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ORIGINAL ARTICLE

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LC-ESI-MS/MS on an ion trap for the determination of LSD, *iso*-LSD, *nor*-LSD and 2-oxo-3-hydroxy-LSD in blood, urine and vitreous humor

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Abstract A method has been developed for the simultaneous determination of lysergic acid diethylamide (LSD), its epimer *iso*-LSD, and its main metabolites *nor*-LSD and 2-oxo-3-hydroxy LSD in blood, urine, and, for the first time, vitreous humor samples. The method is based on liquid/liquid extraction and liquid chromatography-multiple mass spectrometry detection in an ion trap mass spectrometer, in positive ion electrospray ionization conditions. Five microliter of sample are injected and analysis time is 12 min. The method is specific, selective and sensitive, and achieves limits of quantification of 20 pg/ml for both LSD and *nor*-LSD in blood, urine, and vitreous humor. No significant interfering substance or ion suppression was identified for LSD, *iso*-LSD, and *nor*-LSD. The interassay reproducibilities for LSD at 20 pg/ml and 2 ng/ml in urine were 8.3 and 5.6%, respectively. Within-run precision using control samples at 20 pg/ml and 2 ng/ml was 6.9 and 3.9%. Mean recoveries of two concentrations spiked into drug free samples were in the range 60–107% in blood, 50–105% in urine, and 65–105% in vitreous humor. The method was successfully applied to the forensic determination of postmortem LSD levels in the biological fluids of a multi drug abuser; for the first time, LSD could be detected in vitreous humor.

Keywords LSD · Lysergic acid diethylamide · Electrospray · LC-MS · Ion trap

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Introduction

The detection and quantitative determination of the potent hallucinogen L-lysergic acid diethylamide (LSD) in body fluids is still an analytical challenge, due to its physical–chemical properties such as photosensitivity, thermolability, sensitivity to acidic media, ease of isomerization, possibility of adsorptive losses [1–4], and low concentrations (pg–ng/ml) in biological fluids caused by pharmacological and kinetic features.

Reuschel et al. [5] reviewed the most significant progress in the field of LSD analysis covering the period 1992–1999, pointing out that low limits of quantification (LOQ) can be achieved in LSD analysis either by use of a highly selective extraction procedure, or a very sensitive and selective detection method, or a combination of both. Of the various analytical approaches which have been proposed in the last decade, a leading role has been played by those employing multiple mass spectrometry (MS/MS) features which allow the *mise au point* of specific and sensitive methods based either on gas chromatography-multiple mass spectrometry (GC-MS/MS) or liquid chromatography-multiple mass spectrometry (LC-MS/MS) either on triple quadrupole mass spectrometers or on single quadrupoles (by in-source collisional experiments) [6–12]. Poch et al. were the first to propose an LC-MS/MS method in an ion trap (IT) equipped with an atmospheric-pressure chemical ionization (APCI) interface [13] for the determination of O-H-LSD; that method proved suitable for the analysis of the polar metabolite but lacked in specificity for LSD. Because no further investigation was performed to explore the potentials of ion traps in LSD analysis, either in APCI or electrospray ionization (ESI), we thought it of interest to develop an LC-MS/MS method in positive ion electrospray ionization (ESI) conditions to detect LSD, its demethyl metabolite *nor*-LSD, and its nonactive epimer (*iso*-LSD, originating from LSD contamination during synthesis or base-catalyzed epimerization [3]) in biological fluids (see Fig. 1). Because other metabolites which have recently been found in urine at concentrations higher than that of *nor*-LSD, such as 2-oxo-3-hydroxy-LSD (O-H-

