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ORIGINAL ARTICLE

Studies on the phase I metabolism of the new designer drug 3-fluoromethcathinone using rabbit liver slices

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Abstract The metabolism of the novel designer drug 3-fluoromethcathinone (3-FMC), sold as "legal highs", was investigated in vitro via cryopreserved rabbit liver slices. The pharmacological properties and toxicological effects of 3-FMC and its metabolites are not known yet. It can be assumed that 3-FMC will cause effects similar to 4-methylmethcathinone (mephedrone) and methcathinone. For the metabolism studies, pretests were performed with rabbit liver slices incubated with kavain to evaluate optimal conditions. Finally, six known metabolites of kavain were revealed and therefore sufficient information about the suitability of the enzyme system of the rabbit liver slices was obtained. Under optimized conditions, 3-FMC was added to Krebs–Henseleit buffer, pH 7.4 containing NADPH and bicarbonate and incubated with a single rabbit liver slice at 37°C. The metabolism was monitored at 5, 30 and 180 min, respectively. The metabolites formed via the former cryopreserved rabbit liver slices were examined by LC/MS-TOF. Metabolites were identified by their exact masses and isotopic patterns. 3-Fluorocathinone, 3-fluorocathinone-imine, hydroxy-3-fluoromethcathinone and 3-fluoromethcathinone-diol were formed as the main metabolites.

Keywords Designer drug · 3-Fluoromethcathinone · "Legal highs" · Rabbit liver slices · LC/MS-TOF

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Introduction

Cathinones like cathine or norephedrine are natural psychoactive compounds which could be extracted from leaves of the khat plant *Catha edulis* [1]. In recent years, synthetic cathinones like methcathinone, 3-fluoromethcathinone (3-FMC), 4-fluoromethcathinone (flephedrone), or 4-methylmethcathinone (mephedrone) have appeared on the illicit drug market in many countries. Cathinone derivates (beta-keto compounds) are structurally related to amphetamine and cathinone. Fifteen of these synthetic cathinones are currently monitored by the EU early warning system [2]. Up to now, the metabolism of most of these new illicit drugs is still not characterized.

Due to analog structures and the similarity to cathinone, it can be assumed that 3-FMC may cause the same psychostimulant effects as 4-methylmethcathinone (in the illicit market also known as mephedrone, 4-MMC, meow-meow, meph, or MCAT [3]) and methcathinone.

Table 1 Composition for the preparation of Krebs–Henseleit buffer pH 7.4 [6]

Reagent	(g/L)	(mM)
NaCl	6.9	118.0
KCl	0.36	4.8
$MgSO_4 \times 7\ H_2O$	0.295	1.2
$NaHCO_3$	2.0	23.8
KH_2PO_4	0.13	0.95
$CaCl_2 \times 2\ H_2O$	0.498	2.27
D(+)-Glucose	4.5	25.0

3-FMC and other designer drugs are often sold as "legal highs" in headshops or via the internet, e.g., as "bath salts" or "research chemicals". Drug consumers do not really know the ingredients or compounds of these "legal highs". Furthermore, the health risks as well as the addictive potential of the drug consumption are not known and even might be of minor interest for some consumers. Moreover, consumers face problems with wrong identification and consumption, since synthetic cathinones have similar trivial names like mephedrone, methylone, or methedrone [3]. Recent analyses show that most of the sold "legal highs" preparations mainly contain single cathinones or cathinone mixtures. Furthermore, caffeine, lidocaine, procaine and other compounds could be detected in such preparations [4].

In forensic toxicology, it is important to have the analytical data of these compounds at hand—especially those of their metabolites. To obtain these metabolites, one method is the incubation of xenobiotics with liver cells [5]. Another well-established method in metabolism studies is the use of precision-cut tissue slices (such as liver slices). In 1923, Otto Warburg used this method for the determination of respiratory and fermentation processes [6]. Tissue slices can be easily prepared from different organs of all kind of animal species with a various number of cutting procedures (e.g., cryomicrotome or even a razor blade [7]). Tissue slices maintain the intact cell structure, so that intracellular interactions between cells are possible [8]. Furthermore, liver slices can be cryopreserved in liquid nitrogen, which facilitates the storage for long period of time while losing only minimal enzymatic activity [9]. The aim of the present study is the identification of new metabolites of 3-FMC via rabbit liver slices using liquid chromatography/mass spectrometry–time-of-flight (LC/MS-TOF). As a sideline, the study aims to optimize that method.

Materials and methods

Chemicals and reagents

3-FMC-hydrochloride (National Measurement Institute, Australia), kavain (Klinge Pharma Ldt., Germany), methanol LC-MS (Riedel-de-Häen, Germany), acetone for high-performance liquid chromatography (Sigma-Aldrich, Germany), formic acid (50%) and acetonitrile LC-MS were obtained from Fluka Analytical, Germany and nicotinamide adenine dinucleotide phosphate (NADPH) from Roche Diagnostics, USA.

Chemicals for the preparation of Krebs–Henseleit buffer: Salts and glucose for the buffer were obtained from Merck KGaA, Darmstadt, Germany. Hydrochloric acid (37%; Normapur analytical reagent) was obtained from VWR, Germany and carbogen 95% O_2/5% CO_2 from Linde, Germany.

