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# Development and validation of an LC-MS/MS method to quantify LSD, iso-LSD, 2-oxo-3-hydroxy LSD, and nor-LSD and identify novel metabolites in plasma samples in a controlled clinical trial

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**Running title:** LSD and metabolites by LC-MS/MS

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**Abstract**

**Background:** Lysergic acid diethylamide (LSD) is a widely used recreational drug. The aim of the present study was to develop and validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantification of LSD, iso-LSD, 2-oxo-3-hydroxy LSD (O-H-LSD), and nor-LSD in plasma samples from 24 healthy subjects after controlled administration of 100 µg LSD in a clinical trial. In addition metabolites that have been recently described in *in vitro* studies, including lysergic acid monoethylamide (LAE), lysergic acid ethyl-2-hydroxyethylamide (LEO), 2-oxo-LSD, trioxylated LSD, and 13,14-hydroxy-LSD should be identified. **Methods:** Separation of LSD and its metabolites was achieved on a reversed phase chromatography column after turbulent flow online extraction. For the identification and quantification a triple-stage quadrupole LC-MS/MS instrument was used. **Results:** The validation data showed slight matrix effects for LSD, iso-LSD, O-H-LSD, or nor-LSD. Mean intraday and interday accuracy and precision were 105%/4.81% and 105%/4.35% for LSD, 98.7%/5.75% and 99.4%/7.21% for iso-LSD, 106%/4.54% and 99.4%/7.21% for O-H-LSD, and 107%/5.82% and 102%/5.88% for nor-LSD, respectively. The limit of quantification was 0.05 ng/ml for LSD, iso-LSD, and nor-LSD and 0.1 ng/ml for O-H-LSD. The limit of detection was 0.01 ng/ml for all compounds.

**Conclusion:** The method described herein was accurate, precise, and the calibration range within the range of expected plasma concentrations. LSD was quantified in the plasma samples of the 24 subjects of the clinical trial, whereas iso-LSD, O-H-LSD, nor-LSD, LAE, LEO, 13,14-hydroxy-LSD, and 2-oxo LSD could only sporadically be detected but were too low for quantification.

**Keywords**

LSD, LC-MS, lysergic acid diethylamide, metabolism, controlled study, plasma

## 1. Introduction

Lysergic acid diethylamide (LSD) is a psychoactive substance that alters states of consciousness and perception. Its psychedelic effects made it popular as a recreational drug, especially in the 1960s and 1970s, but LSD is still widely used today (1). Additionally, LSD has been reintroduced in psychiatric research (2-16) and investigated as an adjunct to psychotherapy (17; 18). Therefore, information about its metabolism and pharmacokinetics after controlled intake has received increasing interest. Doses that were used in recent clinical studies ranged from 75 µg, i.v. (2-11), to 200 µg, p.o. (12-15; 19), resulting in low blood and urine concentrations (12; 20). Dolder et al. and Steuer et al. recently showed that LSD and its main urinary metabolite 2-oxo-3-hydroxy-LSD (O-H-LSD) were detectable in plasma after controlled intake of 200 µg LSD in 16 healthy subjects (12; 20) and clinical toxicological cases of acute LSD overdose (21). Studies of *in vitro* metabolism have further identified lysergic acid mono-ethyl amide (LAE), lysergic-acid-ethyl-2-hydroxyethylamide (LEO), 2-oxo-LSD, nor-LSD, trioxylated-LSD, and 13,14-hydroxy-LSD as glucuronides (22; 23), but no systematic information is available regarding their presence in human plasma after controlled intake of LSD. However, recent investigations confirmed the presence of 2-oxo-LSD and 13,14-hydroxy-LSD (glucuronides) in plasma samples after controlled intake of 200 µg LSD (20). The aim of the present study was to develop a sensitive turboflow liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify LSD, iso-LSD, O-H-LSD, and nor-LSD and potentially identify LAE, LEO, 2-oxo-LSD, trioxylated-LSD, and 13-14-hydroxy-LSD (glucuronides) in human plasma samples. The method was developed using a triple-stage quadrupole LC-MS/MS instrument in selected reaction monitoring (SRM) mode after

atmospheric pressure ionization (APCI). Our method was established and successfully applied to the analysis of plasma samples from healthy volunteers after the intake of 100 µg LSD in a controlled clinical study.

