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ORIGINAL PAPER

Generation of metabolites by an automated online metabolism method using human liver microsomes with subsequent identification by LC-MS(n), and metabolism of 11 cathinones

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Abstract Human liver microsomes (HLMs) are used to simulate human xenobiotic metabolism in vitro. In forensic and clinical toxicology, HLMs are popularly used to study the metabolism of new designer drugs for example. In this work, we present an automated online extraction system we developed for HLM experiments, which was compared to a classical offline approach. Furthermore, we present studies on the metabolism of 11 cathinones; for eight of these, the metabolism has not previously been reported. Metabolites were identified based on MS^2 and MS^3 scans. Fifty-three substances encompassing various classes of drugs were employed to compare the established offline and the new online methods. The metabolism of each of the following 11 cathinones was studied using the new method: 3,4-methylenedioxy-*N*-benzylcathinone, benzedrone, butylone, dimethylcathinone, ethylone, flephedrone, methedrone, methylone, methylethylcathinone, naphyrone, and pentylone. The agreement between the offline and the online methods was good; a total of 158 metabolites were identified. Using only the offline method, 156 (98.7%) metabolites were identified, while 151 (95.6%) were identified using only the online method. The metabolic pathways identified for the 11 cathinones included the reduction of the keto group, desalkylation, hydroxylation, and desmethylenation in cathinones containing a methylenedioxy moiety. Our method provides a straightforward approach to identifying metabolites which can then be added to the library utilized by our clinical toxicological screening method. The performance of our method compares well with that of an established offline HLM procedure, but is as automated as possible.

Keywords LC-MS · Human liver microsomes · Cathinones · Online extraction · Human metabolism

Abbreviations

APCI	Atmospheric pressure chemical ionization
AU	Arbitrary units
bk	Beta-keto
CYP	Cytochrome P450
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
GST	Glutathione-*S*-transferase
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
HPLC	High-pressure liquid chromatography
HLMs	Human liver microsomes
IU	International units
LC-MS	Liquid chromatography–mass spectrometry
MDPV	Methylenedioxypyrovalerone
$NADP^+$	Nicotinic acid adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinic acid adenine dinucleotide phosphate (reduced form)
NAT	*N*-Acetyltransferase

Introduction

Identifying metabolites is a necessary task during the drug development process as well as in the clinical and forensic toxicological laboratory. Whereas the main focus during drug development is to determine the presence of a

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metabolite, the main focus in the toxicological laboratory is to use the metabolites to prove the intake of a particular drug (e.g., a drug of abuse) when urine samples are analyzed. Therefore, metabolite generation by in vivo and in vitro systems is an important tool in both of these fields of application.

Human liver microsomes (HLMs) have become popular tools for simulating human xenobiotic metabolism in vitro, mainly because they are easy to use and store. Phase I metabolism can be studied using HLMs by simply adding NADPH as a cofactor for the main drug-metabolizing cytochrome P450 enzymes (CYP) [1]. However, there are also some drawbacks of this approach, such as the absence of enzymes such as *N*-acetyltransferase (NAT) or glutathione-*S*-transferase (GST). In other words, metabolic pathways involving those enzymes cannot be studied using HLMs [1].

In the pharmaceutical industry, HLMs are used for metabolite identification and to predict the clearance of drug candidates in vivo, for example [1]. In the field of forensic and clinical toxicology, HLMs are popularly employed to study, for instance, the metabolism of new designer drugs. The aim of such studies is usually to investigate the metabolism of a new designer drug in order to make it detectable in toxicological screening procedures, or to quantify it using dedicated methods. Examples include the metabolism of the synthetic cannabimimetic JWH-018, for which Wintermeyer et al. [2] used HLMs to study its phase I metabolism in vitro. Meyer et al. [3] studied the metabolism of methylenedioxypyrovalerone (MDPV) using HLMs (amongst other methods), and Zaitsu et al. [4] identified several metabolites of butylone and ethylone. The use of mass spectrometry in studies of the metabolism of drugs of abuse and doping agents was recently reviewed, focusing on clinical and forensic toxicology [5].

The process used in HLM experiments can be automated, and this is an especially common approach in the pharmaceutical industry, where a high throughput of experiments is needed. Several methodologies with fully integrated workflows have recently been presented. Drexler et al. [6] developed a sophisticated automated approach to study the metabolic stabilities of drug candidates. This approach involved incubation using HLMs, which was carried out in microplates using an automated liquid handling robot. Similar approaches were presented by Xu et al. [7] and by Jenkins et al. [8], which also used a liquid handling robot to carry out the HLM experiments. Lai et al. [9] described a system where the autosampler was modified to include a heated agitator and the samples were subsequently injected into the online extraction system. The system was used to screen the stability of a prodrug in development. Kool et al. [10] developed an online system using rat liver microsomes to study the affinity of ligands for CYP enzymes.

Regarding the throughput required, assays using, for example, specialized liquid-handling robots are usually overkill for metabolite-generating experiments in the clinical toxicological laboratory, and the high throughput achievable is also not needed.

One possible method for achieving a certain degree of lower-level automation and miniaturization was presented by Nicoli et al. [11] and Curcio et al. [12], which used a capillary as the reaction vessel for HLM experiments. This method included automated metabolite generation, but required manual sample preparation of the incubation mixture.

The main aim of the work presented in this paper was to create an automated method with adequate throughput for our toxicological laboratory to generate phase I metabolites which could then be added to the library utilized by our clinical toxicological screening method [13]. Our approach used a modified, heated autosampler loop for incubation, and the samples were then transferred onto an online extraction LC-MS system. After loading the components for the incubation onto the autosampler, no additional manual steps were needed.

