nicotine metabolites	Sweat, SPE	LC-QQQ (ESI+)	Quantitative	[114]
Buprenorphine, methadone, cocaine, opiates, nicotine, and metabolites,	Oral fluid, PP	LC-QQQ (ESI+)	Quantitative	[117]
Buprenorphine, norbuprenorphine, and glucuronide metabolites	Umbilical cord plasma, SPE	LC-QQQ (ESI+)	Quantitative	[116, 120]
Buprenorphine, norbuprenorphine, and metabolites	Placenta, SPE	LC-QQQ (ESI+)	Quantitative	[119]
Buprenorphine, norbuprenorphine, and metabolites	Meconium, SPE	LC-QQQ (ESI+)	Quantitative	[130]
Methadone, EDDP, EMDP	Plasma, PP	LC-QQQ (APCI+)	Quantitative	[131]
Methadone, cocaine, opiates, and metabolites	Umbilical cord, SPE	LC-QQQ (ESI+)	Quantitative	[121]
Methadone, cocaine, opiates, and metabolites	Placenta, SPE	LC-QQQ (ESI+)	Quantitative	[122]
Opiates + metabolites	Urine	LC-QQQ (ESI+)	Quantitative	[118]
20 DOAs and metabolites	Meconium, SPE	LC-QQQ (ESI+)	Quantitative	[123]
Nicotine, cotinine	Plasma, SPE	LC-QQQ (ESI+)	Quantitative	[125]
Nicotine, cotinine	Oral fluid, SPE	LC-QQQ (ESI+)	Quantitative	[124]
Nicotine and metabolites	Meconium, SPE	LC-QQQ (ESI+)	Quantitative	[127, 128]
Nicotine, opioids, cocaine, and metabolites	Post mortem brain, SPE	LC-QQQ (ESI+)	Quantitative	[126]
Cannabinoids and glucuronides	Whole blood, SPE	LC-QQQ (ESI+)	Quantitative	[115]
MDMA and metabolites	Human and monkey plasma, acid hydrolysis, PP	LC-Q (ESI+)	Quantitative	[133, 135]
MDMA and metabolites	Monkey brain, LLE	LC-Q (ESI+)	Quantitative	[134]
MDMA phase II metabolites	Incubation matrix, PP	Q (ESI+)	Quantitative	[136]
MPBP	Incubation matrix, PP	Q (APCI+)	Quantitative	[137]
PCEPA and PCMPA	Incubation matrix, PP	Q (APCI+)	Quantitative	[138]
PCEEA and PCMEA	Incubation matrix, PP	Q (APCI+)	Quantitative	[24]
DOB, DOC, DOI, MDOB and TMA-2,	Incubation matrix, PP	Q (APCI+)	Quantitative	[139]
Cannabinoids	Incubation matrix, PP	QQQ (ESI+)	Quantitative	[140]
Buprenorphine and norbuprenorphine	Incubation matrix, PP	QQQ (ESI+)	Quantitative	[141]
Psilocin	Incubation matrix, SPE	QQQ (ESI+)	Quantitative	[142]
O-demethyl tramadol	Incubation matrix, SPE	QTOF (ESI+)	Quantitative	[143]
MDPV	Incubation matrix, PP	Orbitrap (ESI+)	Quantitative	[27]
MDMA phase II metabolites	Incubation matrix, PP	Orbitrap (ESI+)	Quantitative	[32, 144, 145]
MDMA phase II metabolites	Incubation matrix, PP	Ion trap (ESI+)	Quantitative	[146]