## 2. Materials and methods

### *2.1 Chemicals and reagents*

Acetonitrile, acetone, methanol, 2-propanol, formic acid, and acetic acid with high-performance liquid chromatography (HPLC)-grade purity were all purchased from Merck (Darmstadt, Germany). HPLC-grade ammonium acetate and ammonium carbonate were obtained from Merck (Darmstadt, Germany). Distilled water was obtained from an in-house installed purifier (ELGA, Bucks, United Kingdom). Drug-free plasma samples (containing lithium-heparin as an anticoagulant) serving as negative control, and blank matrices were obtained from coworkers. LSD and LSD-$d_3$ as 1 mg/ml reference standards in acetonitrile were obtained from Lipomed (Arlesheim, Switzerland). O-H-LSD and iso-LSD as 0.1 mg/ml reference standards in acetonitrile were obtained from Cerilliant (Round Rock, TX, USA). Nor-LSD in powder form was obtained from Toronto Research Chemicals (Toronto, Canada). The non-commercially available metabolites LAE, LEO, 2-oxo-LSD, trioxylated-LSD, and 13-14-hydroxy-LSD (glucuronides) were extracted from pooled 24-h urine samples as described in the results section.

### *2.2 LC-MS/MS analysis*

#### 2.2.1 Equipment

The HPLC system (Transcend TLX1 HPLC, Thermo Scientific, Basel, Switzerland) consisted of two Accela 1250 pumps for loading and eluting. The autosampler and sample extraction system were controlled by the Aria MX 2.1 software (Thermo Scientific, Basel, Switzerland). A cyclone P turboflow column (Thermo Scientific, Basel, Switzerland) was used for extraction, and a Zorbax Eclipse XDB-C8 column (Agilent, Santa Clara, CA, USA) was used for chromatographic separation. The online extraction system was coupled to a TSQ Endura triple stage mass spectrometer (Thermo Scientific, Basel, Switzerland) using APCI in positive mode because of its better performance with regard to matrix effects (24; 25).

### 2.2.2 Liquid chromatography method

For LC, three mobile phases were used in gradient mode for extraction and analytical chromatography. Mobile phase A consisted of 20 mM ammonium acetate in water and 0.1% formic acid. Mobile phase B consisted of 20 mM ammonium acetate in methanol and acetonitrile (1:1) that contained 0.1% formic acid. Mobile phase C was an organic mixture of acetonitrile, acetone, and 2-propanol (1:1:1). Chromatography was run in isocratic mode with 70% mobile phase A and 30% mobile phase B, with a run time of 11 min and 4 additional minutes for flushing and equilibration using mobile phase C.

### 2.2.3 Mass spectrometry conditions

The positive ion discharge current was set to 5 µA. The vaporizer temperature was optimized to 400°C. Sheath and auxiliary gas provided the best results, with flow rates of 15 and 5 arbitrary units, respectively. The temperature of the ion transfer tube was set to 300°C. The system was tuned and optimized for the detection of LSD. LSD and its metabolites were detected

using SRM of the two to three most intense ion transitions. Analytes were identified when quantifier and qualifier ions were present within the given retention time. Structures, transitions, and respective collision energies are shown in Fig. 1.

### *2.3 Standard solutions*

Stock solutions that contained 100 µg/ml LSD, 100 µg/ml LSD-$d_3$, 10 µg/ml iso-LSD, 10 µg/ml O-H-LSD, or 10 µg/ml nor-LSD in acetonitrile were prepared and stored in light-protected brown glass vials at -20°C. All of the solutions were prepared in duplicate to have different sets for quality control (QC) and calibration samples. Working solutions of each analyte at 0.1 µg/ml in purified water/acetonitrile were used for the preparation of QC and calibration samples and matrix and selectivity experiments. Because of the instability of LSD and to minimize possible degradation by various freeze-thaw cycles, 1 ml aliquots of stock and working solutions were prepared.

### *2.4 Sample preparation*

Study samples were sorted according to drug condition (LSD or placebo) and subject (S1-24). Calibrators, controls and subject samples were thawed once, and 100 µl aliquots were taken to minimize the freeze-thaw cycles. To 100 µl of plasma, 110 µl of an acetonitrile/LSD-$d_3$ solution (0.01 µg/ml) was added. The samples were then vigorously vortexed and centrifuged for 10 min at 13,200 × *g*, and the supernatant was then transferred to 96-well plates.