A second aim of this work was to study the metabolism of 11 different cathinones using HLMs; for eight of these, the metabolism has not previously been reported, to our knowledge. Cathinones are a new class of designer drugs that have appeared in the drugs-of-abuse market in recent years. They are reported to inhibit plasma membrane monoamine transporters, thereby increasing the amount of monoamine neurotransmitter in the synaptic cleft [14], which leads to the pharmacological effects of these drugs.

Materials and methods

Chemicals and reagents

Acetonitrile was purchased from Romil (Cambridge, UK), acetone from Merck (Darmstadt, Germany), and methanol and 2-propanol from Seelze (Seelze, Germany); all were of HPLC gradient grade purity. Purified water was produced in-house using a central water purification installation (Burkhalter AG, Worblaufen, Switzerland).

HPLC-grade ammonium acetate and ammonium carbonate were obtained from Scharlau (Taegerig, Switzerland), while analytical grade formic acid and ammonia were from Merck. Analytical grade EDTA, potassium dihydrogen phosphate, potassium hydrogen phosphate, tris base and glacial acetic acid were obtained from Sigma–Aldrich (Buchs, Switzerland). Glycerol Ph. Eur. was purchased from Haenseler (Herisau, Switzerland).

Human liver microsomes (pooled, male and female), β-nicotinamide adenine dinucleotide phosphate hydrate ($NADP^+$), D-glucose-6-phosphate dipotassium hydrate (G6P), and glucose-6-phosphate dehydrogenase (G6PD) from *S. cerevisiae* were purchased from Sigma–Aldrich.

Amitriptyline, chlorpromazine, chlorprothixene, duloxetine, ethylone, fenfluramine, fluoxetine, fluphenazine,

ketamine, methylone, methylphenidate, mianserine, naphyrone, oxycodone, and phencyclidine were purchased from Cerilliant (Round Rock, TX, USA). Bromazepam, butylone, flephedrone, methaqualone, methylfentanyl, psilocin, tilidine, and zolpidem were obtained from Lipomed (Arlesheim, Switzerland). Amlodipine, bisacodyl, desipramine, flupentixol, maprotiline, perphenazine, promazine, propafenone, sertindole, strychnine, and thioridazine were purchased from Sigma–Aldrich. Amisulpride, diclofenac, opipramol, pipamperone, and reboxetine were obtained from TRC (North York, Canada). 3,4-Methylenedioxy-*N*-benzylcathinone, benzedrone, dimethylcathinone, methedrone, methylethylcathinone, and pentylone were purchased from LGC Standards (Wesel, Germany). Aripiprazol, clotiapine, cinnarizine, cyclizine, dextropropoxyphene, doxylamine, fluvoxamine, meclozine, paroxetine, penfluridol, pethidine, pentazocine, remifentanil, sildenafil, thiethylperazine, thioproperazine, tramadol, and zaleplone were provided by their respective manufacturers.

LC-MS analysis

The turbulent flow online extraction HPLC system consisted of an HTC PAL autosampler, one Allegro pump for the online extraction, and one Allegro pump for the analytical chromatography, all controlled by Aria 1.6.3 software (all from Thermo Fisher Scientific, Basel, Switzerland). The autosampler was equipped with a Hamilton 100 μl syringe for CTC autosamplers (Hamilton, Bonaduz, Switzerland).

The detailed conditions for the online extraction and LC-MS analysis are described in our previous publications [13, 15]. In brief, samples were extracted online using chromatography based on turbulent flow under alkaline conditions using ammonium carbonate buffer 10 mM, pH 8.0. A Cyclone and a C18 XL column (both from Thermo Fisher Scientific) were connected in series to achieve sufficient extraction for substances over a wide polarity range. Analytical chromatography was performed under acidic conditions on a Betasil phenyl/hexyl column (100×3 mm, 3 μm particles, Thermo Fisher Scientific) using ammonium acetate 5 mM in water plus 0.1% formic acid and ammonium acetate 5 mM in methanol plus 0.5% formic acid as mobile phases. A mixture of acetonitrile/acetone/2-propanol 1/1/1 (v/v/v) was used as a wash phase. The combination of the two extraction columns and the analytical column could be connected inline and divided again via two six-port switching valves controlled by Aria software.

A LXQ linear ion trap mass spectrometer was used for detection, as controlled by XCalibur 2.0.7 SP1 software (Thermo Fisher Scientific). Ionization was performed under atmospheric pressure chemical ionization (APCI) conditions because of its better performance in terms of ion suppression susceptibility as compared with electrospray ionization (ESI). The vaporizer temperature was fixed at 450 °C, the capillary temperature at 275 °C, the sheath gas flow at 30 arbitrary units (AU), the auxiliary gas flow at 5 AU, and the discharge current was set to 5 μA. Both positive and negative ionization were used, with the polarity constantly switching. Chromatograms were recorded in untargeted data-dependent acquisition mode. In positive ionization mode, MS^2 spectra were recorded for the four most abundant ions, and in negative ionization mode they were recorded for the two most abundant ions. MS^3 spectra were recorded for the most abundant ion from each MS^2 spectrum. The threshold for the acquisition of fragment spectra was set to 100 counts per second. The isolation width was set to 1 amu, while the normalized collision energy for the fragmentation was set to 35%.

For the online metabolism experiment, the injection loop was modified to a 200 μl injection loop placed in a column oven (Thermo Fisher Scientific) that kept the temperature constant at 37 °C.

Metabolism studies

Identification of metabolites

Metabolites were identified using their MS^2 and MS^3 mass spectra. Methods applied were neutral-loss scans and comparisons of their fragmentation patterns to the parent substances. Raw files were examined both manually and assisted by MassFrontier 7 (Thermo Fisher Scientific).