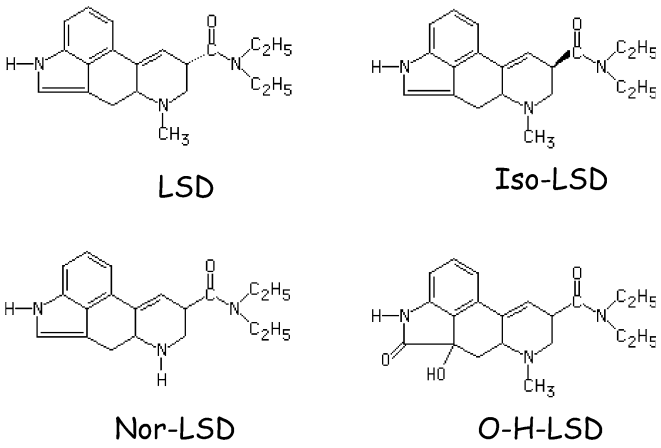

Fig. 1 Molecular structures of LSD, *iso*-LSD, *nor*-LSD, and O-H-LSD

LSD), allow detection of LSD consumption in a larger time-window [9, 10, 13], the method was also able to identify and measure such compounds.

Materials and methods

Reagents and chemicals

LSD, D3-LSD (internal standard, IS), *iso*-LSD, *nor*-LSD, and 2-oxo-3-hydroxy LSD were obtained from Radian International (Austin, TX).

All chemicals were analytical reagent grade and all solvents were high performance liquid chromatography (HPLC) grade.

Stock solutions and calibration standards

Stock solutions were prepared in acetonitrile at concentrations of 1 μg/ml (LSD) and 0.1 μg/ml (*iso*-LSD, *nor*-LSD, 2-oxo-3-hydroxy LSD), and kept for 1 month at −20°C. Working solutions were prepared daily at concentrations of 2 and 100 ng/ml in HPLC grade water. The IS working solution was prepared at 0.1 μg/ml in acetonitrile.

Extraction procedure for urine, blood, and vitreous humor

To 2 ml of urine or whole blood or vitreous humor in a PET tube, 20 μl of IS solution, 100 mg of ammonium carbonate, 10 ml of chloroform (blood, vitreous humor), or 8 ml of chloroform and 2 ml of *i*-propanol (urine) were added. The tubes were vortex-mixed and liquid/liquid extraction was performed for 15 min in the dark. After centrifugation, the organic layer was removed, transferred to a clean PET tube, and dried under nitrogen at room temperature in the dark. The dried residue was dissolved in 40 μl of mobile phase A (see LC experimental conditions). After centrifugation 5 μl of clear supernatant were injected.

HPLC conditions

Chromatographic separation was achieved with a Luna CN (150×2 mm, 5 μm) analytical column (Phenomenex, Torrance, CA). A Spectra System P4000 pump (Thermo-Finnigan, San Jose, CA) was used to perform gradient elution at a constant flow rate of 0.3 ml/min. HPLC solvents were solvent A (water, 0.1% formic acid, ammonium formate 2 mM, pH 3) and solvent B (acetonitrile, 0.1% formic acid, ammonium formate 2 mM). The mobile phase was programmed as follows: original conditions 20% B, linear gradient to 80% B in 8 min, and 80% B hold from 8 to 12 min.

Mass spectrometry conditions

All mass spectrometric measurements were performed on an LCQ Duo (Finnigan, San Jose, CA) ion trap mass spectrometer, equipped with an ESI source working in positive ion conditions. The eluate of chromatographic separation was split to a 1:1 ratio, so as to supply 0.15 ml/min to the ESI interface. ESI parameters were: spray voltage 4.2 kV, capillary temperature 220°C, capillary voltage 20 V, sheath gas (nitrogen) 40 arbitrary units (a.u.), and auxiliary gas (nitrogen) 20 a.u. Collisional induced dissociation (CID) experiments employed a supplementary radio frequency voltage ("tickle voltage") in the range 0–5 V applied to the end caps of the ion trap. Helium was used as target gas, at a pressure of 1.1×10^{-5} torr.