### *2.5 Experiments*

#### 2.5.1 Calibration

Six calibration standards were prepared by spiking plasma samples with LSD, iso-LSD, and nor-LSD to concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml plus blank (matrix only) and zero sample (matrix plus internal standard). Five calibrators were used for O-H-LSD with concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml plus blank (matrix only) and zero sample (matrix plus internal standard). The highest calibration point in plasma was adopted from our previously developed method and pharmacokinetic-pharmacodynamic data (12; 21). The calibration curves were linearly fitted using a weighting factor of $1/x^2$.

### 2.5.2 Selectivity

Following U.S. Food and Drug Administration validation guidelines (26), we collected plasma samples from six different healthy volunteers and tested them for interference to establish selectivity. We further analyzed samples from the placebo condition to confirm the absence of LSD.

### 2.5.3 Matrix effects and recovery

Matrix effects, recovery, and process efficiency were measured and calculated according to Matuszewski et al. (27). In regard of the vulnerability to light and air and because of the online extraction that was used in the present method, the extraction step comprised only protein precipitation. All of the samples were processed through the turbulent-flow extraction column. Five plasma samples were spiked to concentrations between 0.05 and 10 ng/ml for LSD, iso-LSD, O-H-LSD, and nor-LSD. The samples were measured before and after extraction and in neat solution. The peak areas of the spiked samples after extraction were then compared with the area of the spiked mobile phase to calculate matrix effects. Recovery values were calculated as

the areas of standards that were spiked before extraction divided by the areas of standards that were spiked after extraction. The process efficiency was adopted from Matuszewski et al. (27) and calculated as the ratio between the area of the standard spiked before extraction and the areas of the standard in neat solution.

2.5.4 Stability

The determination of long-term stability was based on Li et al. and Klette et al., in which LSD is regarded as stable under storage conditions of -20°C (28; 29). However, LSD is known to be very unstable and vulnerable to air, light, and heat. Even ambient temperature (20-25°C) and normal light conditions can lead to a decrease in LSD concentrations. Therefore, we assessed bench-top stability and autosampler stability with multiple measurements of calibration and QC samples within 24 h. For autosampler stability, the samples were kept in light-protected, sealed, 96-well deep-well plates at 4 °C in the autosampler until injection. During the study, the samples were drawn through an intravenous catheter into lithium-heparin tubes and directly centrifuged, and the plasma was stored at -20°C at the study site before transferring to the laboratory for analysis. Due to the known vulnerability of LSD, calibrators and quality controls were freshly weighted every week and single aliquots were stored at -20°C. A new calibration was run every day and with every study subject.

2.5.5 Lower limits of detection and quantification

Drug-free plasma samples were spiked with different concentrations of LSD, iso-LSD, O-H-LSD, and nor-LSD for determination of the limit of quantification (LOQ) and the limit of detection (LOD). The LOQ concentrations had to give a response at least 5-times greater than the blank. Additionally, precision had to be < 20%, and accuracy had to be 80-120% using at least

five determinations per matrix and concentration. The LOD concentration was determined as the lowest discriminable peak in the region of a signal-to-noise ratio greater than five.

### 2.5.6 Carryover

For the determination of the carryover, different blank plasma samples were run between patient samples, highest calibrations and quality controls.

### 2.5.7 Reproducibility

According to U.S. Food and Drug Administration guidelines (26), the reproducibility of quantification was determined by measuring each QC sample five times in 1 day to establish intraday precision and accuracy. Each QC sample was also measured for 5 consecutive days to determine interday precision and accuracy. All of the values had to meet the criteria of a coefficient of variation (CV) < 15%, response < 20% at the LOQ, and accuracy of 80-120%. To demonstrate the accuracy and precision of the method, we used three QCs (low, medium, high). The QC concentrations were 0.05, 1, and 10 ng/ml for LSD, iso-LSD, and nor-LSD, and 0.1, 1, and 10 ng/ml for O-H-LSD, respectively.

## 3. Results

LSD, LSD-$d_3$, iso-LSD, and the metabolites nor-LSD, LAE, LEO, 2-oxo-LSD, trioxylated-LSD, and 13-14-hydroxy-LSD (glucuronides) eluted between 4 and 11 min. The chromatographic separation of spiked samples and selected metabolites is depicted in Fig. 2A, and the chromatogram of a subject's sample 4 h after LSD administration is presented in Fig. 2B.