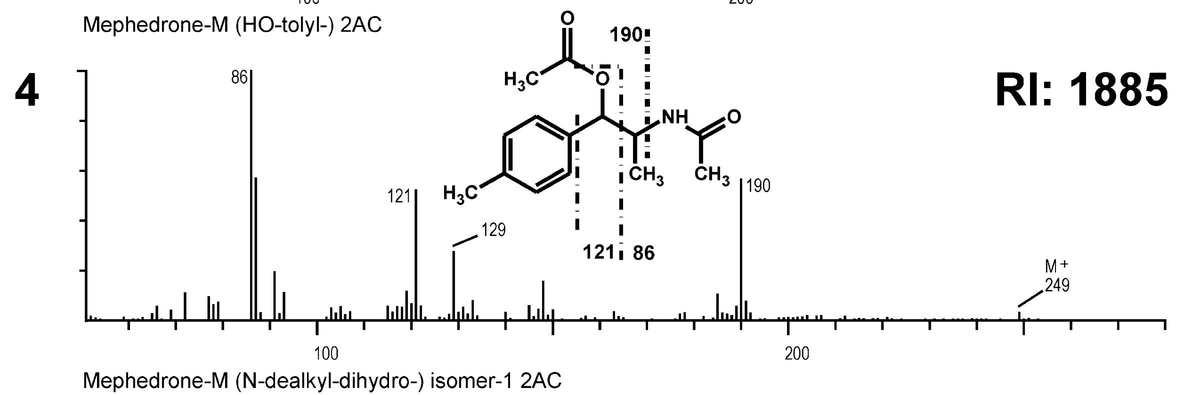
Mephedrone AC



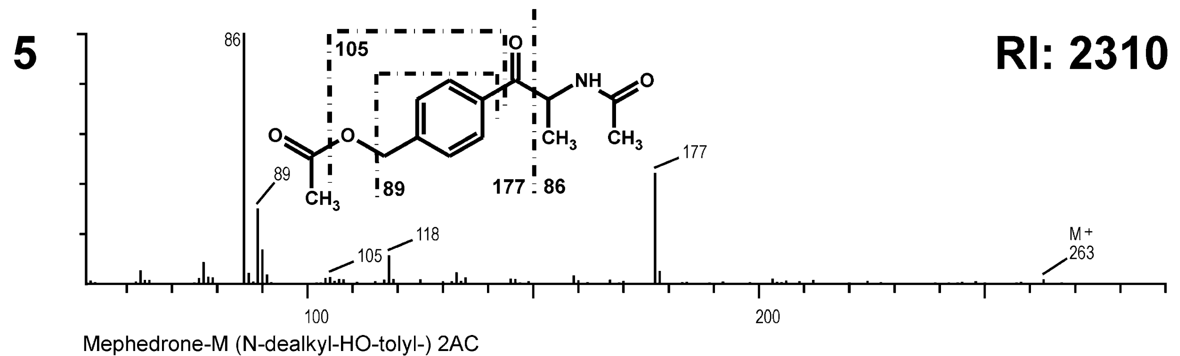
Mephedrone-M (carboxytolyl-dihydro-) 2AC



Mephedrone-M (HO-tolyl-) 2AC



Mephedrone-M (N-dealkyl-dihydro-) isomer-1 2AC



Mephedrone-M (N-dealkyl-HO-tolyl-) 2AC

◀**Fig. 1** EI mass spectra, retention indices (RI), structures, and predominant fragmentation patterns of acetylated mephedrone and some of its metabolites (taken from [96])

alkaloids of the herbal DOA Kratom [62]. Therefore, these approaches are discussed in the LC-MS section.

Metabolism of doping agents

GC-MS is still an established method in doping control, particularly for the determination of steroids or steroid-like compounds [63–67]. For fast in vitro steroid metabolite screening, Wong et al. presented an interesting strategy using homogenized horse liver instead of liver microsomes [68]. After terminating the reaction, the incubation mixtures were centrifuged, extracted and analyzed after trimethylsilylation or acylation using GC-MS. GC-MS was also applied in metabolism and kinetic studies of tramadol in camels to assess drug misuse in racing camels [69]. Thevis et al. used GC-MS to identify and monitor metabolites of compounds others than steroids, such as the ryanodine receptor-based Ca-channel stabilizer S107 [70]. Putative metabolites were analyzed using GC-MS procedures that are common in doping control. Dissociation pathways were elucidated by tandem mass spectrometry and accurate mass measurements using ion trap or sector field mass analyzers. The same group established screening procedures for benfluorex and its major urinary metabolites in doping controls using GC-MS, LC-HRMS, and LC-MS/MS [71]. They showed that GC-MS as well as LC-MS/MS were suitable, but, as expected, the limits of detection were one magnitude lower for the latter.