Solutions

G6P was prepared as a 0.1 M solution in water and $NADP^+$ as a 10 mg/ml solution in water; both were stored as aliquots at −20 °C until use. The G6PD was dissolved in a mixture consisting of 20% 40 mM Tris-acetate buffer containing 1 mM EDTA and 80% glycerol to achieve an activity of 10^3 IU/ml at a protein concentration of 1 mg/ml. Aliquots were stored at −20 °C until use. The HLM suspensions were stored as aliquots at −80 °C until usage.

Stock solutions of the test substances were prepared at 1 mg/ml in water or methanol, depending on the solubility of the substance, and stored at −20 °C until use. Dilutions to 10 mg/l were freshly prepared in phosphate buffer 0.1 M, pH 7.4. The remaining amount of organic solvent was always below 1% (v/v).

Offline method

The method was adapted from that of Sohl et al. [16]. To generate the NADPH needed for the reactions, a NADPH-generating system was used. It consisted of 50 parts 0.1 M G6P, 25 parts 10 mg/ml $NADP^+$, and 1 part G6PD at a

concentration of 1 mg/ml and an activity of 10^3 IU/ml; all solutions were prepared as described above. The mixture was pre-incubated for 1 min before adding it to the incubation mixture to start the reaction.

Five hundred picomoles of total CYP were used in the incubation mixture per incubation. The substances were added diluted in phosphate buffer 0.1 M, pH 7.4 at a concentration of 10 mg/l. The total volume of the incubation mixture was 112 μl.

The mixture was incubated in a water bath at 37 °C for 3 h under occasional gently mixing. After 3 h, the reaction was stopped by adding 110 μl of ice-cold acetonitrile to the incubation mixture. After vigorous vortexing and centrifugation for 5 min at 11,700×*g* and 10 °C, the supernatant was transferred into autosampler vials and stored at 10 °C until injection. One hundred microliters were analyzed as described above.

Online method

The same components (NADPH-generating system, HLM suspension, and substances in phosphate buffer) were used as described above and used for the offline method. The autosampler was modified with a 200 μl injection loop which could be heated to 37 °C using a column oven. The heated autosampler loop served as the incubation vessel. The HLM suspension, the NADPH-generating system, and the phosphate buffer 0.1 M, pH 7.4 containing the substances at a concentration of 10 mg/l were stored separately in the cooled autosampler stack at 10 °C until usage.

To combine the incubation mixture, the autosampler was programmed using the standard options of the Aria software to first pipette the HLM suspension (15 μl), then the NADPH-generating system (10 μl), and finally the phosphate buffer containing the substances (75 μl) into the heated loop. Afterwards, using the installed 100 μl syringe of the autosampler, the whole incubation mixture was slowly mixed twice by aspiration followed by immediate ejection. To ensure that the 100 μl incubation mixture was in the heated region of the loop inside the column oven, a 30 μl air gap was pushed behind the incubation mixture.

The mixture was incubated in the loop for 50 min, and then the injection valve was switched to the active position, which caused the incubation mixture to be transferred onto the online extraction columns. By transferring the incubation mixture onto the online extraction columns, the reaction was stopped by separating the drug-metabolizing enzymes from the small organic molecules.

Intermediate precision of the online method

To check the stability of the HLM suspension and the NADPH-generating system in the cooled autosampler stack, venlafaxine was chosen as a test compound and repeatedly injected over a 6 h period. Stability was evaluated by calculating the ratio of the peak area of *O*-desmethylvenlafaxine to the peak area of venlafaxine.

To check the between-day intermediate precision of the method, including different lots of HLM suspensions, clotiapine was injected on four different days. We checked whether all of the metabolites could be identified on all of the days.

Method comparison

Fifty-three compounds from various drug classes were metabolized with the offline method as well as the online method in order to compare the efficiency of metabolite generation by the new online procedure.

HLM metabolism of 11 cathinones

To study the phase I metabolism of the 11 cathinones (3,4-methylenedioxy-*N*-benzylcathinone, benzedrone, butylone, dimethylcathinone, ethylone, flephedrone, methedrone, methylone, methylethylcathinone, naphyrone, and pentylone), they were metabolized with the new online metabolite generation system, and the resulting chromatograms were analyzed in order to identify metabolites. The structures of the different cathinones studied are shown in Fig. 1.

Results

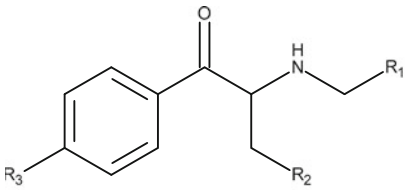
Online method

Using the online method, manual workup was reduced significantly. The only manual steps needed were the loading of the HLM suspension, the substances in phosphate buffer, and the NADPH-generating system onto the autosampler.

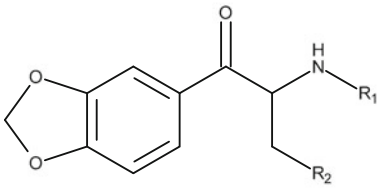
During method development, the poor dispensability of the highly viscous HLM suspension was identified as a problem. This was solved by setting the drawing speed of the autosampler syringe to a low value (5 μl/s) and adjusting the waiting time before the syringe was lifted out of the autosampler vial from 0.1 s to 1 s in order to allow enough time for the viscous suspension to flow into the autosampler syringe.

The remaining volume of HLM suspension that could not be dispensed was reduced to <5 μl by adjusting the penetration level of the autosampler syringe.