### *3.1 Selectivity*

None of the six plasma samples showed any interference within the measured mass range and time frame (Fig 4). Furthermore, none of the measured plasma samples from the placebo condition showed any interference.

### *3.2 Matrix effects and recovery*

The plasma matrix effects were 125 % for LSD, 119 % for iso-LSD, 103 % for O-H-LSD, and 118 % for nor-LSD at concentrations of 10 ng/ml, consistent with a slight ion enhancement for LSD, iso-LSD and nor-LSD. Recoveries were calculated as 70-90 % for all substances at 10 ng/ml. Process efficiencies were 113 % for LSD, 86 % for iso-LSD, 77 % for O-H-LSD, and 93 % for nor-LSD.

### *3.3 Stability*

The concentrations of the processed samples decreased up to -60% within 24 h at ambient temperature (20-23°C). The concentrations of the extracted and sealed plasma samples that were stored within the closed autosampler at 4°C were stable up to 24 h.

### *3.4 Lower limits of detection and quantification*

The LOQ was 0.05 ng/ml for LSD, iso-LSD, and nor-LSD. For O-H-LSD the respective concentration was 0.1 ng/ml. The LODs were 0.01 ng/ml for all compounds.

### *3.5 Carryover*

No carryover was found for LSD, iso-LSD, O-H-LSD, or nor-LSD in the plasma samples. Despite these results as a preventive measure a consecutive blank was always run after the highest calibrator (10 ng/ml) and QC (10 ng/ml) during method development and the measurement of the study samples.

### *3.6 Linearity*

Calibration curves in plasma were linear over the respective calibration ranges, with a mean correlation coefficient ($R^2$) of 0.99. The calibration curves (mean ± SEM) are shown in Fig. 3.

### *3.7 Reproducibility*

All of the substances fulfilled the accuracy and precision criteria. The mean intraday accuracy and precision were 105 % and 4.81 % for LSD, 98.7 % and 5.75 % for iso-LSD, 106 % and 4.54 % for O-H-LSD, and 107 % and 5.82 % for nor-LSD, respectively. The mean interday accuracy and precision were 105 % and 4.35 % for LSD, 99.4 % and 7.21 % for iso-LSD, 99.4 % and 7.21 % for O-H-LSD, and 102 % and 5.88 % for nor-LSD, respectively.

### *3.8 Identification of non-commercially available LSD metabolites*

LSD metabolites were extracted by liquid-liquid extraction from pooled LSD-positive 24-h urine samples (8 L) to reach high concentrations. One part of the concentrated metabolites was kept for eventual quantification, and the second part was extracted using industrial separation by automated thin-layer chromatography and purification. Separation was performed with generous support from Camag (Muttenz, Switzerland). Parent masses and selected transitions for LC-MS

were adopted from Cai et al. (22) and Canezin et al. (23) and replicated by injecting a mixture of the concentrated, extracted metabolites. All of the identified metabolites from concentrated urine samples (LAE, LEO, 2-oxo-LSD, trioxylated-LSD, and 13,14-hydroxy LSD) were added to the quantification method before validation, for qualitative screening of the study samples.

### *3.9 Samples*

LSD (100 μg) and placebo were administered to 24 healthy subjects (12 women, 12 men) in a double-blind, randomized, placebo-controlled, cross-over study. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice (ICH-GCP) and approved by the Ethics Committee Northwest Switzerland and Swiss Federal Office for Public Health, Bern, Switzerland. The study was registered at ClinicalTrials.gov (NCT02308969). Plasma samples were collected at baseline and 1, 2, 3, 4, 6, 8, 10, 12, 16, and 24 h after LSD administration. Maximum LSD plasma concentrations of 1.3 ± 0.17 ng/ml (mean ± SEM) were determined. Nor-LSD could only be quantified in two subjects (3 and 4 h post-administration), and LAE, LEO, 2-oxy LSD, and 13,14-hydroxy LSD were detected in some of the samples. 13,14-hydroxy glucuronides were undetectable because they were cleaved during ionization. Detailed study descriptions, pharmacokinetic data, and pharmacokinetic-pharmacodynamic analyses will be published elsewhere.