Chemicals for the preparation of the storage medium: Waymouth MB 752/1 and fetal calf serum (FCS) were obtained from Sigma-Aldrich, Co., USA and dimethyl sulfoxide (DMSO) from Merck, Darmstadt, Germany.

Preparation of Krebs–Henseleit buffer pH 7.4

The Krebs–Henseleit buffer pH 7.4 was prepared as shown in Table 1. The pH value of the buffer was adjusted with sodium hydroxide or hydrochloric acid to pH 7.4 and purged with 95% O_2/5% CO_2 for 10 min [6].

Preparation of the medium for the storage of the rabbit liver slices

Fourteen grams of powdered Waymouth MB 752/1 was dissolved in 1 l distilled water by stirring gently. Seven hundred milliliters of the medium was mixed with 200 ml FCS and 100 ml DMSO. The rabbit liver slices were put into 1 ml medium (glass vial) for 30 min until they were treated with liquid nitrogen.

Preparation of the NADPH solution

One milligrams of NADPH was dissolved in 1 ml distilled water and kept into a fridge (1–4°C) until required. A fresh solution was prepared every day.

Preparation of freshly and cryopreserved rabbit liver slices

The liver of a freshly slaughtered rabbit from the Bonnen Spargelhof in Neuss, Germany was immediately excised,

bled and placed into the ice-cold Krebs–Henseleit buffer (pH 7.4). The liver was cut into pieces of 1–2 cm^3. According to the method of Deutsch [7], slices were freshly prepared by cutting the liver pieces with a common razor blade, while fixing the liver pieces between two microscopic slides. The slices were immediately placed in Krebs–Henseleit buffer at 4°C and a gas mixture (Carbogen) was bubbled through the buffer until required. The freshly prepared liver slices were placed into glass vials containing the storage medium, cryopreserved by direct immersion in liquid nitrogen and kept refrigerated at −30°C until required.

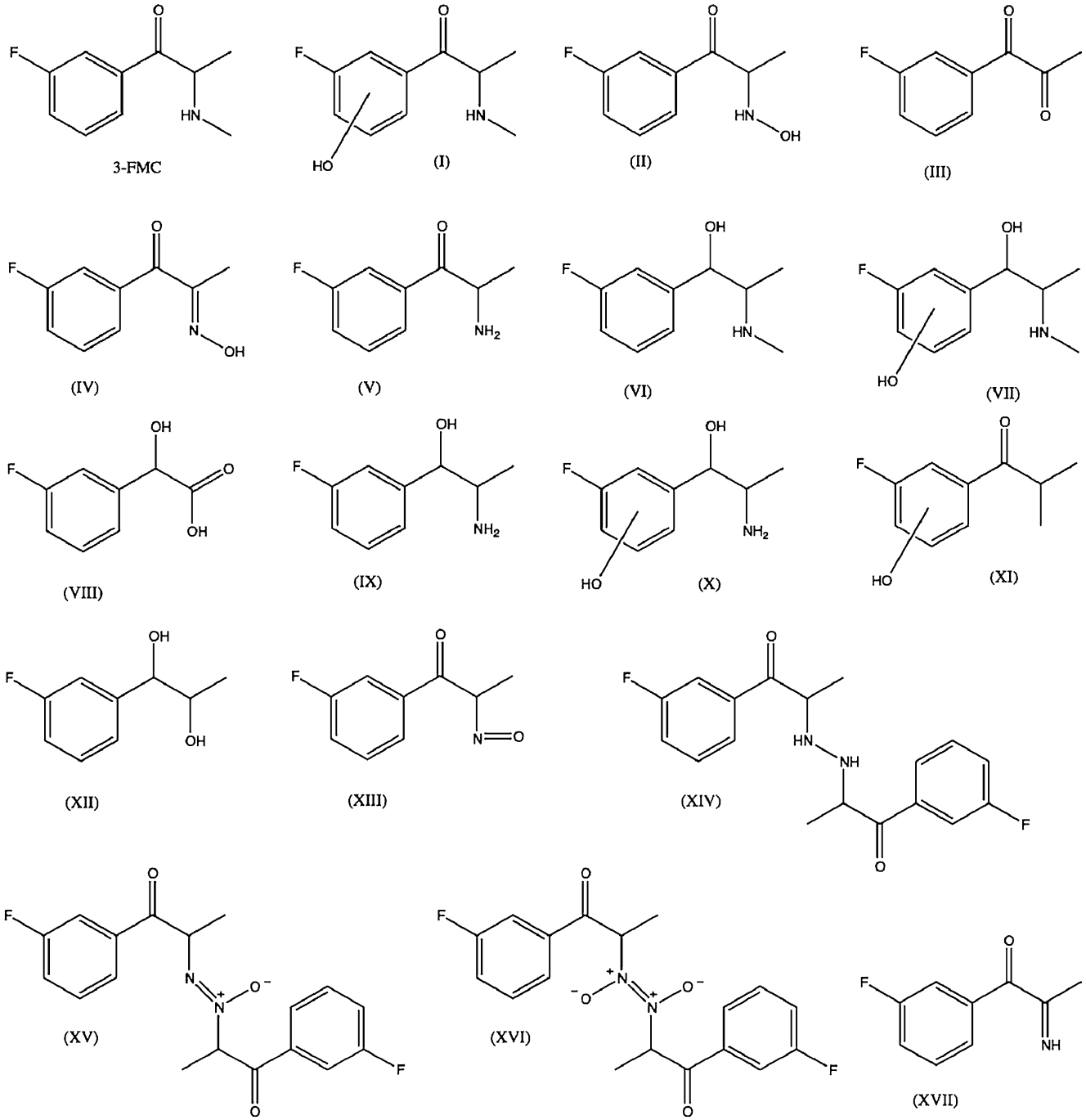

Fig. 1 Proposed structures for the phase I metabolites of 3-FMC

Table 2 Identified metabolites of 3-FMC with their chemical formulas, scanned masses (M + nH), the error (in parts per million) values of their C^{12}-signals and the error (in parts per million) values of their isotopes after the incubation with cryopreserved rabbit liver slices (explanation given below)