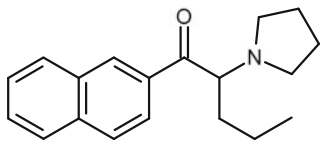
The incubation duration of 50 min was a compromise between the speed and efficiency of metabolite generation; both shorter and longer incubation times are possible with this system. The chromatographic run after the 50 min incubation took about 33 min. However, a new incubation



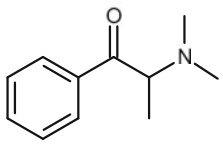
	R1	R2	R3
Benzedrone		H	CH_3
Flephedrone	H	H	F
Methedrone	H	H	OCH_3
Methylethcathinone	CH_3	H	CH_3



	R1	R2
3,4-Methylenedioxy-N-benzylcathinone		H
Butylone	CH_3	CH_3
Ethylone	CH_2CH_3	H
Methylone	CH_3	H
Pentylone	CH_3	$CH_2CH_2CH_3$



Naphyrone



Dimethylcathinone

Fig. 1 Structures of the 11 different cathinones studied

could be started 3 min after the chromatography of the previous incubation was initiated. When a series of different drugs were tested, the time needed for single substances could therefore be reduced to only 50 min (because the previous incubation was analyzed during the incubation of the next sample) instead of 83 min, which represents the sum of the incubation time plus the chromatographic time.

Intermediate precision of the online method

The autosampler stability experiment using the demethylation of venlafaxine to its main metabolite, *O*-desmethylvenlafaxine, showed that the HLM suspension and the NADPH-generating system were stable enough to be used over a time period of at least 6 h when kept in the cooled autosampler stack at 10 °C; the *O*-desmethylvenlafaxine to venlafaxine peak ratio was still 50% of its initial value after 6 h. This is acceptable considering the goal of the method, which was to generate metabolites which would then be added to the library of our toxicological screening method.

In the interday experiment using the metabolites of clotiapine and different lots of HLM suspensions, the same six metabolites (desmethyl-, MI, MII, MIII, MIV, and MV, as shown in Table 1) were identified on all four days, demonstrating that the method achieves reproducible results.

Method comparison

The phase I metabolites of the 53 test compounds identified with the offline and the online methods are shown in Table 1. A chromatogram for pipamperone and its metabolites as well as the corresponding mass spectra of the metabolites can be seen in Fig. 2.

Using the offline method, a total of 156 metabolites were identified. Using the online method, a total of 151 metabolites were identified, among which two metabolites were identified that were not identified by the offline method, and seven metabolites identified by the offline method were not identified using the online method. Therefore, a total of 158 metabolites were identified using both methods, among which 156 (98.7%) were identified by the offline method and 151 (95.6%) by the online method. The seven metabolites that were not identified using the online method were dehydronorketamine, didesmethyltramadol, didesmethyldoxylamine, didesmethylchlorpromazine, cyclic dinordextropropoxyphene, and two oxidation products of thioridazine. The two metabolites identified solely by the online method were dehydrosertindole and an oxidation product of thioproperazine.

Not all of the metabolites could be identified unambiguously using only LC-MS. For example, the differentiation of *N*-oxide- and hydroxy metabolites was not always possible

Table 1 Comparison of the offline and online methods. MI, MII, etc. refer to metabolite I, metabolite II, etc. Different chemical structures are possible for those metabolites based on the acquired MS spectra, as listed in the final column

Substance	Metabolites found with the offline method	Metabolites found with online method	Comment
Amisulpride	Desethyl-, dehydro-, nitro-, MI	Desethyl-, dehydro-, nitro-, MI	MI: hydroxy- or *N*-oxide
Amitriptyline	Desmethylhydroxy-, desmethyl-, didemethyl-, MI, MII	Desmethylhydroxy-, desmethyl-, didemethyl-, MI, MII	MI, MII: hydroxy- or *N*-oxide
Amlodipine	Dehydro-	Dehydro-	
Aripiprazole	Dehydro-, dihydroxy-, MI, MII	Dehydro-, dihydroxy-, MI, MII	MI, MII: hydroxy- or *N*-oxide
Bisacodyl	Didesacetyl-	Didesacetyl-	
Bromazepam	Hydroxy-	Hydroxy-	
Chlorpromazine	Desmethyl-, didesmethyl-, MI, MII, MIII, MIV	Desmethyl-, MI, MII, MIII, MIV	MI, MII, MIII, MIV: hydroxy-, sulfoxide, or *N*-oxide
Chlorprothixene	Desmethyl-, didesmethyl-, *N*-oxide, MI, MII	Desmethyl-, didesmethyl-, *N*-oxide, MI, MII	MI, MII: hydroxy- or sulfoxide
Cinnarizine	MI	MI	MI: hydroxy- or N-oxide
Clotiapine	Desmethyl-, MI, MII, MIII, MIV, MV	Desmethyl-, MI, MII, MIII, MIV, MV	MI: demethyl- plus hydroxy-, *N*-oxide or sulfoxide, MI and MIV: hydroxy-, *N*-oxide or sulfoxide, MII/MV: dihydroxy-, sulfoxide plus *N*-oxide, hydroxy- plus *N*-oxide, or hydroxy- plus sulfoxide
Cyclizine	Desmethyl-	Desmethyl-	
Desipramine	Desmethyl-, MI, MII, MIII	Desmethyl-, MI, MII, MIII	MI, MII: hydroxy- or *N*-oxide, MIII: iminodibenzyl-
Dextropropoxyphene	Desmethyl-, MI	Desmethyl-	MI: cyclic dinor-
Diclofenac	Hydroxy-	Hydroxy-	
Doxylamine	Desmethyl-, didesmethyl-	Desmethyl-	
Duloxetine	Hydroxy-	Hydroxy-	
Fenfluramine	Nor-	Nor-	
Fluoxetine	Desmethyl-	Desmethyl-	
Flupenthixol	Nor-, MI, MII	Nor-, MI, MII	MI: hydroxy-, *N*-oxide or sulfoxide: MII: nor- plus hydroxy-, *N*-oxide or sulfoxide
Fluphenazine	MI, MII	MI, MII	MI, MII: hydroxy-, *N*-oxide or sulfoxide
Fluvoxamine	Desmethyl-, MI	Desmethyl-, MI	MI: hydroxy-, *N*-oxide or sulfoxide
Ketamine	Dehydronor-, desmethyl-, hydroxy-	Desmethyl-, hydroxy-	
Maprotiline	Desmethyl-, MI, MII, MIII	Desmethyl-, MI, MII, MIII	MI–MIII: hydroxy-
Meclozine	Nor-, MI	Nor-, MI	MI: hydroxy- or *N*-oxide
Melitracene	Desmethyl-	Desmethyl-	
Meperidine	Desmethyl-, MI, MII	Desmethyl-, MI, MII	MI: meperidinic acid, MII: normeperidinic acid
Methaqualone	MI, MII, MIII, MIV	MI, MII, MIII, MIV	MI, MII, MIII, MIV: hydroxy-
Methylfentanyl	MI	MI	MI: hydroxy- or *N*-oxide
Methylphenidate	Ritalinic acid	Ritalinic acid	
Mianserine	Desmethyl-, MI	Desmethyl-, MI	MI: hydroxy- or *N*-oxide
Opipramol	Nor-, MI, MII, MIII, MIV, MV, MVI, MVII	Nor, MI, MII, MIII, MIV, MV, MVI, MVII	MI, MII: hydroxy- or *N*-oxide; MIII, MIV, MV, MVI: dihydroxy- or *N*-oxide plus hydroxy-
Oxycodone	MI, MII	MI, MII	MI, MII: desmethyl-
Paroxetine	Hydroxy-	Hydroxy-	
Penfluridol	Nor-, MI	Nor-, MI	MI: hydroxy- or *N*-oxide