## 4. Discussion & Conclusion

With mean maximum plasma concentrations of LSD of ~1 ng/ml, the development of analytical methods for quantification remains a challenge and brings LC-MS technologies to

their limits. For separation of the different analytes, various columns have been used. Especially the separation of LSD and iso-LSD was challenging, and only achieved using the Zorbax Eclipse XDB-C8 column. However, the method was only developed to chromatographically separate LSD, iso-LSD, nor-LSD and O-H-LSD. The non-commercially available metabolites were not available in sufficient amounts for extensive experiments. Further, to improve sensitivity, different sample preparation procedures (e.g., liquid-liquid extraction using chlorobutane and tert-butyl-methylether) have been performed but have not led to significant changes in the LOQ. Considering the light sensitivity, the thermal instability of LSD and the manual workload that is caused by liquid-liquid extraction or solid-phase extraction, simple and fast protein precipitation has been favored instead. APCI was equally to ESI regarding signal intensity but gave slightly better results regarding matrix effects and was therefore favored. Overall, quantifying plasma samples between 12 and 24 h after LSD administration requires techniques that provide precise and sensitive measurements within the low picogram range. This poses a challenge to quantifying LSD concentrations and also makes it impossible to quantify or even identify new metabolites in plasma samples after controlled intake of 100 µg LSD. In our recent investigations (12), we detected quantifiable plasma levels of O-H-LSD after the administration of 200 µg LSD. Steuer et al. additionally identified O-H-LSD and 13-14-hydroxy LSD (glucuronides) (20). We did not expect to detect quantifiable concentrations of LSD metabolites after the administration of 100 µg LSD. The metabolites did not reach the LOD of our or other methods. Nevertheless, we sporadically detected the presence of metabolites in some plasma samples and could confirm the presence of O-H-LSD, nor-LSD, LEO, LAE, and 13,14-hydroxy LSD in plasma. To investigate the metabolism of LSD more comprehensively, further studies that use

higher doses of LSD are required and metabolites need to be commercially available to develop comprehensive analytical methods for their quantification.

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## Tables

Table 1 gives the measured plasma concentrations (Cmax) and the corresponding timepoints (Tmax) following oral administration of 100 µg LSD in 24 healthy subjects.

**Table 1. Measured LSD plasma concentrations in 24 subjects**

| Subject | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cmax (ng/ml) | 1.7 | 1.2 | 1.2 | 2.0 | 2.1 | 1.5 | 1.4 | 1.9 | 3.3 | 2.0 | 0.8 | 3.2 | 0.8 | 0.9 | 1.4 | 0.8 | 1.9 | 1.3 | 0.3 | 1.8 | 1.9 | 1.1 | 1.4 | 1.2 |
| Tmax (h) | 1 | 1 | 2 | 1 | 3 | 1 | 2 | 2 | 1 | 2 | 3 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 1 | 2 | 2 | 3 | 2 | 3 |