Compound	Chemical formula + H	Mass (M + nH) m/z	Error (ppm)	Error (ppm) isotopes
Hydroxy-3-fluoromethcathinone (I)	$C_{10}H_{12}FNO_2$	198.0924	+1.9	5.7
3-Fluorocathinone (V)	$C_9H_{10}FNO$	168.0819	−0.4	−34.7
3-Fluoromethcathinone-diol (VII)	$C_{10}H_{14}FNO_2$	200.1081	−0.6	–
3-Fluorocathinone-imine (XVII)	C_9H_8FNO	166.0662	−1.0	−1.7

Metabolism of 3-fluoromethcathinone with cryopreserved rabbit liver slices

The cryopreserved liver slices were thawed on ice and preincubated in a total volume of 1.7 ml Krebs–Henseleit buffer pH 7.4 in addition of 2 μg NADPH (single slice per experimental approach). The preincubation was carried out at 37°C on a shaker for 1 h. Preincubated liver slices were placed into 1.7 ml fresh Krebs–Henseleit buffer containing 2 μg NADPH and 200 μg 3-FMC. The experiment was started by adding the liver slice into the medium. The incubations were carried out at 37°C on a shaker for 5, 30 and 180 min, respectively [10]. Every incubation period represented a single experimental appendage. The medium was initially oxygenated with 95% O_2/5% CO_2 and every subsequent hour for several minutes (to maintain optimal conditions). The incubation was stopped by adding 10 ml of an acetone/methanol mixture (90:10, *v*/*v*) to the medium. The flasks were placed into an ultrasonic bath for 5 min. The liver slices were removed and the solutions were centrifuged for 10 min at 2,500 rpm. The supernatants were filtered, evaporated under a stream of argon to a volume of approximately 1.7 ml and analyzed by LC/MS-TOF (injection volume 2.5 or 5 μl). Controls were subjected to the same incubation conditions.

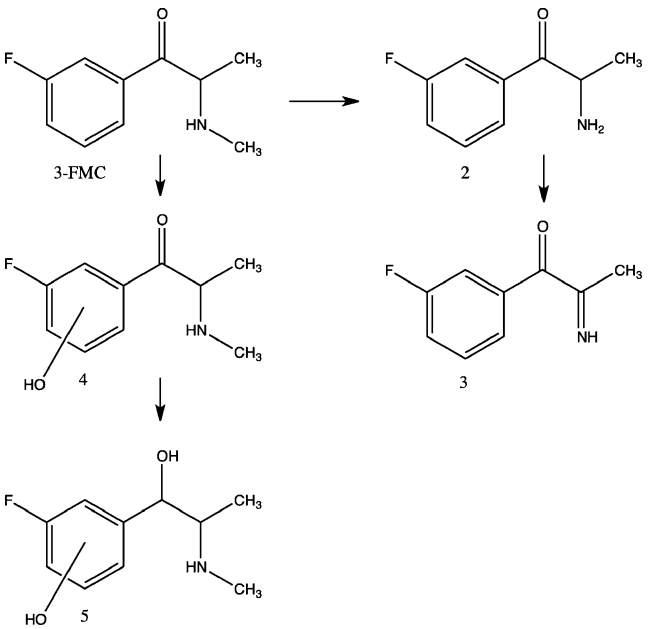

Fig. 2 Scheme of the proposed phase I metabolism of 3-FMC in rabbit liver

LC/MS-TOF

Analyses were carried out using an Agilent system LC 1200 Series with a stainless steel YMC-Pack 3 μm, ODS-AQ 150×2 mm column at a temperature of 30°C. As eluent, 30% acetonitrile/70% water containing 0.05 vol.% formic acid at a flow rate of 0.2 ml/min was used. Mass spectrometer was supplied from Bruker (MS Bruker microTOF-Q II) using ESI mode at a capillary voltage of 4,500 V and a scan range of 50–1,000 amu. The identification of 3-FMC and its metabolites was carried out via their exact masses and isotopic patterns using Bruker Compass Software Version 1.3 (Smart Formula Manually).