Metabolite quantification in body samples for pharmacokinetic studies

Huestis and coworkers published a series of papers on method development and application for pharmacokinetic studies of DOAs (e.g., amphetamines, cannabinoids, or opioids) and their metabolites after controlled administration using GC-MS [72–82] or LC-MS approaches (see below). The analytes, monitored body samples, workup, and MS device used are summarized in Table 1. For sensitive detection in very complex biomatrices, they used two-dimensional GC [72–75, 77, 81, 83].

In order to perform an enantioselective elimination study of 3,4-methylenedioxy-methamphetamine (MDMA) and its phase I and II metabolites in urine, Schwaninger et al. had to develop three methods [32]. LC-HRMS was applied for the stereoselective determination of glucuronides and the achiral determination of the intact sulfate conjugates. As the enantiomers of the sulfates could not be separated by LC, they were determined after selective cleavage by GC negative-ion chemical ionization (NICI) MS after chiral derivatization with *S*-heptafluorobutyrylprolyl chloride (*S*-HFBPCl). This method was also used to determine the unconjugated metabolites without cleavage. This nicely illustrates that, even for enantioselective kinetic studies of sulfate conjugates, GC-MS was the only suitable technique.

Metabolite quantification for in vitro enzyme kinetic studies

Several in vitro approaches are in use to determine the contributions of particular phase I and II metabolizing isoenzymes [84]. Either the depletion of the substrate or the formation of the product must be monitored in order to calculate the enzyme kinetics and the contribution to the hepatic clearance. While deproteinization is mostly sufficient for LC-MS determination, GC-MS always demands workup of the incubation mixture. To avoid the formation of artifacts (e.g., of reactive catechols), Meyer et al. used direct derivatization in the aqueous medium before extraction [85–89]. As already mentioned above, chiral derivatization with *S*-HFBPCl followed by NICI-MS allowed the separation and sensitive determination of the enantiomers of the metabolites formed in order to elucidate the enzyme kinetics of each individual enantiomer. Another GC-MS application was described by Spanakis et al. for the simultaneous determination of dextromethorphan and its metabolites in biological matrices and its application to in vitro CYP2D6 and CYP3A4 inhibition studies [90]. Lenz et al. monitored the degradation of 1,4-butanediol to gamma-hydroxybutyric acid (GHB) in cytosolic supernatant of human liver by GC-MS [91]. Furthermore, the authors examined the effects of ethanol and acetaldehyde on 1,4-butanediol metabolism. They concluded that ethanol inhibited the conversion of 1,4-butanediol to GHB competitively, and that the co-ingestion of 1,4-butanediol and ethanol may increase the concentrations and the effects of 1,4-butanediol itself, whereas acetaldehyde accelerated the formation of GHB. All antidotes showed the ability to inhibit the formation of GHB. Analytes were monitored after silylation using GC coupled to ion trap MS.

In conclusion, all of these examples show that GC-MS still plays a role in in vitro and in vivo metabolism studies, but LC-MS is mandatory for low-dosed and larger drugs, and of course for the analysis of phase II metabolites.

LC-MS for studies of the metabolism of DOAs and doping agents

Today, LC-MS is widely used to study drug metabolism in vitro and in vivo, with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) employed. Fragment-rich spectra for structure elucidation can be produced by in-source fragmentation or by collision-induced dissociation (CID) using ion trap or triple

quadrupole instruments [92]. Although aqueous body or enzyme incubation samples can be injected directly or after simple protein precipitation (PP) for LC-MS analysis, more or less sophisticated sample workup may be necessary, particularly to reduce the risk of matrix effects or to increase the analyte concentration. Cleaving conjugates may also be a sensible step if the phase I metabolites must be identified first.

