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Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) in toxicological analysis

Studies on the detection of clobenzorex and its metabolites within a systematic toxicological analysis procedure by GC–MS and by immunoassay and studies on the detection of α- and β-amanitin in urine by atmospheric pressure ionization electrospray LC–MS

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Abstract

GC–MS is the method of choice for toxicological analysis of toxicants volatile in GC while non-volatile and/or thermally labile toxicants need LC–MS for their determination. Studies are presented on the toxicological detection of the amphetamine-like anorectic clobenzorex in urine by GC–MS after acid hydrolysis, extraction and acetylation and by fluorescence polarization immunoassay (FPIA, TDx (meth)amphetamine II). After ingestion of 60 mg of clobenzorex, the parent compound and/or its metabolites could be detected by GC–MS for up to 84 h or by FPIA for up to 60 h. Since clobenzorex shows no cross-reactivity with the used immunoassay, the N-dealkylated metabolite amphetamine is responsible for the positive TDx results. The intake of clobenzorex instead of amphetamine can be differentiated by GC–MS detection of hydroxyclobenzorex which is detectable for at least as long as amphetamine. In addition, the described GC–MS procedure allows the simultaneous detection of most of the toxicologically relevant drugs. Furthermore, studies are described on the atmospheric pressure ionization electrospray LC–MS detection of α- and β-amanitin, toxic peptides of amanita mushrooms, in urine after solid-phase extraction on RP-18 columns. Using the single ion monitoring mode with the ions m/z 919 and 920 the amanitins could be detected down to 10 ng/ml of urine which allows us to diagnose intoxications with amanita mushrooms.

Keywords: Clobenzorex; Amanitin; Amphetamine; Toxicological analysis

1. Introduction

Clinical toxicological analysis is usually a single-case analysis which must be available on a 24-h

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basis. The method should be as rapid and precise as necessary for clinical diagnosis and therapy. Ideally, one single procedure should allow the detection of all relevant potential toxicants. Forensic toxicological analysis should also provide high reliability and accuracy in order to be defensible in court. Therefore, GC–MS is the method of choice for detection and quantification of toxicants volatile under GC conditions, whereas non-volatile toxicants require LC–MS.

The first step of the toxicological analysis is the identification of the usually unknown drugs and poisons in body fluids. A systematic toxicological analysis (STA) procedure is necessary that allows the simultaneous detection of as many toxicants as possible in biosamples. Unspecific chromatographic methods, even if several were combined [1], proved to be insufficient for these purposes, because merely the same chromatographic behavior of two compounds is not evident for their identity [2]. Besides, all chromatographic peaks or spots have to be identified because any of them may represent a potential poison. An STA procedure using GC–MS was developed that allows the simultaneous screening and identification of most of the toxicologically relevant drugs and their metabolites [3]. New drugs or poisons should be integrated in this STA procedure.

Clobenzorex [*S*-(+)-N-(2-chlorobenzyl)-amphetamine] is a widely used anorectic which is metabolized to the pharmacologically active *S*-(+)-amphetamine [4]. For two reasons, it is of clinical and forensic interest: abuse of clobenzorex as a stimulant is known and therapeutic use of clobenzorex may mislead to suspect amphetamine abuse when urine samples of the patients are tested for drug abuse [5]. Therefore, according to the standard guidelines in toxicological analysis a screening and a confirmation procedure are necessary for detection of an abuse or for differentiation of a therapeutic use of clobenzorex from an amphetamine abuse. A GC–MS procedure for the detection of sympathomimetic drugs [6] only allows the detection of clobenzorex itself, while detection of its metabolites is necessary for differentiation in the later phase of excretion. In the following, studies are presented on the toxicological detection of clobenzorex and its metabolites in urine by GC–MS and fluorescence polarization immunoassay (FPIA) and on the duration of detectability by these procedures.