Validation protocol

For method validation, samples were prepared by adding the working standards to drug-free urine, blood (collected from laboratory personnel), and vitreous humor (collected from deceased not suspected of being intoxicated or poisoned with psychoactive agents) at the following concentrations: 10, 20, 50, 100, and 200 pg/ml, 2, 5, and 10 ng/ml (LSD, *iso*-LSD and *nor*-LSD); 200 and 400 pg/ml, and 1, 2, and 10 ng/ml (O-H-LSD). To determine matrix effects and ion suppression effects, spike-after-extraction samples were prepared by extracting LSD-free blood, urine, and vitreous humor (obtained as detailed above) and adding the analytes to the sample extracts at concentrations equivalent to those of standard solutions. Recoveries were determined at 20 and 200 pg/ml for LSD, *iso*-LSD and *nor*-LSD, and at 400 pg/ml and 2 ng/ml for O-H-LSD, by comparison of the chomatographic peak areas obtained for spiked samples to those obtained for spike-after-extraction samples at the same concentration. Intraassay and interassay precision [as relative standard deviation (R.S.D.)] were determined by assay of four replicates of spiked samples at 20 and 200 pg/ml for LSD, *iso*-LSD, and *nor*-LSD on three different days. Accuracy [as relative error (R.E.), i.e., percentage deviation of the mean from the true value] was determined on the basis of the total data set (n=8). The lowest limit of quantitation

(LLOQ) was the concentration below which R.E. was >20% and CV was >20%. The limit of detection (LOD) was the concentration with signal-to-noise ratio of 3.

Results

MS/MS

It must be noticed that all the CID spectra available in the literature for LSD and related substances, either in APCI or ESI conditions, have been obtained by triple quadrupoles or by single quadrupoles by in-source CID, while no ion trap collisional experiments have been previously discussed for these molecules with the exception of O-H-LSD [13]. To optimize the experimental conditions of the CID experiments, aqueous solutions of the analytes at a concentration of 100 ng/ml were directly infused into the ESI interface of LCQ, while multiple mass spectrometry experiments were conducted by varying the amplitude and time of the tickle voltage. Figure 2 (top) shows the CID spectrum of MH^+ of LSD obtained at 28% of maximum tickle voltage (5 V) at a collision time of 30 ms. The observed fragment ions are listed in Table 1 (MS^2 spectrum), together with their relative abundances. As previously reported in triple quadrupole CID experiments [6] and according to the even-electron rule, MH^+ mainly fragments through neutral losses of diethylamine ($[MH\text{-}C_4H_{11}N]^+$) and diethylformamide ($[MH\text{-}C_4H_{11}NCO]^+$) to yield ion species at m/z 251 and 223, respectively. Abundant ions at m/z 281 are produced through cleavage of two bonds in the pyrimidine ring, producing a radical loss of C_2H_5. Also, less abundant ions are due to radical losses: $[MH\text{-}CH_3]^{+\cdot}$ at m/z 309 and $[MH\text{-}C_2H_5]^{+\cdot}$ at m/z 293 but neither were observed in ESI and CID conditions in a triple quadrupole [6, 10]. To detect further fragmentation pathways, MS^3 experiments were also performed on CID-generated ion species at m/z 281 and 223 (see Table 1) at tickle voltage of 28 and 35%, respectively.

When MH^+ ions of the epimer *iso*-LSD are collided under the same conditions, the same product ions are produced, but their relative abundances are different, as may be seen in Table 1; by varying the tickle voltage at a fixed tickle time and monitoring the absolute abundance of MH^+ and product ions at m/z 281 and 223, the two stereoisomers are clearly distinguished over a wide range of collisional conditions (data not shown) and ion trap multiple mass spectrometry, due to its step-by-step energy deposition on preselected ions [14], once again proves to be a powerful diagnostic tool in stereoisomer characterization. Canezin et al. also found analogous results on a triple quadrupole [10], under collisional conditions which are, however, different from those of an ion trap.

CID experiments were then performed on MH^+ of the two metabolites *nor*-LSD and O-H-LSD, whose product ion spectra are also listed in Table 1. The former exhibits fragmentation pathways similar to those already described for the parent compound [6]; in the latter, the most prominent product species is that of water loss at m/z 338, whereas all the other fragment ions probably originate from the base peak through secondary processes. These secondary processes are informative of the LSD-like structure of the metabolite because they produce loss of diethylamine and diethylformamide, and cleavage of the pyrimidine ring. Consequently, in the LC-MS/MS run described below, potential interference in the MS confirmation of O-H-LSD would be expected when monitoring $[MH\text{-}H_2O]^+$ ions, due to the poor specificity of this fragmentation pathway. Vice versa, the use of a more specific fragment, such as $[MH\text{-}H_2O\text{-}C_4H_{11}N]^+$ at m/z 265, yielded cleaner chromatograms free of any interference and with high s/n, as shown.