Sample workup

Just as they are for GC-MS, LLE, offline and online SPE, dilution and filtration, and PP are very commonly employed for LC-MS applications, as summarized in Table 2. Derivatization can also be useful for LC-MS in order to improve structure elucidation and confirmation or the ionization efficiencies of the analytes. Philipp et al., for example, determined the positions of glucuronic or sulfuric acid in mitragynine phase II metabolites by selective methylation using diazomethane [42]. Other authors used 1,2-dimethylimidazole-4-sulfonyl chloride as a tool to determine the site of conjugation of the glucuronide of morphine and formoterol [93] using derivatization reagents for ionization enhancement. An overview of the chemical derivatization methods that have been applied to small molecules in the field of LC-MS was recently provided by Iwasaki et al. [94].

Another important point that must be considered in the context of sample preparation is the cleavage of conjugates prior to analysis. When investigating phase II metabolism, samples should ideally be injected untreated using, for example, PP [27, 95]. However, to increase the sensitivity of phase I metabolite analysis, conjugate cleavage may be conducted [27]. Nevertheless, reproducible procedures should be used, especially in quantitative analysis, as previously mentioned [30–33].

Metabolism of herbal drugs

In recent years, so-called herbal drugs of abuse have entered the drug abuse scene. For example, the Thai medicinal plant *Mitragyna speciosa* (Kratom) is being widely misused as herbal drug. A new herbal blend, named Krypton, has also appeared on the DOA market. This is a mixture of *O*-demethyl tramadol (ODT) and Kratom [62]. As previously mentioned, only the main phase I metabolites can be detected by GC-MS [62]. Therefore, Philipp et al. used low-resolution linear ion trap and high-resolution Orbitrap technology to elucidate the complex phase I and II metabolism in rats and humans of six alkaloids: mitragynine and its diastereomers speciogynine, speciociliatine, and mitraciliatine, as well as paynantheine and its diastereomer isopaynantheine [42, 96–99]. As exemplified in Fig. 2 for the paynantheine 9-*O*-demethyl metabolite, the LIT of the HRMS Orbitrap apparatus produces the same fragments at different stages with accurate masses, thus allowing their elemental compositions to be confirmed, thereby elucidating the structure of the metabolite [96].

Metabolism of synthetic cannabinoids

Again, GC-MS allowed the identification of only some of the major metabolites of the synthetic cannabinoids [59, 61]. More detailed studies were performed using LC coupled to triple-quadrupole MS [59, 61] or quadrupole–time-of-flight hybrid (QTOF) MS [59]. Beuck et al. synthesized five potential in vivo metabolites of JWH-018 in order to confirm the metabolites postulated after CID and Orbitrap analysis [60]. Using an authentic urine sample analyzed on an LC–quadrupole–linear ion trap hybrid (QTRAP) MS, they identified four of the synthetic JWH-018 analogs as true in vivo metabolites.

Metabolism of doping agents

In the field of doping control, the qualitative metabolism of potential doping agents is also extensively investigated using LC coupled to different analyzers such as QTRAP [60, 100], QTOF [101], triple-quadrupole [102], and Orbitrap-based [15, 71, 100, 103] analyzers.

For example, Thevis et al. developed an LC-MS/MS assay to detect the doping agent methoxyphenamine (an amphetamine derivative) after direct injection of urine, allowing differentiation from the isomeric designer drug (PMMA). In a study aimed at the detection and structural investigation of metabolites of stanozolol (an anabolic steroid) in human urine, the authors reported that the application of the method to a single human excretion study revealed that one of the stanozolol metabolites was detected in negative ionization mode for a longer period than those commonly used to screen for stanozolol misuse in doping analysis [101]. Lootens et al. and Pozo et al. described the application of LC-MS to steroid metabolism in urine [102] or chimeric mouse liver [104]. While the urinary metabolites of the doping agent S107 were studied using GC-MS [70], the metabolic fate of S107 due to human microsomal and S9 liver enzymes was tested by LC-QTRAP and LC-HRMS Orbitrap because they have better identification power than GC-MS [103].