As mentioned above, non-volatile toxicants cannot be detected by GC–MS. Therefore, we are developing LC–MS procedures using an atmospheric pressure ionization electrospray interface for those compounds. Among the non-volatiles, α- and β-amanitin, toxic peptides of amanita mushrooms, show the greatest relevance in clinical toxicology. Highly specific detection of amanitins in body fluids is necessary for an early diagnosis of an intoxication entailing a large scale of invasive and expansive therapy. In 1992 Dorizzi et al. [7] have critically reviewed methods published for the determination of amatoxins in biological matrices. Determination of amanitins in urine by a commercially available radioimmunoassay (RIA) is possible but the tracer is only stable for 1–2 months and the assay is not available throughout the year. Moreover, interferences with urine matrix are possible so that confirmation of the RIA results by alternative techniques is required [7]. Several of the reviewed HPLC methods are suitable for separation of the amanitins. However, electrochemical or UV detection requires a complex protocol of extraction and purification steps often followed by column switching techniques. Therefore, we have developed a LC–MS method for the detection of α- and β-amanitin in human urine after a simple solid-phase extraction.

2. Experimental

2.1. Detection of clobenzorex and its metabolites in urine by GC–MS and FPIA

2.1.1. Chemicals and reagents

All chemicals used were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade. Clobenzorex was obtained from Roussel Uclaf (Paris, France).

2.1.2. Urine samples for GC–MS

After informing them according to the declaration of Helsinki and obtaining written consent, three healthy volunteers received a single oral dose of 60 mg of clobenzorex. Urine samples were collected every 4 h for 8 days. All samples were stored at

−20°C before analysis. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

2.1.3. Sample preparation of urine for GC–MS

A 10-ml volume of urine was refluxed with 3 ml of 30% hydrochloric acid for 15 min. Following hydrolysis, the sample was basified with 4 ml of 10 mol/l aqueous sodium hydroxide and the resulting solution was mixed with 10 ml of 30% aqueous ammonium sulphate to obtain a pH between 8 and 9. This solution was extracted with 10 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v). After phase separation by centrifugation (5 min, 1500 *g*), the organic layer was evaporated to dryness. The residue was derivatized by acetylation with 100 μl of acetic anhydride–pyridine (3:2, v/v) for 30 min at 60°C. After evaporation of the derivatization mixture, the residue was dissolved in 100 μl of methanol and 0.5–1 μl were injected into the gas chromatograph.

2.1.4. Gas chromatography–mass spectrometry

Clobenzorex and its metabolites were separated and identified in the acetylated urine extracts using a Hewlett-Packard (HP, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP MS Engine 5989 Series A mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m× 0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280°C, carrier gas, helium; flow-rate, 1.9 ml/min; column temperature, programmed from 100–310°C at 30°C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode; ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C. For detection of clobenzorex and its metabolites, mass chromatography with the selected ions *m/z* 86, 118, 168 and 170 was used. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros (the macros can be obtained from the authors). The identity of the peaks in the mass chromatograms was confirmed by computer library search of the underlying mass spectra [8].

2.1.5. Fluorescence polarization immunoassays (FPIA)

The urine samples from the volunteers were used for immunological determination. The TDx system of Abbott (Irving, TX, USA) with the amphetamine–methamphetamine II assay (AM/MA II) was applied. The cut-off value and the detection limit in urine recommended by the manufacturer were 300 and 100 ng/ml, respectively. Blank urine samples were spiked with clobenzorex at a concentration of 8000 ng/ml for determination of its cross-reactivity with the assay.

2.2. Detection of α- and β-amanitin in urine by LC–MS

2.2.1. Chemicals and reagents

Erythromycin and α- and β-amanitin were obtained from Sigma-Aldrich (Deisenhofen, Germany). Methanol, water (both HPLC grade), ammonium acetate, acetic acid (both analytical grade) and the LiChrolut RP-18 (500 mg) cartridges for the solid-phase extraction (SPE) were obtained from E. Merck.

2.2.2. Urine samples for LC–MS

Blank urine samples were collected from healthy volunteers. Authentic urine samples were obtained from patients recently intoxicated by amanita mushrooms.

2.2.3. Sample preparation of urine for LC–MS

A 5-ml volume of urine was first diluted 1:1 with distilled water. The SPE columns were washed with ten reservoir volumes of aqueous acetic acid (1%), activated with 3 ml of methanol and rinsed with 3 ml of water. The sample was loaded onto the column, washed with 3 ml of aqueous acetic acid (1%) and then eluted with 6 ml of methanol. The eluate was evaporated to dryness at 65°C. The residue was redissolved in 100 μl of mobile phase and 20 μl were injected into the HPLC system with complete loop filling mode.