## Figure Legends

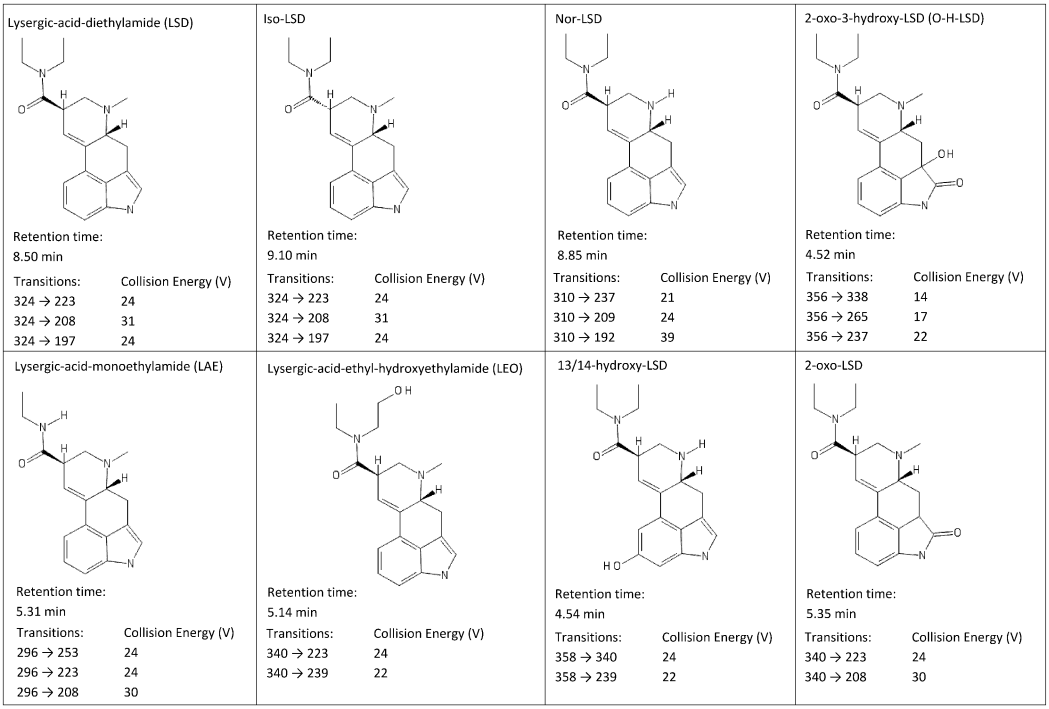



Figure 1. Structure, retention time, ion transitions and collision energies of LSD and selected metabolites.

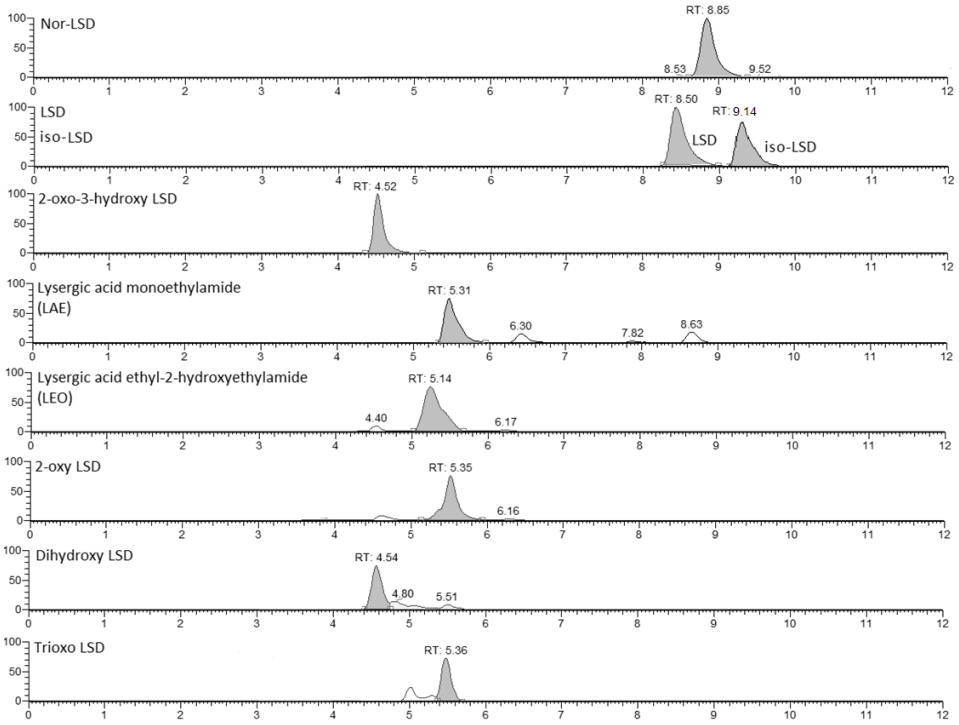
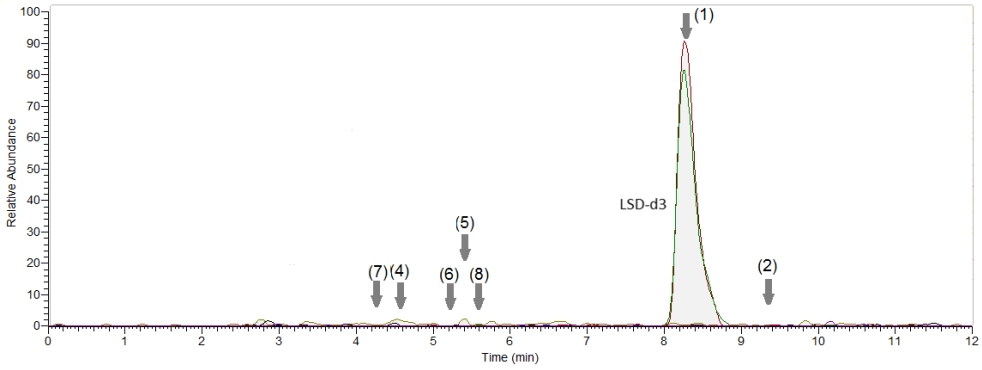



Figure 2. (A) Chromatogram of selected metabolites. LSD, iso-LSD, nor-LSD and 2-oxo-3-hydroxy-LSD are spiked at 1 ng/ml in plasma, the concentration of lysergic-acid monoethylamide, lysergic-acid-ethyl-2-hydroxyethylamide, dihydroxy-LSD, and 2-oxo-LSD is unknown.