Software tools for metabolite identification

Software solutions can be used to screen for new metabolites; for example, in the so-called neutral loss scan for, say, phase II metabolites such as glucuronides that result in a neutral loss of *m/z* 176 or sulfates of *m/z* 80 [42]. In the context of a

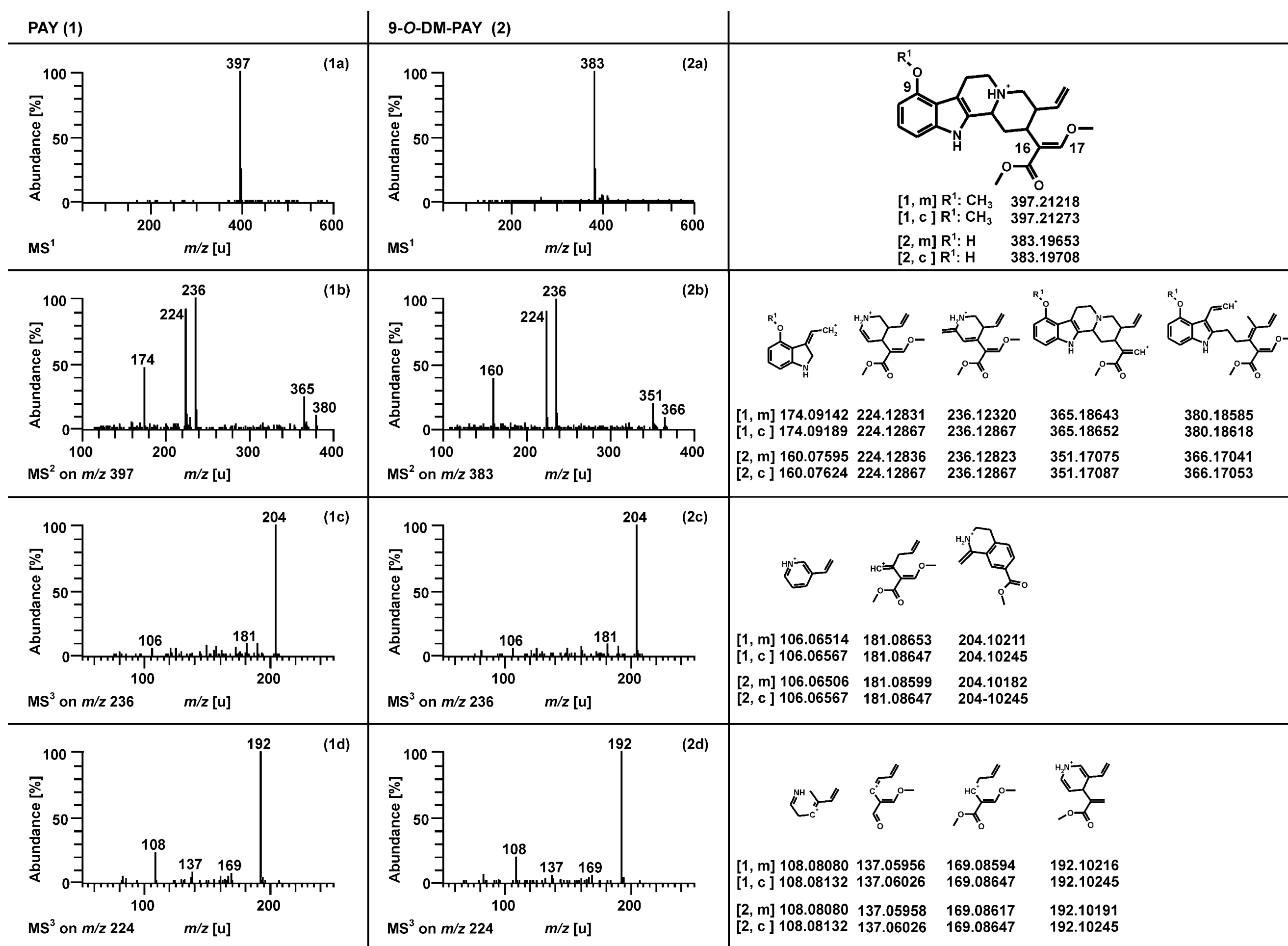


Fig. 2 MS^1 spectra of paynantheine and its 9-*O*-demethyl metabolite, MS^2 spectra of the protonated molecular ions in the MS^1 spectra, MS^3 spectra of the two most abundant fragments in the MS^2 spectra, and the structures of the fragments as well as their accurate masses (*m* measured, *c* calculated) (taken from [96])