2.2.4. Liquid chromatography–mass spectrometry

The amanita toxins α- and β-amanitin were separated and identified in urine extracts using a Hewlett-Packard API-ES LC–MS including an electrospray interface (HP 59987A) and an HP-MS Engine 5989 Series B. The MS conditions were as follows: single ion monitoring (SIM) mode with the ions *m/z* 919 and 920 for α- and β-amanitin, respectively. Tuning of the MS was performed in three steps: first tuning with an erythromycin solution (100 μg/ml of methanol–water (1:1, v/v), tuning ions *m/z* 558, 716 and 734). Fine tuning with a β-amanitin solution (10 μg/ml of mobile phase, tuning ion *m/z* 920). These two tuning steps were done using a syringe pump (Harvard, South Natick, MA, USA) at a flow-rate of 75 μl/min. Last performance check by injection of a standard solution of α- and β-amanitin (5 μg/ml of mobile phase, respectively) into the LC–MS under normal LC–MS conditions. A Valco six-port injection valve was used to connect either the syringe pump or the HPLC system to the electrospray chamber. The HPLC consisted of an HP 1050 equipment with a manual injector, a 20-μl sample loop and a variable-wavelength detector and an electrospray interface. The isocratic separation was achieved on a Chromcart CC 125/2 Kromasil RP-18 narrowbore column (125×2 mm I.D.) with 5 μm particle size and a Kromasil guard column from Macherey-Nagel (Düren, Germany). The HPLC mobile phase was a 22:78 mixture (v/v) of methanol–ammonium acetate (0.02 mol/l, adjusted to pH 5). Before use, the mixture was degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed with helium. The flow-rate for the LC–MS was 75 μl/min. UV detection was performed at 302 nm before the efluent entered the electrospray interface.

3. Results and discussion

3.1. Detection of clobenzorex and its metabolites in urine by GC–MS within a STA procedure and by FPIA.

3.1.1. Sample preparation

For sample preparation, a procedure was used that has proved to be successful for systematic toxicological analysis [3]. Since the hydroxy metabolites of clobenzorex are excreted as conjugates and hydroxyclobenzorex is the main metabolite, rapid acid hydrolysis was performed before isolation and derivatization. Derivatization was essential for sensitive detection of the polar clobenzorex metabolites. Acetylation has been approved for the identification of numerous drugs and their metabolites as described in review articles [3,9]. It leads to stable derivatives with good gas chromatographic properties. The acetylation mixture can be evaporated before analysis so that the resolution power of capillary columns does not decrease in contrast to other derivatization reagents. The molecular mass does not increase very much, in contrast to silylation or perfluoroacylation, so that compounds with relatively high molecular mass and several derivatizable groups can be measured with low-priced mass selective detectors with a mass range only up to 650 u. The analytical recovery of clobenzorex and its metabolite amphetamine determined at urine concentrations of 1000 ng/ml was 85±10 and 57±6%, respectively.

3.1.2. Detection by GC–MS

The full mass spectra recorded during temperature-programmed GC were evaluated using mass chromatography. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu (Fig. 1) which executes the user-defined macros. The selected ions *m/z* 86, 118, 168, 170 were used for indicating the presence of acetylated clobenzorex and its metabolites in urine: amphetamine (86, 118; peaks 1 in Fig. 1), norephedrine and hydroxyamphetamine (86; peaks 2–3 in Fig. 1) or clobenzorex and its non-dealkylated metabolites (168, 170; peaks 4–7 in Fig. 1). Clobenzorex itself is not indicated in Fig. 1, because this urine sample was taken at a time when clobenzorex was no longer being excreted (20 h after ingestion). The identity of the peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectra with reference spectra recorded during this study [8]. The reference spectra, the gas chromatographic retention indices (RI) and the structures of acetylated clobenzorex and its main metabolites are shown in Fig. 2. The numbers of the spectra correspond to those of the peaks in Fig. 1. The retention indices were recorded during the GC–MS procedure (Section 2.1.4.) and calculated in