Results and discussion

Pretests demonstrated that kavain is metabolized by the rabbit liver cells indicating a functional enzyme system. Most of the kavain metabolites described before [5] such as hydroxykavain, 6-phenyl-5-hexen-2, 4-dione, 5,6-dehydrokavain, 12-hydroxy-5,6-dehydrokavain and/or 11-hydroxy-5,6-dehydrokavain, cinnamyl-acetone and/or 6-phenyl-3-hexen-2-on and 4-hydroxy-6-hydrophenyl-5-hexen-2-on could be detected in the incubation medium. The advantages of using liver slices rather than hepatocytes, microsomes (only membrane-bound enzymes), cDNA, or cloned

enzymes lie in the preservation of the biological cell structure (united cell structure), which guarantees the cell–cell and cell–matrix interactions and thus simulates a system in vivo. In addition, examinations on the phase I and phase II metabolism are possible. The disadvantages of using tissue slices lie in the insufficient reproducibility of making precision cuts, which can cause major differences in the various concentrations, especially if minimal deviations of the tissue slice thickness coupled with insufficient oxygen supply exist. Besides, the preparation of precision cut slices requires the availability of fresh tissue, and the storage of cryopreserved liver slices might be difficult over long periods of time [8] if not liquid nitrogen is used [9].

Identification of 3-fluoromethcathinone metabolites

Ephedrine and norpseudoephedrine are formed from methcathinone [11] while the parent substance cathinone metabolizes to norephedrine and norpseudoephedrine [12]. Therefore, it was assumed that 3-FMC may metabolize in a similar way to the corresponding compounds. The possible phase I metabolites of 3-FMC are exposed in Fig. 1. The

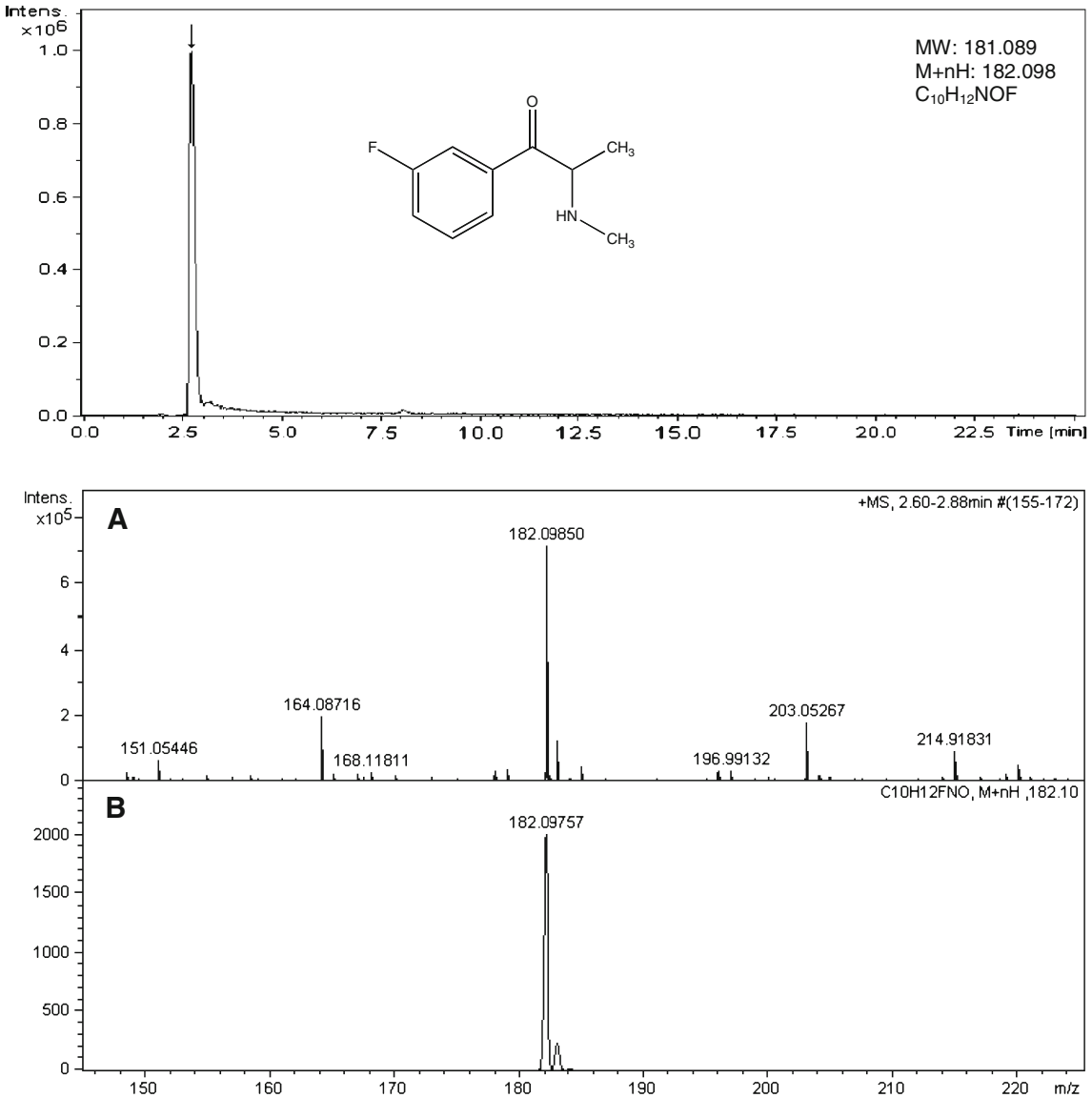

Fig. 3 LC/MS-TOF chromatogram (*above*) and spectra (*below*) of 3-FMC after 3 h of incubation with a cryopreserved rabbit liver slice. The signal shows the C^{13} mass of 3-FMC. **a** 3-FMC mass spectrum measured after the incubation. **b** Spectrum of the calculated 3-FMC mass

proposed structures were created according to the known metabolism of methcathinone, cathinone, amphetamine and methamphetamine [11–14].