screening procedure using LC-QTOF-MS [105], Broecker et al. presented software that assisted metabolite identification using the tool "Find Metabolites" (Agilent, Waldronn, Germany). They demonstrated the power of this tool for tramadol. As shown in Fig. 3, 19 metabolites were proposed to result from demethylation, didemethylation, tridemethylation, hydroxylation, the formation of the *N*-oxide, combinations of these, and their combination with glucuronidation. Another option for assisted phase I and II metabolite identification was described by Wissenbach et al. using the SmileMS (Genebio, Geneva, Switzerland) software [106]. Figure 4 shows LC chromatograms indicating phase I and II metabolites of JWH-250 in an authentic human urine sample after PP, selected using SmileMS. The corresponding MS^2 and MS^3 library spectra of the most abundant JWH-250 metabolite, III, are depicted in Fig. 4.

Aside from these recent examples, other solutions such as MassLynx (Waters, Eschborn, Germany), LightSight (AB Sciex, Darmstadt, Germany), and MetaboliteID or MetWorks (Thermo Scientific, Dreieich, Germany) are available for the software-assisted identification of metabolites in human biosamples [107–113].

Metabolite quantification in body samples for pharmacokinetic studies

As already mentioned, Huestis and coworkers published a series of papers on the pharmacokinetics of DOAs after controlled administration, where the DOAs and their metabolites were determined in various body samples (blood, urine, oral fluid, etc.) using LC-MS approaches [114–131] (see Table 2). They developed, validated and applied assays that could be used to determine buprenorphine and its metabolites in urine [129], sweat [114], oral fluid [117], umbilical cord plasma [116, 120], placenta [119], or meconium, [130], with the aim being to elucidate the pharmacokinetics and obtain data that could be employed to interpret toxicological results in clinical and