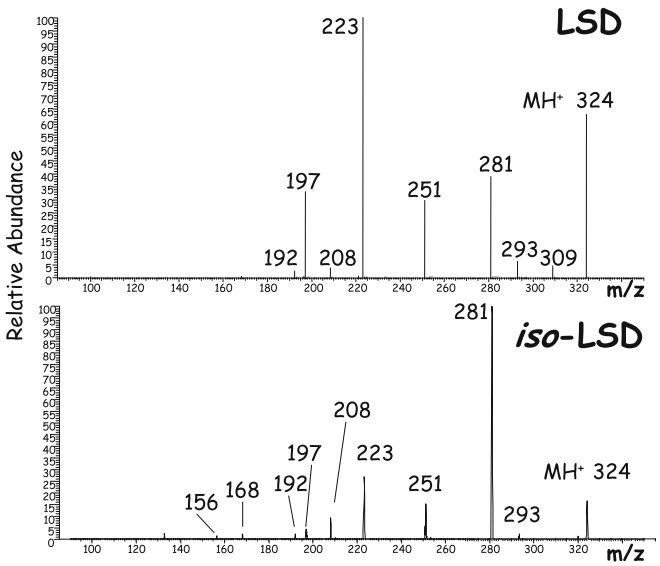

Fig. 2 Product ion spectrum obtained by ion trap CID on ESI-generated MH^+ of LSD and *iso*-LSD

LC-MS/MS

After optimum MS/MS conditions had been found for each analyte, chromatographic separation was optimized by injection of pure standard solutions. The choice of a slightly polar phase (cyanopropyl) allowed the use of a mixed reversed-phase and normal-phase separation mechanism, so that *nor*-LSD, LSD, and *iso*-LSD could be efficiently separated by gradient elution in a relatively short time (less than 12 min). In addition, the polar metabolite O-H-LSD, which tends to elute early, close to the front of the solvent in pure reversed-phase conditions, could be delayed to a retention time (RT) of 3.6 min.

Transitions monitored for the LC-MS/MS runs are listed in Table 2, together with RTs and ions chosen for quantification. As may be seen, three ions (one quantifier and two qualifiers) were monitored for each analyte with the exception of O-H-LSD, to provide sufficient identification of drugs. The percent deviation of relative intensities of ions were determined by duplicate analysis of six different urine samples spiked with 20 pg/ml of LSD, *iso*-LSD and *nor*-LSD, and 400 pg/ml of O-H-LSD performed on two separate days (n=12).

Table 1 MS^2 product ion spectra obtained by CID on ESI-generated MH^+ ions, and MS^3 of CID-generated ion species at m/z 281(**a**) and 223 (**b**) for LSD

	LSD	*iso*-**LSD**	*nor*-**LSD**	**O-H-LSD**
MH+ precursor ion	m/z 324	m/z 324	m/z 310	m/z 356
Percent tickle voltage	28%	28%	28%	35%
Product ions	m/z (relative abundance)			
Surviving MH^+ precursor ion	324 (64)	324 (17)	310 (5)	
$[MH\text{-}CH_3]^+$	309 (6)		293 (3)	
$[MH\text{-}C_2H_5]^+$	293 (7)	293 (1)		
$[MH\text{-}RNCH_2]^+$ **a**	281 (40) R=CH_3	281 (100) R=CH_3	281 (7) R=H	313 (45) R=CH_3
$[MH\text{-}C_4H_{11}N]^+$	251 (30)	251 (16)	237 (100)	
$[MH\text{-}C_4H_{11}NCO]^+$ **b**	223 (100)	223 (27)	209 (77)	
$[MH\text{-}RNCH_2\text{-}C_4H_{11}N]^+$	208 (5)	208 (9)		
$[MH\text{-}C_4H_{11}NCOCH_2CH]^+$	197 (33)	197 (5)	183 (22)	
$[MH\text{-}C_4H_{11}NCO\text{-}NH_2R\ ]^+$	192 (3)	192 (2)		
$[MH\text{-}H_2O]^+$				338 (100)
$[MH\text{-}H_2O\text{-}RNCH_2]^+$				295 (11)
$[MH\text{-}RNCH_2\ \text{-}HCN]^+$				286 (35)
$[MH\text{-}H_2O\text{-}C_4H_{11}N]^+$				265 (79)
$[MH\text{-}H_2O\text{-}C_4H_{11}NCO]^+$				237 (17)
$[\mathbf{a}\text{-}C_4H_{11}N]^+$	208 (39)			
$[\mathbf{a}\text{-}C_4H_{11}NCO]^+$	180 (10)			
$[\mathbf{b}\text{-}CH_3]^+$	208 (100)			
$[\mathbf{b}\text{-}HCN]^+$	196 (7)			
$[\mathbf{b}\text{-}NCH_2]^+$	194 (10)			
$[\mathbf{b}\text{-}NHCH_3]^+$	192 (15)			
$[\mathbf{b}\text{-}CH_3CN]^+$	182 (20)			