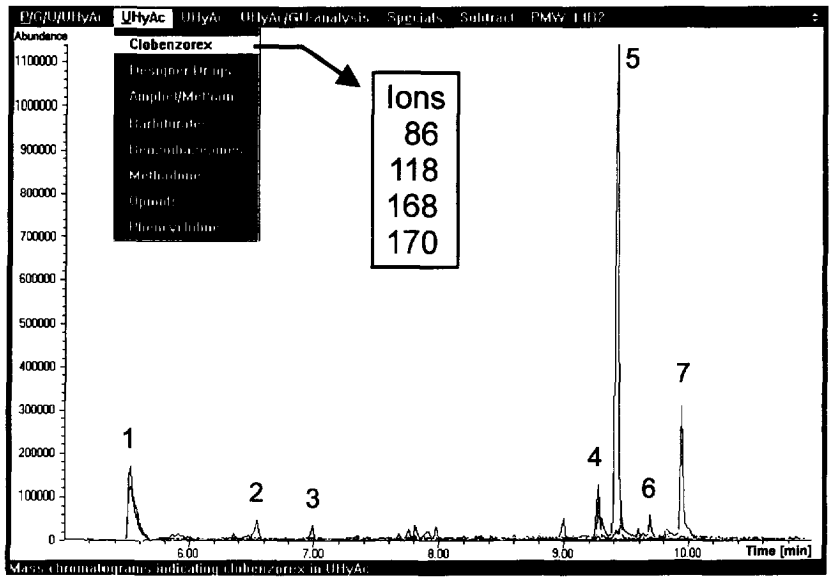

Fig. 1. Typical mass chromatograms with the ions *m/z* 86, 118, 168 and 170 indicating the presence of acetylated clobenzorex metabolites in urine 20 h after ingestion: amphetamine (1), norephedrine (2), hydroxyamphetamine (3), isomer 1 of hydroxyclobenzorex (4), isomer 2 of hydroxyclobenzorex (5), hydroxymethoxyclobenzorex (6) and dihydroxyclobenzorex (7). The merged mass chromatograms can be differentiated by their colours on a colour screen.

correlation with the Kovats' indices [10] of the components of a standard solution of typical drugs which was measured daily for testing the GC–MS performance [11,12]. The minor metabolites of clobenzorex are not essential for the screening procedure. As given in the legend to the spectra, the clobenzorex metabolites amphetamine, hydroxy amphetamine and norephedrine are either drugs or common metabolites of further stimulants or anorectics. However, the detection of the non-dealkylated metabolites, especially hydroxyclobenzorex (spectrum no. 5 in Fig. 2) allowed the differentiation of the drugs. If racemic amphetamine, commonly used as a street drug, was taken in addition, differentiation from the clobenzorex metabolite *S*-(+)-amphetamine could be performed by enantioselective detection [13]. Interferences by biomolecules or further drugs could be excluded because of mass spectral identification. The mass spectra of all the endogenous biomolecules detectable using the described procedure are included in our library and handbook [8,14]. The limit of detection in urine was 50 ng/ml of clobenzorex and 100 ng/ml of the metabolite amphetamine ($S/N=3$).

3.1.3. Detection by immunoassay

Clobenzorex shows no cross reactivity with the AM/MA II assay because of the large chlorobenzyl substituent in the side chain. Even at the high concentration level tested (8000 ng/ml) the measured values were not higher than those of the blank urine samples. For lack of reference substance of the main metabolite (conjugated) hydroxyclobenzorex, its cross reactivity could not be determined. Due to the intact side chain it can be concluded that it also shows no cross reactivity with the TDx AM/MA II. Since clobenzorex is metabolized to amphetamine the assay gives positive results in urine after ingestion of clobenzorex. The TDx values measured during our excretion study ranged between 100 and 10 000 ng/ml (cross reactivity value of amphetamine: 36% at a concentration level of 1000 ng/ml).