Table 1 (continued)

Substance	Metabolites found with the offline method	Metabolites found with online method	Comment
Pentazocine	MI, MII, MIII	MI, MII, MIII	MI, MII, MIII: hydroxy-
Perphenazine	Nor-, MI, MII, MIII	Nor-, MI, MII, MIII	MI, MII: hydroxy-, *N*-oxide or sulfoxide; MIII: nor- plus hydroxy-, *N*-oxide or sulfoxide
Phencyclidine	MI, MII	MI, MII	MI, MII: hydroxy- or *N*-oxide
Pipamperone	Dehydro-, dihydro-, MI	Dehydro-, dihydro-, MI	MI: hydroxy- or *N*-oxide
Promazine	Desmethyl-, MI, MII	Desmethyl-, MI, MII	MI, MII: hydroxy-, *N*-oxide or sulfoxide
Propafenone	Hydroxy-, Nor-	Hydroxy-, Nor-	
Psilocine	HIAA	HIAA	HIAA: hydroxyindoleacetic acid
Reboxetine	Desethyl-, hydroxy-, MI	Desethyl-, hydroxy-, MI	MI: oxidation of morpholino ring
Remifentanil	MI, MII	MI, MII	MI: GI-94219; MII: GI-90291
Sertindole	Hydroxy-	Dehydro-, hydroxy-	
Sildenafil	Desmethyl-, MI, MII, MIII	Desmethyl-, MI, MII, MIII	MI: hydroxy- or *N*-oxide, MII: opening of piperazine ring, MIII: cleavage of piperazine moiety
Strychnine	MI, MII, MIII	MI, MII, MIII	MI, MII: hydroxy- or *N*-oxide, MIII: dihydroxy- or hydroxy- plus *N*-oxide
Thiethylperazine	Desmethyl-, MI, MII, MIII, MIV, MV, MVI, MVII, MVIII	Desmethyl-, MI, MII, MIII, MIV, MV, MVI, MVII, MVIII	MI, MII: hydroxy-, N-oxide or sulfoxide; MIII, MIV, MV: dihydroxy- or combinations of *N*-oxide, sulfoxide and hydroxy-; MVI: opening of piperazine ring; MVII, MVIII: desmethyl- plus hydroxy-, *N*-oxide or sulfoxide
Thioproperazine	Desmethyl-I, desmethyl-II, didesmethyl-I, didesmethyl-II, MI, MII, MIII, MIV, MV	Desmethyl-I, desmethyl-II, didesmethyl-I, didesmethyl-II, MI, MII, MIII, MIV, MV	MI, MII: hydroxy- or *N*-oxide or sulphoxide; MIII: didesmethyl- plus hydroxy-, *N*-oxide or sulfoxide; MIV, MV: desmethyl- plus hydroxy-, *N*-oxide or sulfoxide
Thioridazine	MI, MII, MIII, MIV, MV, MVI, MVII, MVIII, MIX	MI, MII, MIII, MIV, MV, MVI; MIX	MI–MIII: hydroxy-, *N*-oxide or sulfoxide, MIV–MVI: dihydroxy- or combinations of hydroxy-, *N*-oxide or sulfoxide, MVII, MVIII: trihydroxy- or combinations of hydroxy-, *N*-oxide or sulfoxide
Tilidine	Desmethyl-, didesmethyl	Desmethyl-, didesmethyl	
Tramadol	Desmethyl-, desmethyl-II, didesmethyl-	Desmethyl-, desmethyl-II	
Zaleplone	Desethyl-, oxo-, oxodesethyl-	Desethyl-, oxo-, oxodesethyl-	
Zolpidem	Hydroxy-I, hydroxy-II, MI	Hydroxy-I, hydroxy-II, MI	MI: oxidation of methyl group to carboxylic acid

using the acquired MS spectra only. In Table 1, those metabolites are denoted using the naming scheme of M plus a Roman numeral, and the different chemical structures are mentioned in the final column. To elucidate the exact structure of each metabolite, complementary techniques need to be applied.