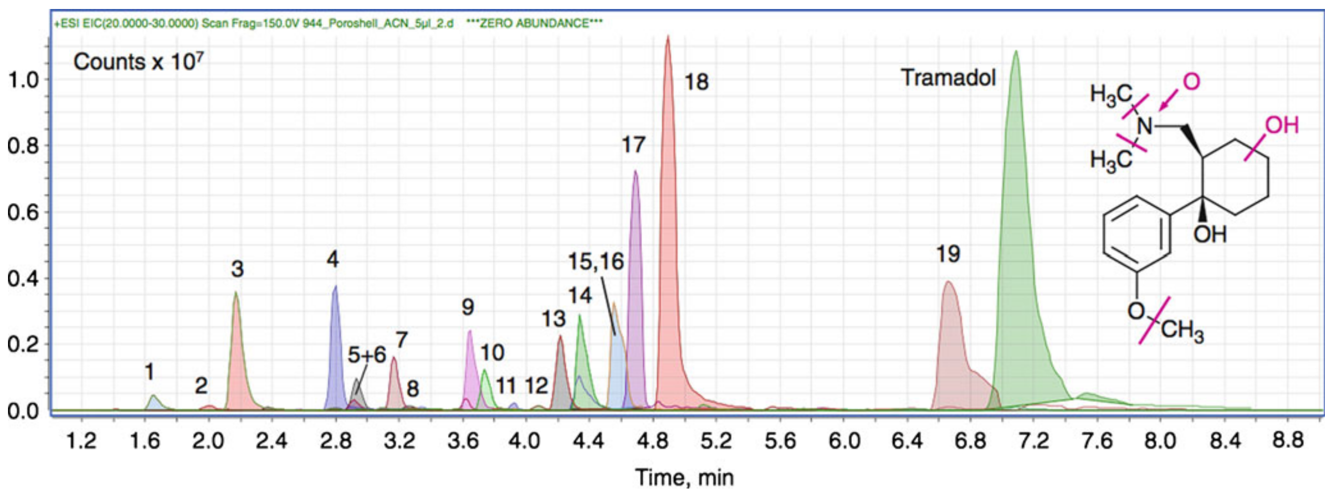


Fig. 3 LC chromatogram with peaks identified by the "Find Metabolites" tool as those of tramadol (T), *1, 3, 10, 14* = isomeric hydroxydemethyl-T; *2, 12* = isomeric hydroxydidemethyl-T; *4* = glucuronide of demethyl-T; *5, 7* = glucuronides of didemethyl-T; *6* = glucuronide of hydroxydemethyl-T; *8* = glucuronide of hydroxydidemethyl-T; *9* = glucuronide of hydroxy-T; *11* = *N,O*-didemethyl-T, *13, 18* = hydroxy-T; *15* = tridemethyl-T, *16* = *N,N*-didemethyl-T; *17* = *O*-demethyl-T; *19* = *N*-demethyl-T, and T-*N*-oxide (taken from [105])

forensic toxicology. One issue that they focused on was the in utero exposure of DOA. Similar procedures were published for methadone and its metabolites in plasma [131], umbilical cord [121], placenta [122], for nicotine and its metabolites in oral fluid [124], meconium [127, 128], post mortem brain [126], for cannabinoids and their glucuronides in whole blood [115], and finally for other opioids and metabolites in urine [118] or meconium [123]. Comparing the outcomes of combined screening and quantification procedures, Shakleya et al. reported the differential pattern of opiate and cocaine biomarkers observed after LC–MS as compared to GC-MS analysis

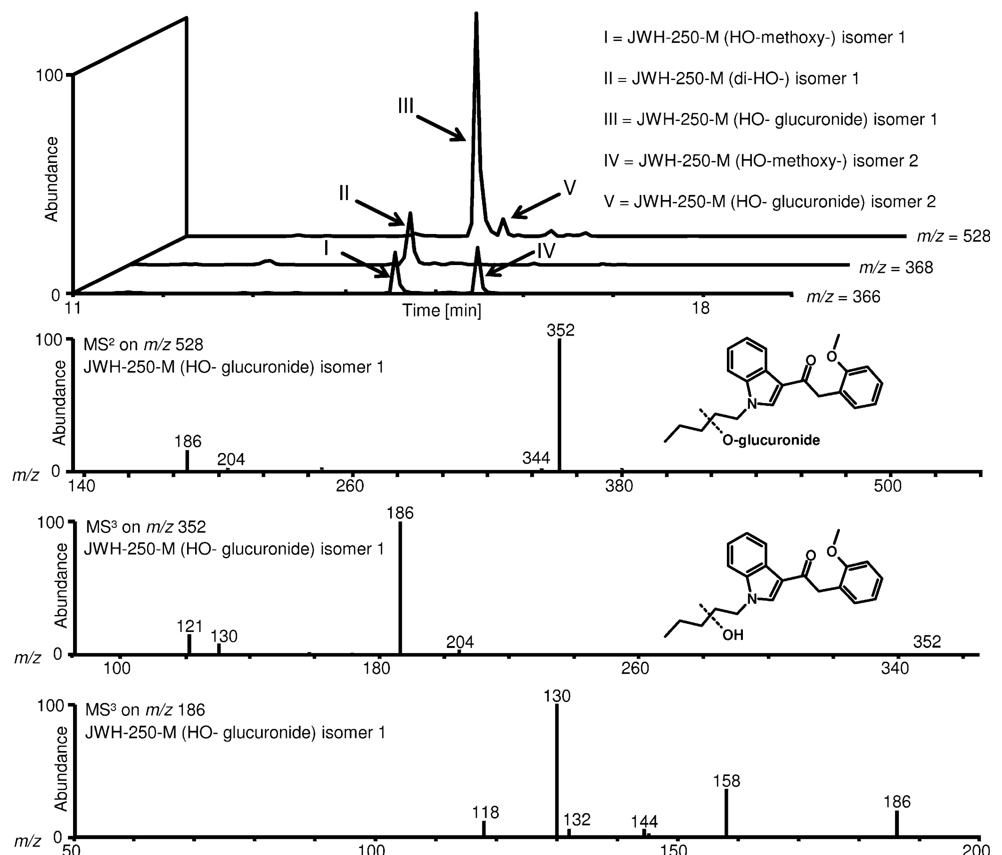


Fig. 4 LC chromatograms indicating phase I and II metabolites of JWH-250 in an authentic human urine sample after PP. The corresponding MS^2 and MS^3 library spectra of the most abundant JWH-250 metabolite, III, are depicted below (taken from [95])

[118]. For instance, they discussed the possible pitfall of falsely interpreting the presence of anhydroecgonine methyl ester (AEME) after GC-MS analysis as a marker for crack consumption. AEME can also be a product of thermal decomposition, and it does not form during LC-MS.