The incubation of 3-FMC was carried out by applying different incubation times to detect possible metabolic pathways and metabolic intermediates, respectively, as presented in Fig. 1. Samples were not derivatized and the metabolism studies were repeated at least twice. While metabolites akin to norephedrine and norpseudoephedrine were not found, other fluorinated metabolites could be detected after the incubation of 3-FMC with the cryopreserved rabbit liver slices.

Metabolites formed by the rabbit liver slices were separated by HPLC. The identification of the proposed 3-FMC metabolites was performed via their exact masses using internal calibration with lithium formate. Other than that, they were identified via intensity and exact masses of their isotope signals compared to the theoretical isotopic

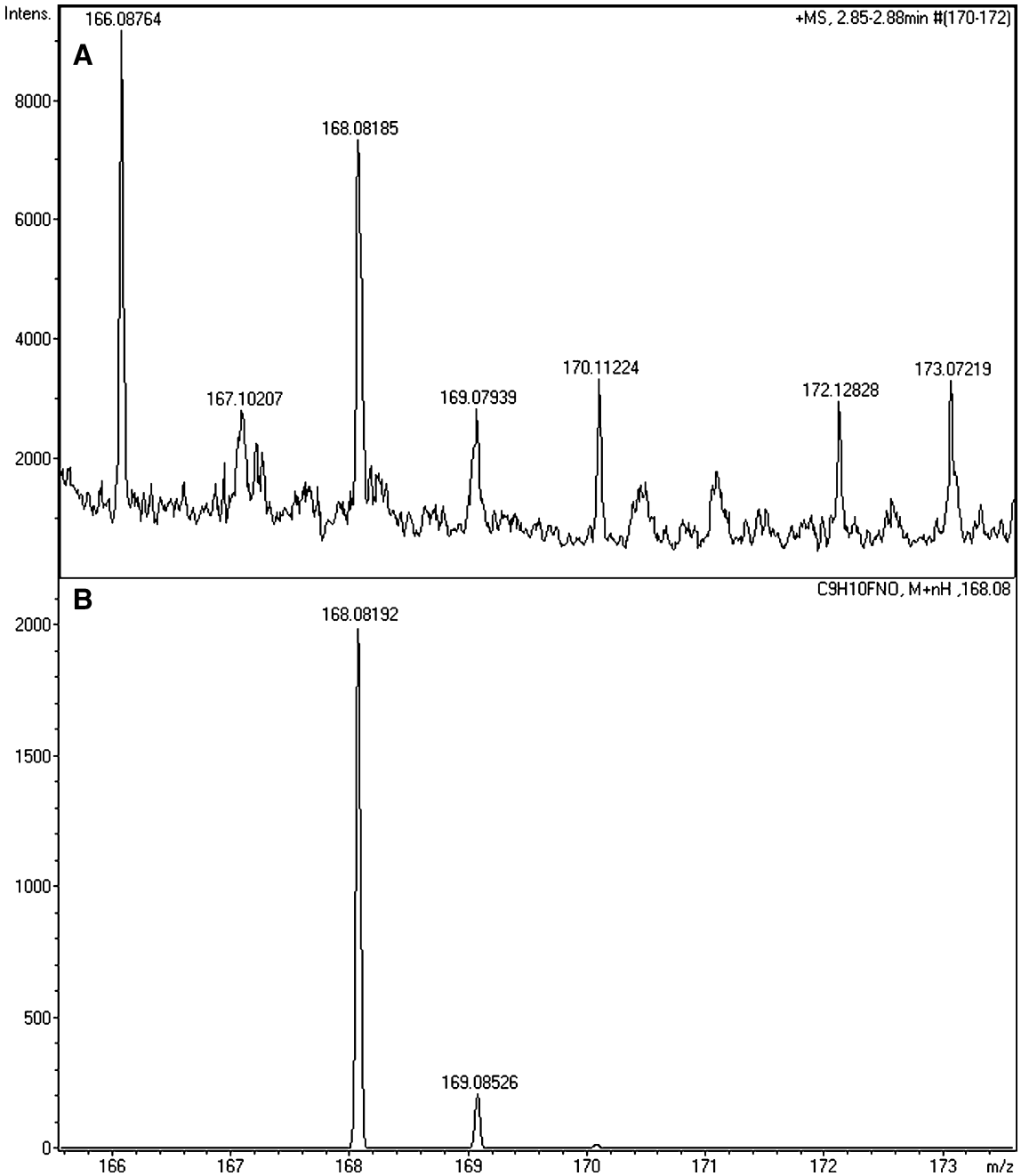

Fig. 4 LC/MS-TOF chromatogram (*above*) and spectra (*below*) of 3-fluorocathinone formed after 30 min of incubation of 3-FMC with a cryopreserved rabbit liver slice. The signal shows the C^{12} mass of the metabolite. **a** 3-Fluorocathinone mass spectrum after the incubation. **b** Spectrum of the calculated 3-fluorocathinone mass

patterns. The incubation solution as well as raw cryopreserved cell slices were regularly checked for the absence of these metabolites.