3.1.4. Duration of detectability of clobenzorex and its metabolites in urine by GC–MS and FPIA

The duration of detectability of clobenzorex and its metabolites in urine by GC–MS and FPIA is shown in Fig. 3. After a single oral dose of 60 mg of clobenzorex the AM/MA II assay gave positive

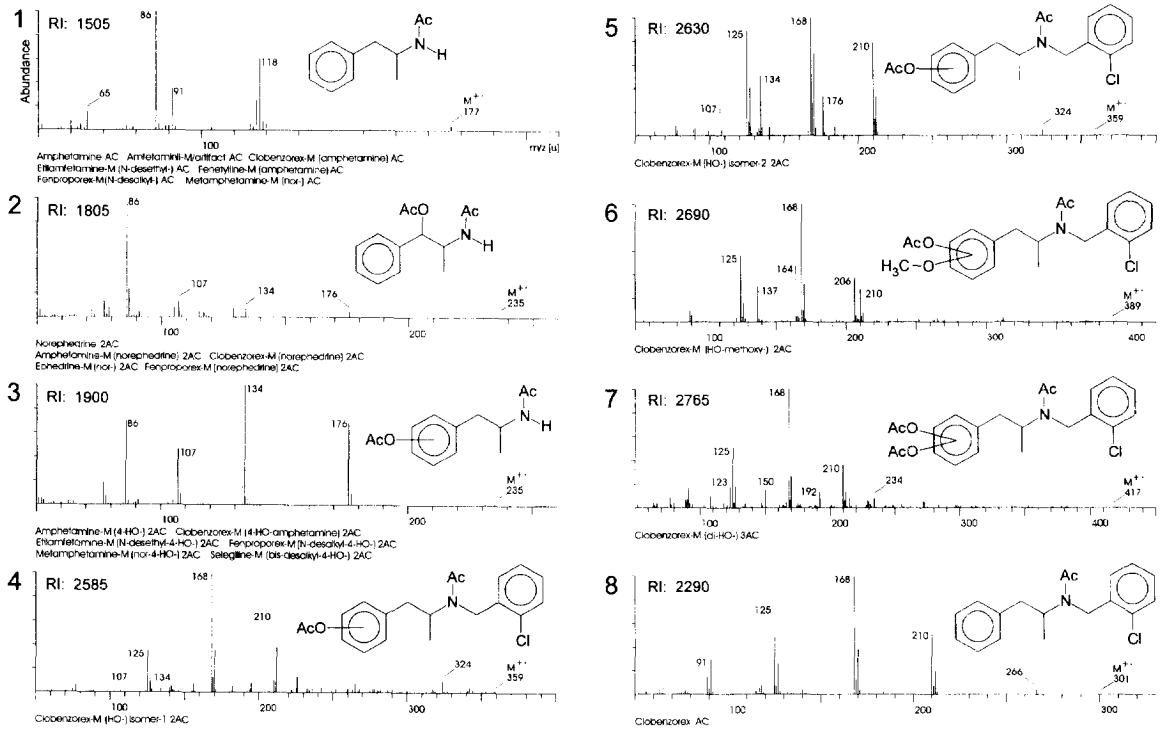

Fig. 2. Mass spectra, GC retention indices (RI) and structures of acetylated (Ac) clobenzorex and its main metabolites for precise identification. All the molecular ions (M^+) are present with low abundance. The numbers of the spectra correspond to those of the peaks in Fig. 1.

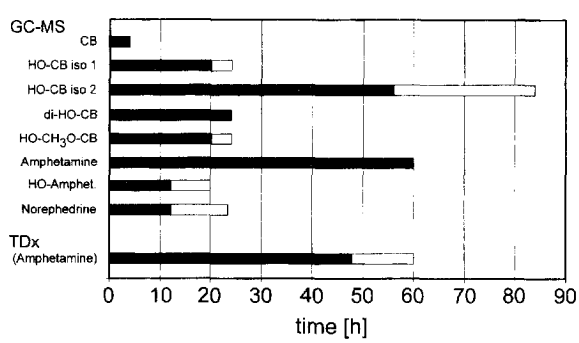

Fig. 3. Duration of detectability of clobenzorex and its main metabolites (hours after ingestion) by the TDx assay AM–MA II and GC–MS in urine samples after ingestion of 60 mg of clobenzorex ($n=3$).