In the context of neurotoxicity studies [10, 132], Mueller et al. developed LC-MS procedures to investigate the pharmacokinetics of MDMA and its metabolites in human plasma and in monkey plasma and brain [133–135]. As already mentioned above, aside from GC-MS assays, Schwaninger et al. developed an LC-HRMS approach for the stereoselective determination of glucuronides and the achiral determination of the intact sulfate conjugates of MDMA [32]. This study showed that both techniques have a place in the modern drug metabolism laboratory.

Metabolite quantification for in vitro enzyme kinetic studies

LC-MS is the method of choice for quantification in the context of in vitro enzyme kinetics, particularly when analyzing phase II conjugates. Using LC-MS, extensive sample preparation can be avoided in most cases, leading to direct injection of the incubation supernatant after centrifugating when the reaction has terminated. Studies using single quad devices have been reported for the enzyme kinetics of the following designer drugs using incubation of the recombinant human isoenzymes MDMA [136], 4-methylpyrrolidinobutyrophenone (MPBP) [137], *N*-(1-phenylcyclohexyl)-3-ethoxypropanamine (PCEPA) and *N*-(1-phenylcyclohexyl)-3-methoxypropanamine (PCMPA) [138], *N*-(1-phenylcyclohexyl)-2-ethoxyethanamine (PCEEA), and *N*-(1-phenylcyclohexyl)-2-methoxyethanamine (PCMEA) [24], as well as 4-bromo-2,5-dimethoxyamphetamine (DOB), 4-chloro-2,5-dimethoxyamphetamine (DOC), 4-iodo-2,5-dimethoxyamphetamine (DOI), 4-bromo-2,5-dimethoxymethamphetamine (MDOB), and 2,4,5-trimethoxyamphetamine (TMA-2) [139]. Analyses were performed using APCI in the positive selected-ion monitoring mode.

Mazur et al. used tandem mass spectrometry to characterize human hepatic and extrahepatic uridine-5′-diphosphoglucuronosyltransferases (UGT) involved in the metabolism of classic cannabinoids [140]. Rouguieg et al. used the same technique to assess the contributions of UGT isoforms to buprenorphine and norbuprenorphine metabolism [141]. Further studies were conducted on the glucuronidation of psilocin by 19 recombinant human UGTs of the subfamilies 1A, 2A, and 2B [142]. Lehtonen et al. developed a LC-QTOF-based method to separate, analyze, and quantify the diastereomeric phenolic *O*-glucuronides of *O*-demethyl tramadol after enzyme incubation [143].

In other studies, Orbitrap devices were used in the full-scan mode for metabolite detection and quantification [27, 32, 144, 145]. Schwaninger et al. used HRMS for the quantification of MDMA phase II metabolites associated with in vitro kinetics after the incubation of recombinant enzymes, as well as for excretion studies in human urine [32, 144, 145]. Finally, using ion trap technology, MDMA metabolites were quantified in order to determine the kinetics of glucuronide formation [146].

Conclusions and perspectives

Hyphenated mass spectrometry plays a major role in laboratories studying in vitro and/or in vivo metabolism. The examples reviewed above show that GC-MS still has some advantages over LC-MS. However, LC-MS is the method of choice for these tasks, particularly for low-dosed and larger drugs, and of course for the analysis of phase II metabolites. One important side effect of such metabolism studies of drugs of abuse is the generation of a huge collection of reference spectra of drug metabolites. This huge collection of spectra is required when drug testing is performed using GC-MS [147] or LC-MS [95] in clinical and forensic toxicology as well as doping control.

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