The error (in parts per million) values (deviation) describing the difference between the measured metabolite mass *m/z* (*M* + nH) and the calculated mass *m/z* (*M* + nH) divided by the calculated mass *m/z* (*M* + nH) multiplied with one million (Table 2) were fairly well inside the calculated limits (5 ppm).

The detection of 3-fluorocathinone (V) and 3-fluoromethcathinone-diol (VII) isotopes was complicated due to similar elution time with the surplus parent substance, but hydroxy-3-fluoromethcathinone (I) and 3-fluorocathinone-imine (XVII) were well separated by liquid chromatography resulting in the best accordance to the theoretically given values (Table 2). The relative intensities of the metabolite isotopes matched the calculated values in all isotopes that could be separated from the matrix. The detected exact masses of the isotopes were inside acceptable ranges but not as precise as their main signals because the low concentration of a metabolite in the examined incubation media results in a decline of precision. Therefore, the chemical structure of the different expected metabolites was also taken into consideration.

According to Meyer and Maurer [15, 16], using human liver microsomes, beta-keto drugs are metabolized to their

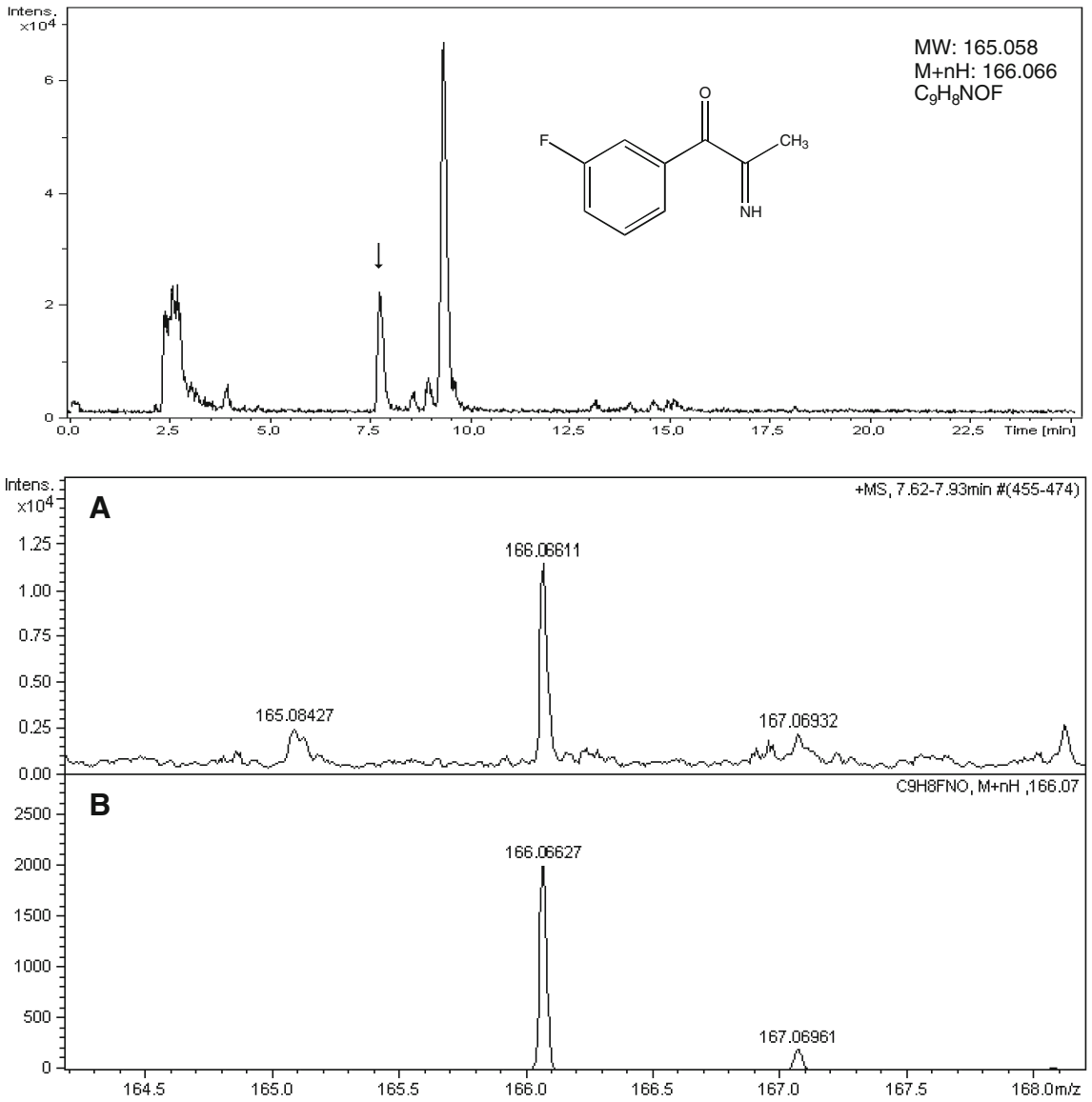

Fig. 5 LC/MS-TOF chromatogram (*above*) and spectra (*below*) of 3-fluoromethcathinone-imine formed after 30 min of incubation of 3-FMC with a cryopreserved rabbit liver slice. The signal shows the C^{12}-mass of the metabolite. **a** Measured 3-fluoromethcathinone-imine mass spectrum after the incubation. **b** Spectrum of the calculated 3-fluoromethcathinone-imine mass

corresponding alcohols or are N-demethylated to their corresponding amines [15, 16]. These results correlate with our results (Fig. 2, *2*).