HLM phase I metabolism of 11 cathinones

The proposed structures of the identified phase I metabolites are shown in Figs. 3, 4, and 5. The general metabolism pathways seen in several substances include desalkylation of the amine group (cf. Fig. 1). This metabolite was seen in all cathinones tested apart from naphyrone, which contains a tertiary amine included in a 5-ring instead of the secondary amine present in all of the other tested substances. Other metabolites observed for more than one substance include those resulting from the reduction of the keto group and hydroxylation products. In cathinones containing a methylenedioxy moiety, this moiety can be desmethylenated to the respective diol, a metabolite which exhibited quite a high intensity in our experiments. Combinations of the mentioned phase I metabolites were also observed for some substances, indicating subsequent metabolism steps.

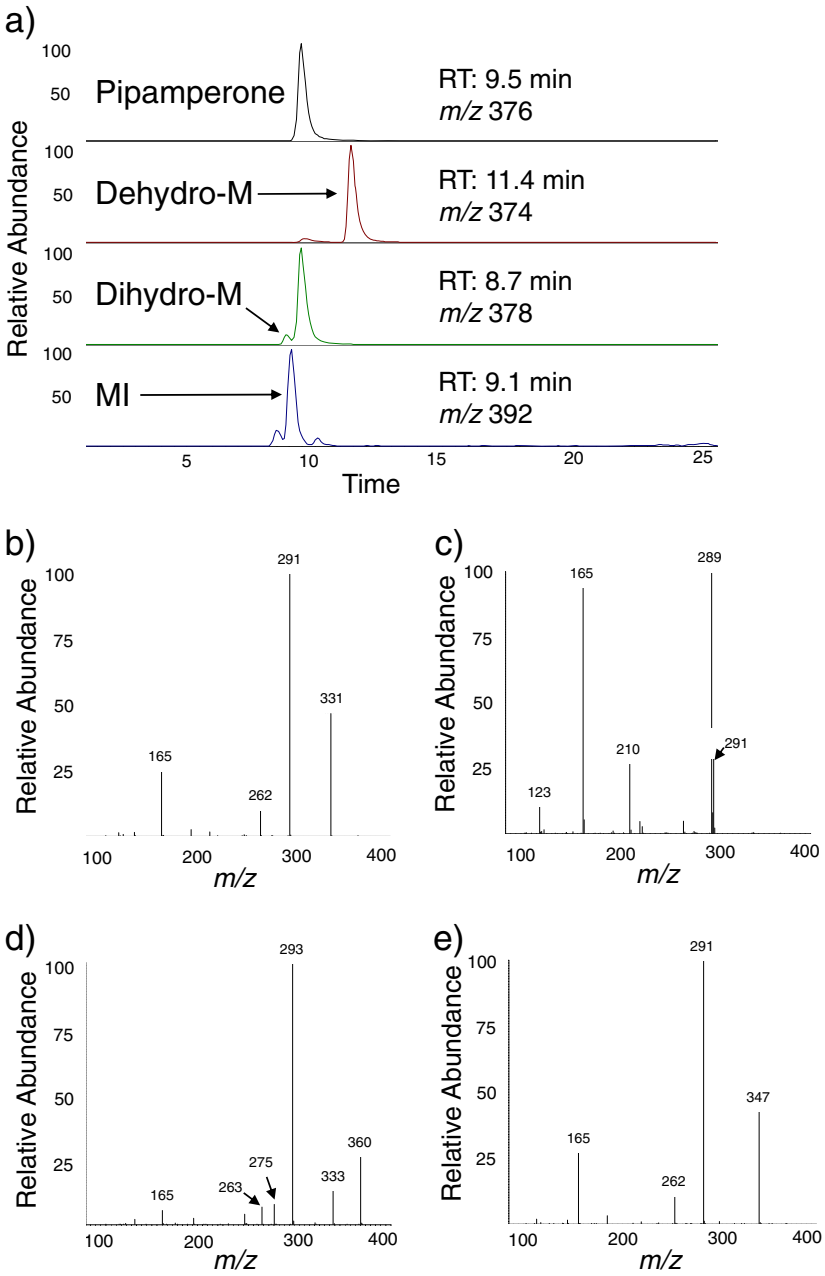


Fig. 2a–e Chromatogram and mass spectra for pipamperone and its metabolites. **a** Example of a chromatogram of pipamperone and its metabolites. Extracted ion chromatograms obtained in positive mode are shown. The corresponding retention times (RTs) as well as the mass-to-charge ratios (*m/z*) are indicated. **b** Mass spectrum of pipamperone. **c** Mass spectrum of the dehydro metabolite of pipamperone. **d** Mass spectrum of the dihydro metabolite of pipamperone. **e** Mass spectrum of metabolite I (MI) of pipamperone. As indicated in Table 1, the hydroxy metabolite or the *N*-oxide metabolite cannot be distinguished