results in urine for 40 to 60 h taking into consideration the cut-off value. The following metabolites could be detected in urine by the described GC–MS procedure during the given time: unchanged clobenzorex (CB) for only 4 h, hydroxy clobenzorex (HO–CB) for 56 to 84 h and amphetamine for about 60 h. The other metabolites described here were detectable for 12 to 24 h. All the immunological results could be confirmed by GC–MS detection of amphetamine (Fig. 3). The intake of clobenzorex instead of amphetamine can be differentiated by detection of hydroxyclobenzorex which is detectable at least as long as amphetamine. However, it should be stated clearly that cleavage of conjugates and derivatization are indispensable for the long term detection of hydroxyclobenzorex. Detection of unchanged clobenzorex recommended by Franceschini et al. [6] is not suitable because clobenzorex is only detectable in minor amounts within the first few hours after ingestion while amphetamine is detectable for up to

40 to 60 h. The variation in the time of excretion of clobenzorex may be caused by inter-individual differences e.g. relative body mass and/or renal function.

3.2. Detection of α- and β-amanitin in urine by LC–MS

3.2.1. Sample preparation for LC–MS

SPE has proved to be suitable for the extraction of α- and β-amanitin from biological matrices [7]. The Merck LiChrolut RP-18 columns used gave better results than several other tested columns. The ten times washing of the cartridges with 1% aqueous acetic acid led to more reproducible results. Addition of acetic acid to the washing solution was necessary to prevent the carboxy group of β-amanitin from deprotonation and elution from the column during the washing step. Concentration by a factor of 50 was necessary to improve the detection at low concentrations. The analytical recovery of α- and β-amanitin determined at urine concentrations of 100 ng/ml was 65±7 and 63±5%, respectively.

3.2.2. Detection by LC–MS

The coupling of HPLC with MS is a powerful tool in biomedical analysis, since this detector provides high specifity and sensitivity. Today, the electrospray interface seems to be the most universal interface for LC–MS, providing the widest range of applications. Unfortunately, it is not possible to adopt every HPLC method for the use in LC–MS. There are several restrictions: only volatile buffers or volatile ion pairing reagents can be used, the flow-rate is limited. The more water or the less organic solvent is in the mobile phase the less flow is allowed. In our system, the optimal flow-rate was 75 μl/min. Using the described method we were able to separate α- and β-amanitin in urine extracts. The applied electrospray LC–MS technique allowed the ionization and detection of both amatoxins in the positive mode. In Fig. 4 the electrospray mass spectra, structures, empirical formulas and molecular masses of α- and β-amanitin are shown. Since both spectra contain the ions m/z 919 and 920 in different abundances, both ions were selected as diagnostic ions for detection of the amatoxins in the SIM mode (Fig. 5, bottom). Blank urine samples were analysed to show that there were no interferences (Fig. 5, top). The limit of detection for both toxins was 10 ng/ml of urine ($S/N=3$).

Symptoms of an intoxication with amanita mushrooms do not appear before a lag time of about 8 to 24 h. In our experience, urine concentrations of α- and β-amanitin at this time range between 50 to 500 ng/ml. Therefore, the detection of the described