But instead of finding N-demethylated metabolites with a simultaneous reduction of the ketone, we found metabolites with a reduction of the carbonyl group and metabolites with an oxidation of the phenyl moiety (Fig. 2, *5*). In addition, we found metabolites with a reduction of the amino group (Fig. 2, *3*) or metabolites with only an oxidation of the phenyl moiety (Fig. 2, *4*). A possible explanation is either resonance structures via mesomeric effects of the fluorine that might facilitate the rapid formation of phenyl oxidation products or the metabolic pathways in rabbit liver differ from those in rat or human liver due to diverse enzyme activities especially due to the deaminase as well as CYP activity. Based on the mentioned metabolites, the scheme of the phase I metabolism of 3-FMC in rabbit liver presented in Fig. 2 is proposed.

The determination of diastereomers as well as the determination of the position of the hydroxyl group on the

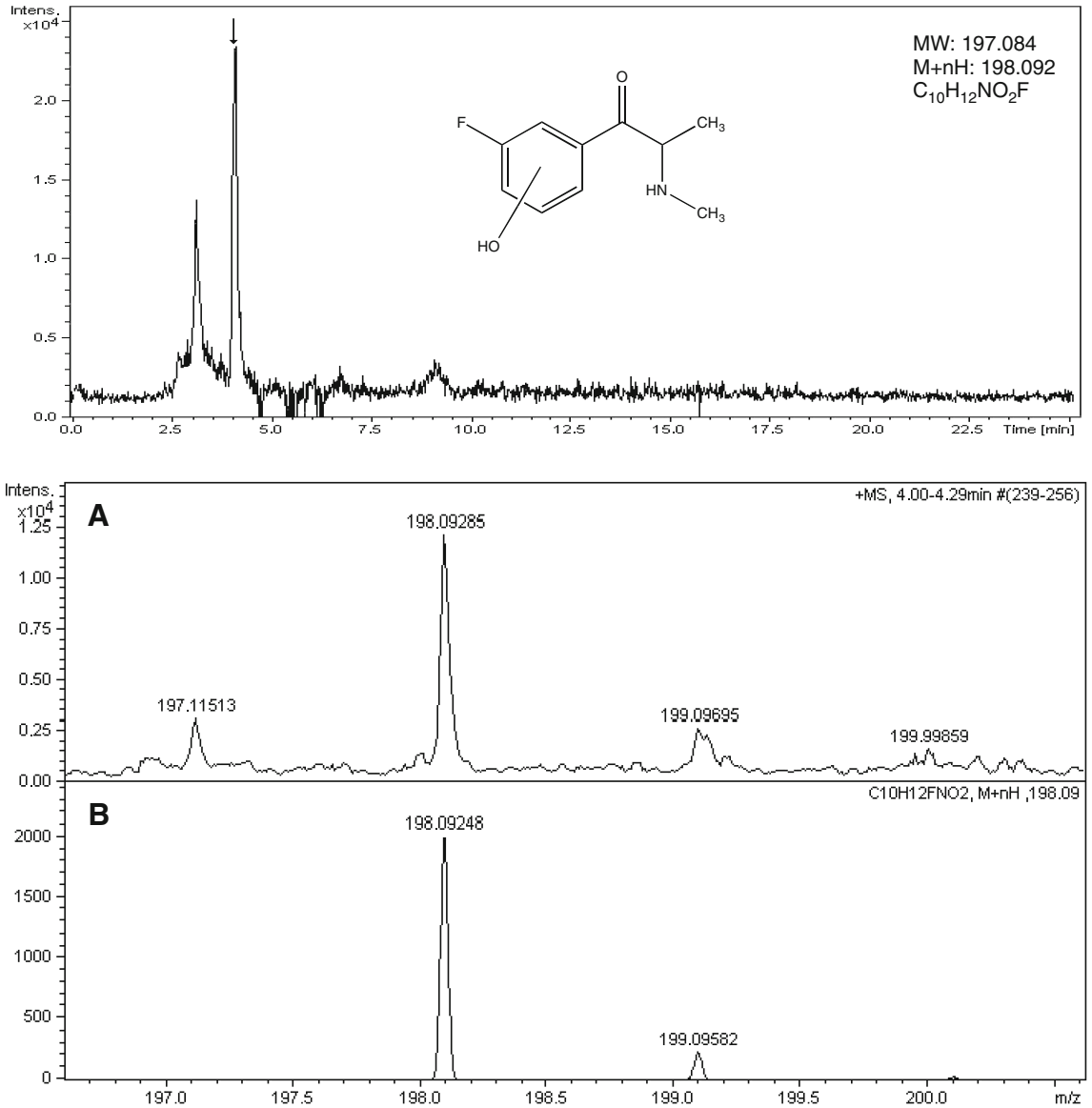

Fig. 6 LC/MS-TOF chromatogram (*above*) and spectra (*below*) of hydroxyl-3-fluoromethcathinone formed after 30 min of incubation of 3-FMC with a cryopreserved rabbit liver slice. The signal shows the C^{12}-mass of the metabolite. **a** Measured hydroxy-3-fluoromethcathinone mass spectrum after the incubation. **b** Spectrum of the calculated hydroxy-3-fluoromethcathinone mass