Extraction of urine, blood, and vitreous humor

Several extractive procedures have been proposed to determine LSD and its metabolites in body fluids: liquid/liquid extraction at basic pH with varying solvents [3, 4, 7, 10], immunoaffinity purification procedures [8, 15, 16], solid-phase extraction on different sorbents [17–19], or a combination of these methods [1, 6, 9, 13]. In our experiments, liquid/liquid extraction seemed to be the simplest and least expensive procedure, considering that a single extraction step is sufficient for a LC-MS/MS procedure in which the high specificity of the detection balances possible partial purification of the analytes from the matrix.

Among the various solvents which were tested to extract spiked urine, blood, and vitreous humor samples, chloroform turned out to give the best recoveries and was finally chosen for blood and vitreous humor while chloroform/*i*-propanol 85/15 was used for urine to improve recovery of the polar metabolite O-H-LSD. Chloroform was chosen in spite of its toxicity because the volume required for extraction is small and the frequency of suspected LSD intoxication cases, requiring confirmation, is low. The mean (n=3) recoveries obtained for the analytes in different biological fliuds are reported in Table 3.

Table 2 Analytes, retention times (RT), MH^+ precursor ions, and product ions monitored in LC-MS/MS method

Analyte	RT min	Surviving MH^+ m/z (%)	Product ion 1 m/z (%)	Product ion 2 m/z (%)
LSD	7.10	324 (64±11)	281 (40±14.8)	**223** (100±7.4)
iso-LSD	7.91	324 (17±18)	**281** (100±8.5)	223 (27±22)
nor-LSD	6.72	310 (5±32)	**237** (100±6.2)	209 (77±9.6)
O-H-LSD	3.80	–	338 (100±22)	**265** (79±9.8)
IS (D_3-LSD)	7.11	327 (65±10)	**226** (100±8)	–

Percent relative abundance in the MS/MS spectrum and standard deviation as determined with n=12 at one concentration level (in bracket)
Quantifier (bold type)

Validation

Because the matrix effect is a noticeable problem while determining compounds in biological fluids, the specificity of the method was determined by running six different blank samples for each specimen (blood, urine, vitreous humor). In every case, s/n values were well below three for selected ion traces. To evaluate the possibility of ion suppression in the present experiments, chromatographic peak areas of the analytes from the spike-after-extraction samples (see experimental) at low (20 pg/ml) and high (2 ng/ml) concentration levels were compared to those obtained for standard solutions at the same concentrations

Table 3 Mean (n=3) recoveries obtained for the analytes at two concentration in different biological fluids

Analyte	Blood[a] 20 pg/ml (%)	Blood[a] 200 pg/ml (%)	Urine[b] 20 pg/ml (%)	Urine[b] 200 pg/ml (%)	Vitreous humor[a] 20 pg/ml (%)	Vitreous humor[a] 200 pg/ml (%)
LSD	60	85	50	80	65	95
iso-LSD	80	90	65	87	88	98
nor-LSD	100	107	99	105	98	105
			Urine[b] 400 pg/ml (%)	Urine[b] 2 ng/ml (%)		
O-H-LSD	–	–	65	80		