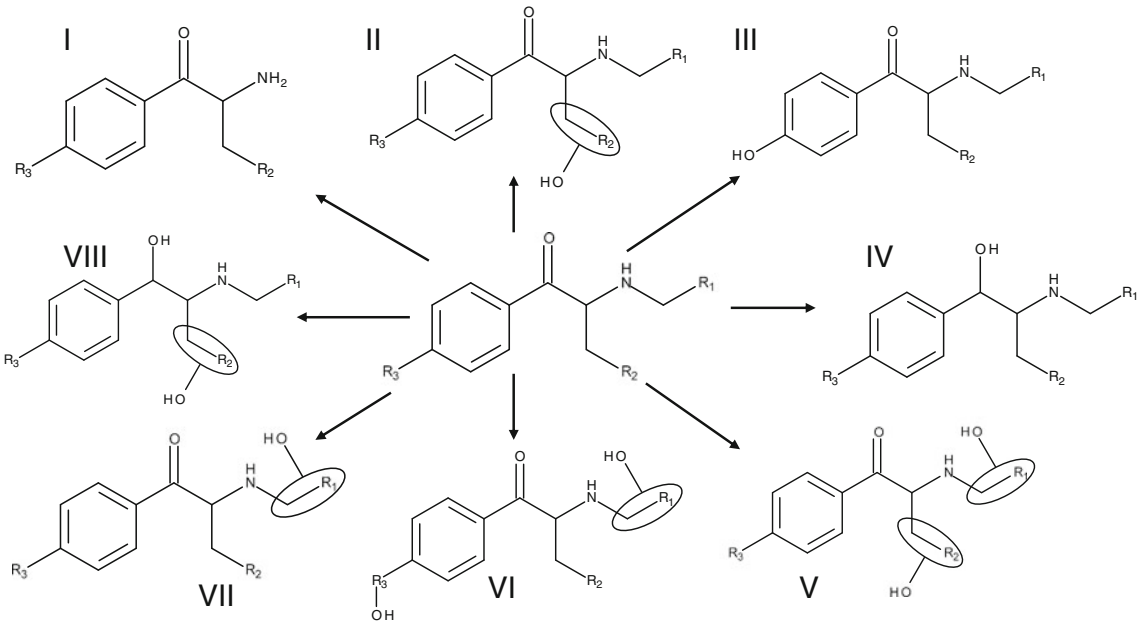
Discussion

The majority of the metabolites identified using the offline method (95.6% of them) could be identified using the online method. A possible issue for the other seven metabolites that could not be identified using the online method may be the difference in incubation times. In the offline method, substances were incubated for 3 h, but this was reduced to 50 min in the online method to speed up the process.

As sertindole is light sensitive, the lack of identification of dehydrosertindole by the offline method could be due to the instability of the parent compound. In the opaque loop used for online incubation, the incubation mixture was always protected from the light, which was not the case in the Eppendorf vials that were used for the offline method.

The online method minimizes the manual preparation steps required compared to the offline method. This helped to speed up the whole process and to minimize errors. The autosampler modifications required were minimal and could be done in a few minutes. The system can therefore be used sequentially for clinical toxicological analysis and for metabolism experiments.

In 2005, a method using similar instrumentation was published. Lai et al. [9] used an automated online extraction system to study pro-drug stability. The autosampler was modified and included a heated agitator for sample incubation.



	R1	R2	R3	Metabolites identified
Benzedrone		H	CH_3	I, II, V, VI, VII, VIII
Flephedrone	H	H	F	I, II
Methedrone	H	H	OCH_3	I, III
Methylethcathinone	CH_3	H	CH_3	I, II, IV

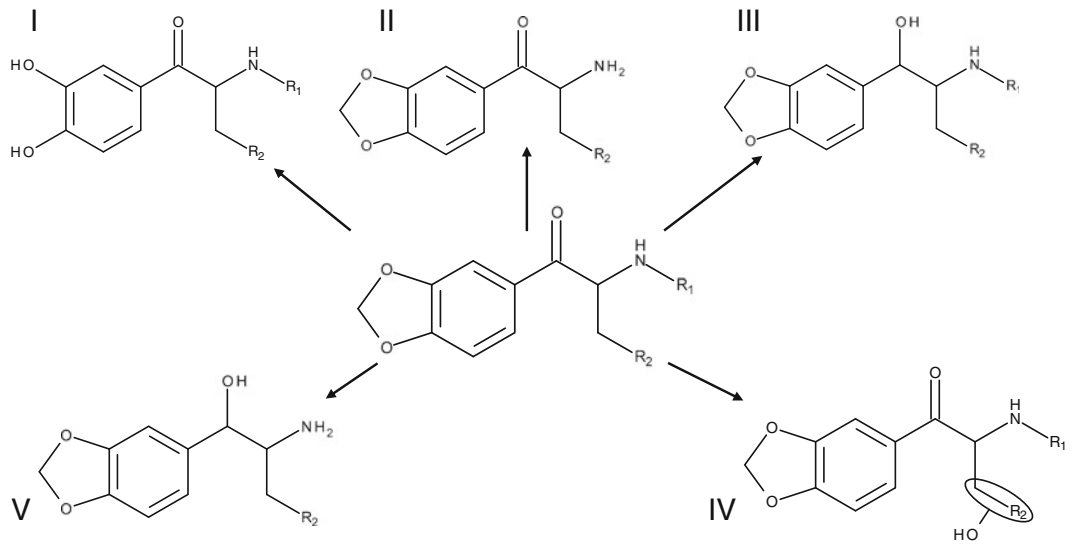
Fig. 3 Structures of the proposed metabolites for benzedrone, flephedrone, methedrone, and methylethcathionone

Incubation vials containing the biological matrix (e.g., S9 fraction) diluted with buffer were stored in the cooled autosampler stack. The autosampler was programmed to add the cofactors (e.g., the NADPH-generating system) to this vial and subsequently transport the whole vial to the heated agitator. After a pre-incubation, the test substance was added. At programmed time points, the autosampler withdrew an aliquot for analysis, which was subsequently extracted online using turbulent flow chromatography. Compared with our method, the autosampler needed more modifications (e.g., the heated agitator). In our approach, only the loop needed to be changed in daily operation. Also, fewer manual steps were needed, since the autosampler adds not only cofactors and the test substance to the incubation mixture but also the HLM suspension. However, our method was slower and did not allow sampling at different time points, since the aim of the method was a different one. We were not interested in kinetic experiments, only the end points.