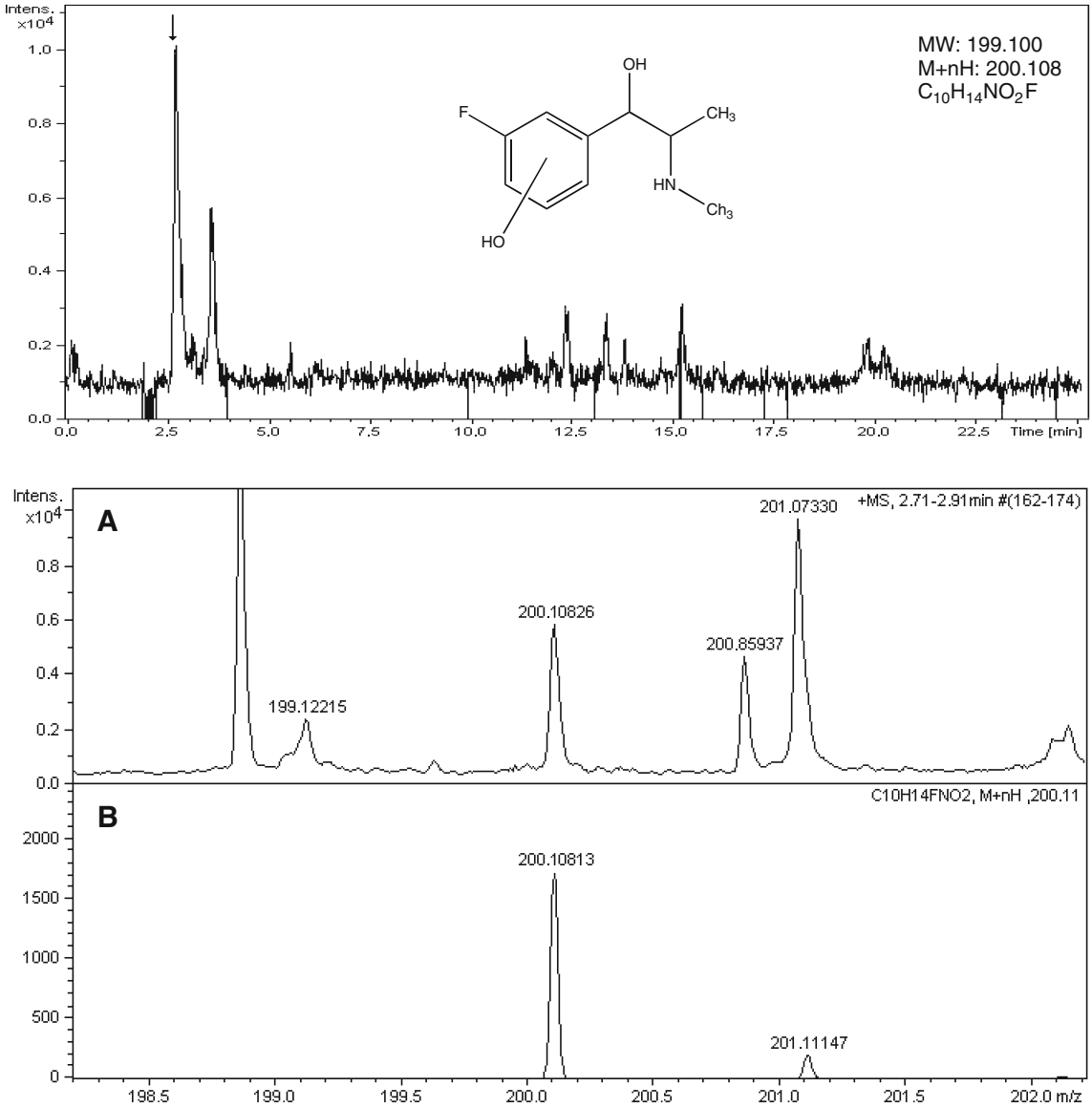

Fig. 7 LC/MS-TOF chromatogram (*above*) and spectra (*below*) of 3-fluoromethcathinone-diol formed after 30 min of incubation of 3-FMC with a cryopreserved rabbit liver slice. The signal shows the C^{12}-mass of the metabolite. **a** Measured 3-fluoromethcathinone-diol mass spectrum after the incubation. **b** Spectrum of the calculated 3-fluoromethcathinone-diol mass

phenyl moiety was not possible. The LC/MS-TOF chromatograms and spectra of 3-FMC and its metabolites are shown in Figs. 3, 4, 5, 6 and 7.

Conclusion

We could demonstrate that after the incubation with cryopreserved rabbit liver slices, the new "legal high" 3-FMC is metabolized into four phase I compounds. These are: 3-fluorocathinone, 3-fluoromethcathinone-diol, 3-fluoromethcathinone-imine and hydroxy-3-fluoromethcathinone.

Ethical standards We declare that the present study complies with the current German law. The rabbit was slaughtered by the butcher and sold for food consumption. Usually, the liver would have been sold together with the slaughtered rabbit, the study is therefore not declared as an animal test.

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Conflict of interest The authors declare that they have no conflict of interest.

Animal welfare No animal experiment was carried out.

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