(B) Chromatogram of a healthy volunteer 4 hours after administration of 100 µg LSD. Arrows are indicating peaks of LSD (1), iso-LSD (2), nor-LSD (3) and 2-oxo-3-hydroxy-LSD (4), lysergic-acid monoethylamide (5), lysergic-acid-ethyl-2-hydroxyethylamide (6), dihydroxy-LSD (7), and 2-oxo-LSD (8).

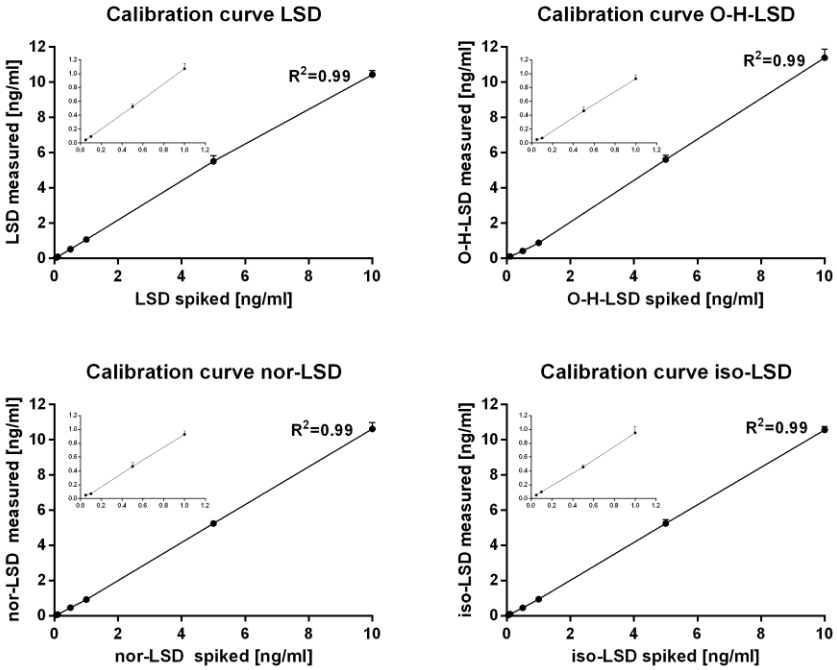



Figure 3. Calibration curves of LSD, iso-LSD, nor-LSD, and 2-oxo-3-hydroxy-LSD in human plasma.

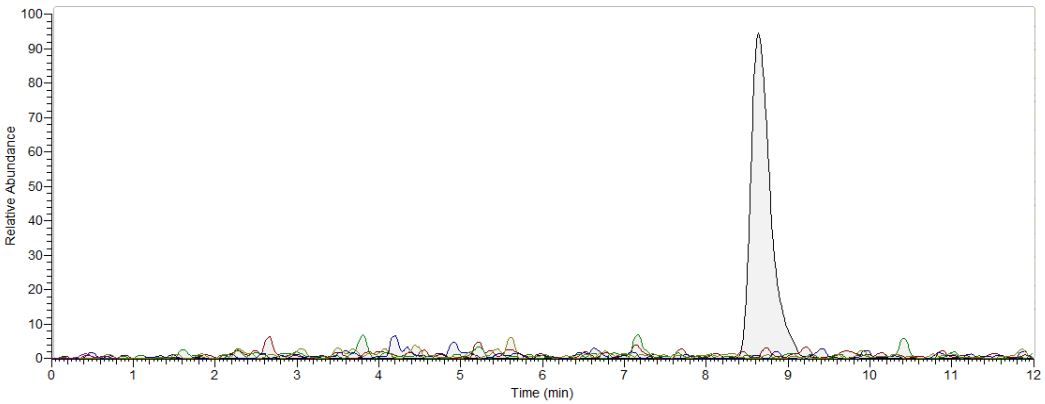



Figure 4. Chromatogram of 6 blank plasma samples from 6 different subjects, and a blank sample containing LSD-d3.