[a]Extracted by chloroform
[b]Extracted by chloroform/*i*-propanol 85/15

in clean solvent. After evaluating six different lots of blood and urine and two lots of vitreous humor at each concentration level, percent nominal concentrations determined were within acceptable limits (90–105%) for LSD, *iso*-LSD, and *nor*-LSD. The same evaluation was performed for the deuterated IS and no significant peak area differences were observed. For the polar metabolite O-H-LSD, percent nominal concentrations were determined in six lots of urine at two levels (400 pg/ml and 2 ng/ml) and were in the range 65–80%. As a result, ion suppression or enhancement from matrix was negligible for LSD, *iso*-LSD, and *nor*-LSD in the present experimental conditions, and these findings are consistent with the fact that they elute far from the polar LC front [20]. For O-H-LSD, which elute early, a certain degree of ion suppression has been noticed, and can be responsible for the higher LLOQ and LOD determined for this analyte.

In LC-MS/MS of urine samples spiked with LSD, *iso*-LSD, *nor*-LSD, and O-H-LSD, each at a concentration of 20 pg/ml, O-H-LSD could not be detected; for the other analytes, good separation was achieved (more than 1 min between the RTs of LSD and *iso*-LSD), with signal-to-noise ratios higher than 25 (ratio of peak height between the RT of the analyte, and peak height at the chromatographic baseline in the corresponding ion current profile).

Figure 3a shows chromatograms obtained by spiking drug-free urine with LSD, *iso*-LSD, *nor*-LSD, and O-H-LSD, each at a concentration of 100 pg/ml. Although in this case O-H-LSD could be detected, as anticipated, the trace for $[MH\text{-}H_2O]^+$ species at m/z 338, due to its poor specificity, had a very low signal-to-noise ratio, whereas the more specific fragment at m/z 265 gave rise to a cleaner trace which allowed detection. The areas of this second ion species were thus chosen for quantification purposes. At higher concentration levels (≥400 pg/ml), the trace for m/z 338 definitely improved, in terms of both peak shape and signal-to-noise ratio.

Calibration curves were then constructed with five concentrations over the range 20 pg/ml−10 ng/ml in urine, blood and vitreous humor for LSD, *iso*-LSD, *nor*-LSD, and over the range 400 pg/ml−10 ng/ml for O-H-LSD in urine. The correlation coefficients of all individual curves exceeded 0.995. The LODs were 200 pg/ml for O-H-LSD in urine, 10 pg/ml for LSD and *nor*-LSD in urine, blood, and vitreous humor, and 5 pg/ml for *iso*-LSD in in urine, blood, and vitreous humor. The LLOQ, considered as the