Compared to the method of Nicoli et al. [11] and Curcio et al. [12], where a capillary was used as reaction vessel for the HLM experiments, our method coupled the use of a modified injection loop online to an online extraction system. No manual sample pretreatment was necessary.

Compared to fully integrated and automated solutions, as presented by Drexler et al. [6], Xu et al. [7], and Jenkins et al. [8] for instance, our method had a much lower throughput. The aim of this work was not a fully integrated high-throughput method but a method that is as automated and makes it as easy as possible to generate metabolites that can be added to the library of our toxicological screening method, and to study the metabolism of new designer drugs.

To our knowledge, among the 11 cathinones studied, only butylone, ethylone, and methylone have had their metabolisms published already [4, 17, 18], and these have always been based on the use of HLMs. For butylone and ethylone, Zaitsu et al. [4] identified the normetabolite, produced by the reduction of the keto group to the corresponding alcohol and demethylenation with subsequent methylation by the catechol-*O*-methyltransferase. Meyer et al. [18] were also able to detect those metabolites for butylone using their standard toxicological GC-MS screening procedure. In our experiments, we also detected the normetabolites for butylone and ethylone. Since we did not add the co-substrate needed for the methylation (*S*-adenosylmethionine; SAM) to the incubation mixture, we could not detect the methylated derivatives of both cathinones, only the demethylenyl-diol-metabolites.



	R1	R2	Metabolites identified
3,4-Methylenedioxy-N-benzylcathinone		H	I, II, III
Butylone	CH_3	CH_3	I, II, IV
Ethylone	CH_2CH_3	H	I, II, IV
Methylone	CH_3	H	II, III, IV
Pentylone	CH_3	$CH_2CH_2CH_3$	I, II, III, V

Fig. 4 Structures of the proposed metabolites for 3,4-methylenedioxy-*N*-benzylcathinone, butylone, ethylone, methylone, and pentylone

Interestingly, we did not observe the products of keto-group reduction, but we were able to identify an additional hydroxy metabolite for both butylone and ethylone.

The metabolism of methylone was studied by Kamata et al. [17]. They identified the normetabolite and the demethylenation products, which were methylated by the catechol-*O*-methyltransferase. Meyer et al. [18] identified an additional metabolite, the demethylenated normetabolite, which was again methylated by the catechol-*O*-methyltransferase. We also detected the normetabolite, but

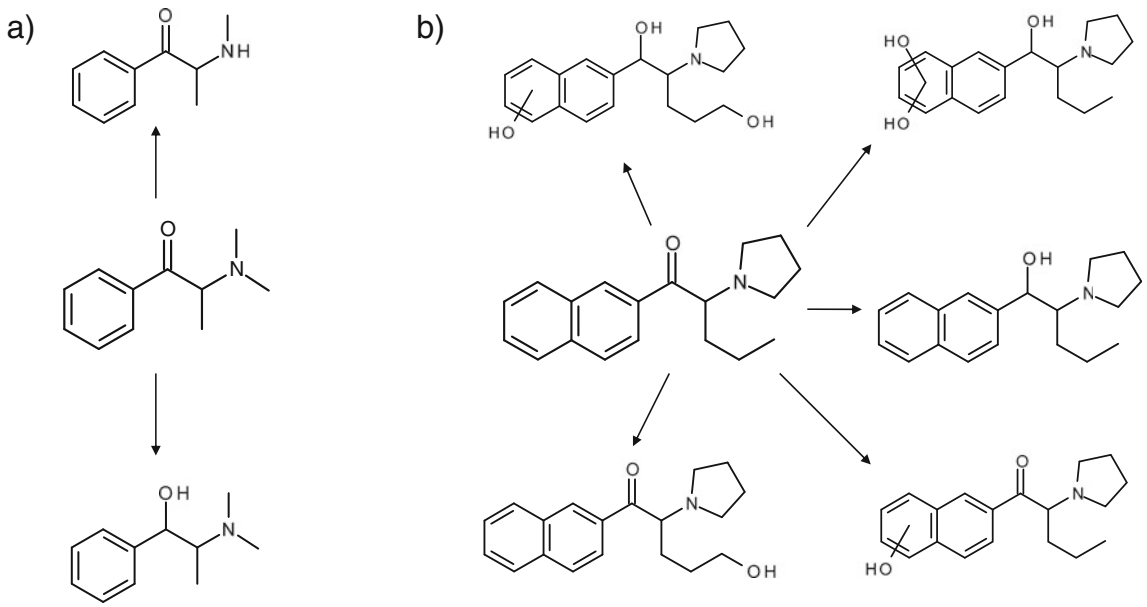


Fig. 5a–b Structures of the proposed metabolites for **a** dimethylcathinone, and **b** naphyrone

no demethylenation products. Instead, we detected a metabolite due to the reduction of the keto group plus a hydroxy metabolite.

No information on the metabolism of the other cathinones could be found in the literature. However, the metabolic pathways described for butylone, ethylone, and methylone were also observed in most other cathinones.

Conclusions

The method presented in this paper provides an easy way to identify metabolites (which can then be added to the library of our clinical toxicological screening method) at an adequate throughput. The performance of the method compared well with that of an established offline HLM procedure, but it requires less manual work.

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