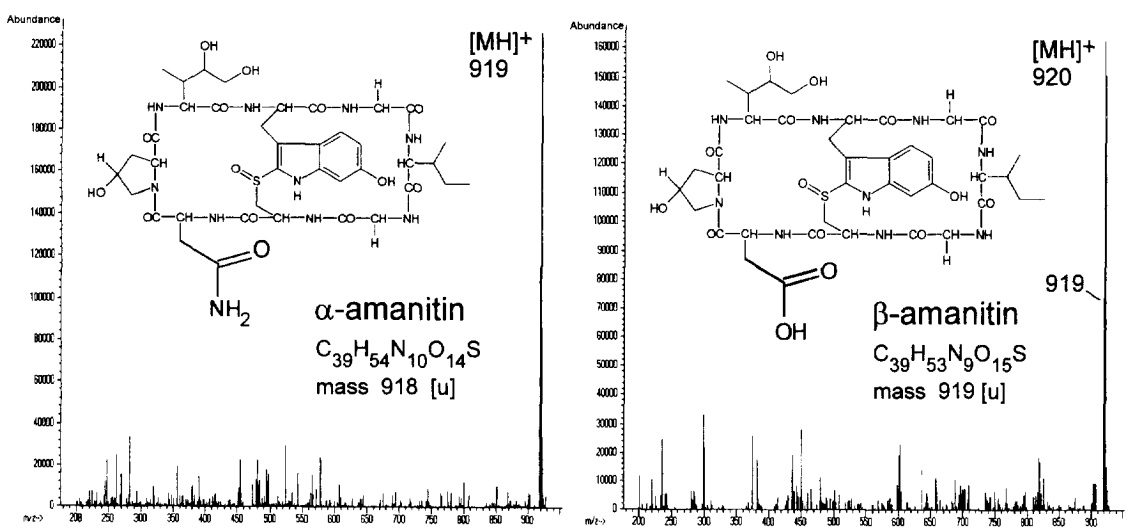

Fig. 4. Electrospray mass spectra, structures, empirical formulas and molecular masses of α- and β-amanitin.

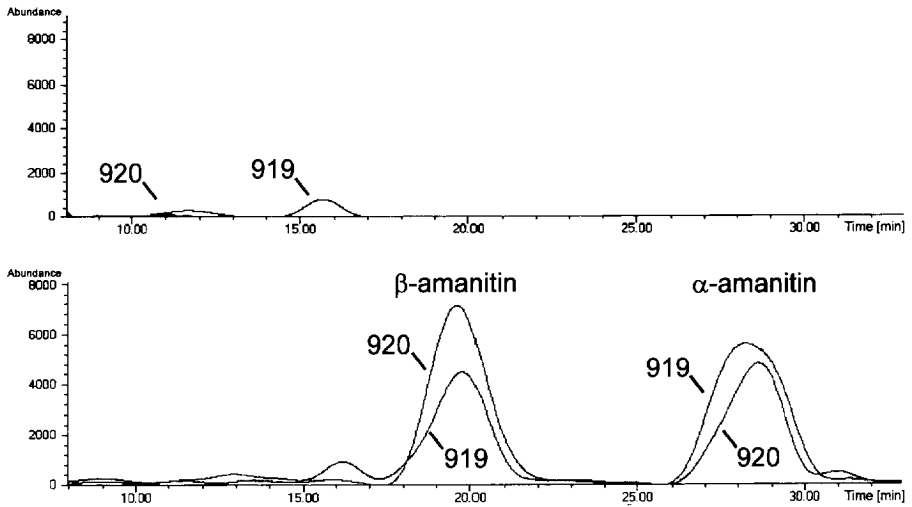

Fig. 5. Smoothed mass fragmentograms with the ions m/z 919 and 920 of extracts of a blank urine sample (top) and of a blank urine sample spiked with 50 ng/ml of α- and β-amanitin (bottom).

LC–MS procedure is suitable to diagnose an intoxication. In the meantime, we successfully applied our procedure in authentic clinical cases in which the RIA results could be confirmed by LC–MS.

4. Conclusions

The GC–MS procedure described here allows the precise and sensitive detection of clobenzorex and its metabolites in urine after therapeutic doses of clobenzorex within a systematic toxicological analysis. Other amphetamine derivatives as well as most of the toxicologically relevant drugs like barbiturates and other sedative–hypnotics, anticonvulsants, benzodiazepines, antidepressants, phenothiazine and butyrophenone neuroleptics, central stimulants, designer drugs, phencyclidine, opioids, non-opioid analgesics, antihistamines, antiparkinsonians, beta-blockers, antiarrhythmics, laxatives and their metabolites [3] could also be detected and differentiated within the same procedure by clicking the corresponding pull down menu (e.g. "amphetamine class") followed by library search of the spectra underlying the peaks.

The LC–MS procedure presented here allows the precise and sensitive detection of α- and β-amanitin in urine, thus allowing the specific diagnosis of an intoxication with amanita mushrooms.

These examples show that GC–MS is the method of choice for systematic toxicological analysis of GC volatile toxicants and that LC–MS is the method of choice for the detection of non-volatile toxicants.

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