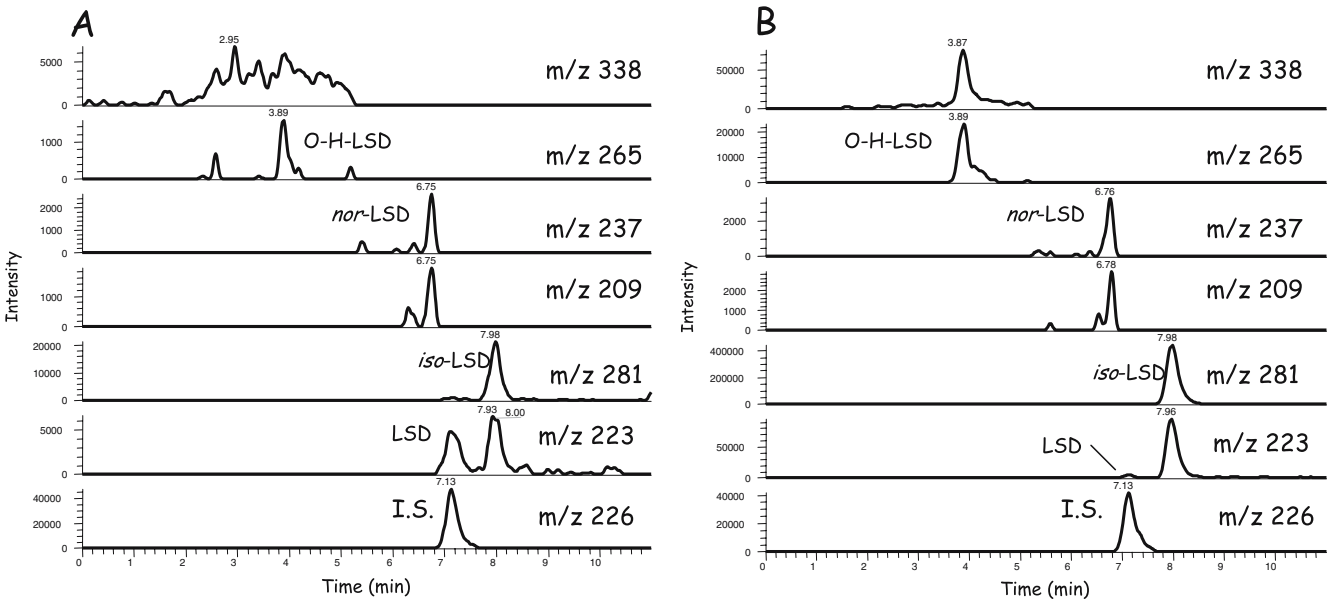

Fig. 3 Chromatograms obtained by LC-MS/MS of: **a** urine sample spiked with all analytes at 100 pg/ml. **b** urine sample positive for LSD and metabolites

Table 4 Postmortem blood, urine, and vitreous humor levels of LSD, *iso*-LSD, *nor*-LSD, and O-H-LSD (ND = not detected)

Biological fluid	Blood ng/ml	Urine ng/ml	Vitreous humor ng/ml
Analyte			
LSD	3.2	91.0	2.9
iso-LSD	ND	2.9	ND
nor-LSD	4.2	108.1	2.2
O-H-LSD	ND	430.2	ND

smallest concentrations which could be measured on the standard curves with acceptable reproducibility, were 20 pg/ml for LSD and *nor*-LSD and 10 pg/ml for *iso*-LSD in urine, blood, and vitreous humor, and 400 pg/ml for O-H-LSD in urine.

Precision and accuracy were satisfactory at the two concentrations studied as detailed above. The intraassay standard deviations using control samples at 20 pg/ml and 2 ng/ml in urine were, respectively, 8.3 and 4.2% for LSD, 7.9 and 6.4% for *nor*-LSD, while the interassay reproducibilities were 10.3 and 5.6% for LSD, 9.9 and 5.6% for *nor*-LSD, with an accuracy (R.E percentage) below 12.

The method can easily be applied on a routine basis as a confirmation procedure for the detection of LSD in urine. Figure 3b shows, as an example, chromatograms obtained by extracting a positive urine sample. The described LC-MS/MS method is currently employed in clinical and forensic cases when there is suspicion of LSD consumption. For example, it was successfully applied to confirm the postmortem presence of LSD and its metabolites in biological fluids of a multidrug abuser (Table 4).

Conclusions

Electrospray ionization conditions and multiple mass spectrometry on an ion trap allowed to develop a LC-MS/MS method which is suitable for simultaneous qualitative and quantitative determination of LSD, its epimer *iso*-LSD, and its main metabolites *nor*-LSD and O-H-LSD in blood, urine, and vitreous humor samples. Its main features are summarized below:

- Rapid, low-cost sample preparation (liquid/liquid extraction of analytes from biological fluids with chloroform or chloroform/*i*-propanol);
- Use of a deuterated internal standard (D_3-LSD);
- efficient chromatographic separation (including adequate retention of the O-H-metabolite) in a relatively short time;
- High specificity and sensitivity obtained by optimized collisional experiments on electrospray-produced MH^+ precursors and by monitoring of MH^+ plus two product ions for each analyte in the analytical scan function;
- Lack of significant interference by biological fluid extracts;
- Linearity over a range of 20 pg/ml−10 ng/ml for LSD in blood, urine, and vitreous humor.

When compared with existing methods for LSD analysis, the simpler, faster, highly sensitive, and selective multianalyte method presented in this study may be conveniently applied for forensic toxicology purposes.

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