Received: 22 December 2016 | Accepted: 25 April 2017

DOI: 10.1002/jcla.22265

RESEARCH ARTICLE

WILEY

Development and validation of an LC-MS/MS method to quantify lysergic acid diethylamide (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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Funding information
This work was supported by the Swiss National Science Foundation (grant no. 320030_170249 to ML).

Background: Lysergic acid diethylamide (LSD) is a widely used recreational drug. The aim of this 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
controlled study, LC-MS, LSD, lysergic acid diethylamide, metabolism, 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] In addition, 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 monoethylamide (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 this 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. Distilled water was obtained from an in-house installed purifier (ELGA, Bucks, UK). 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 noncommercially 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 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). A cyclone P turboflow column (Thermo Scientific) 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) 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 mmol/L ammonium acetate in water and 0.1% formic acid. Mobile phase B consisted of 20 mmol/L 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 minutes and four 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 Figure 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 was 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 minutes at 13 200 *g*, and the supernatant was then transferred to 96-well plates.

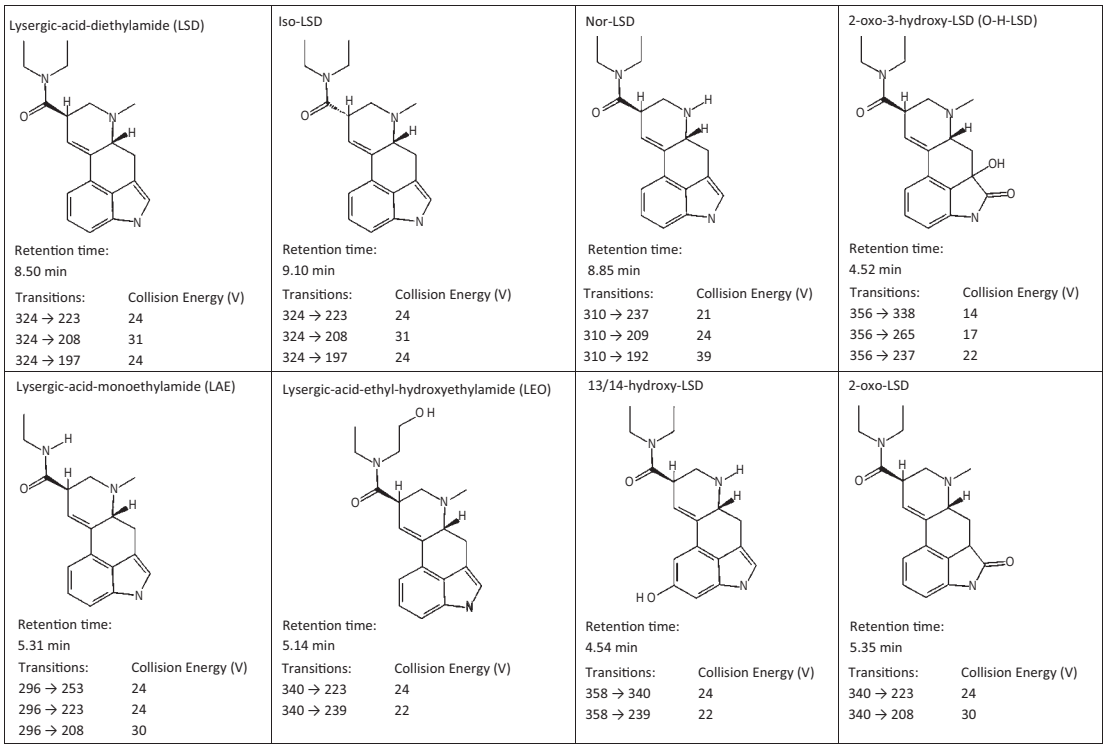

FIGURE 1 Structure, retention time, ion transitions, and collision energies of lysergic acid diethylamide (LSD) and selected metabolites

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

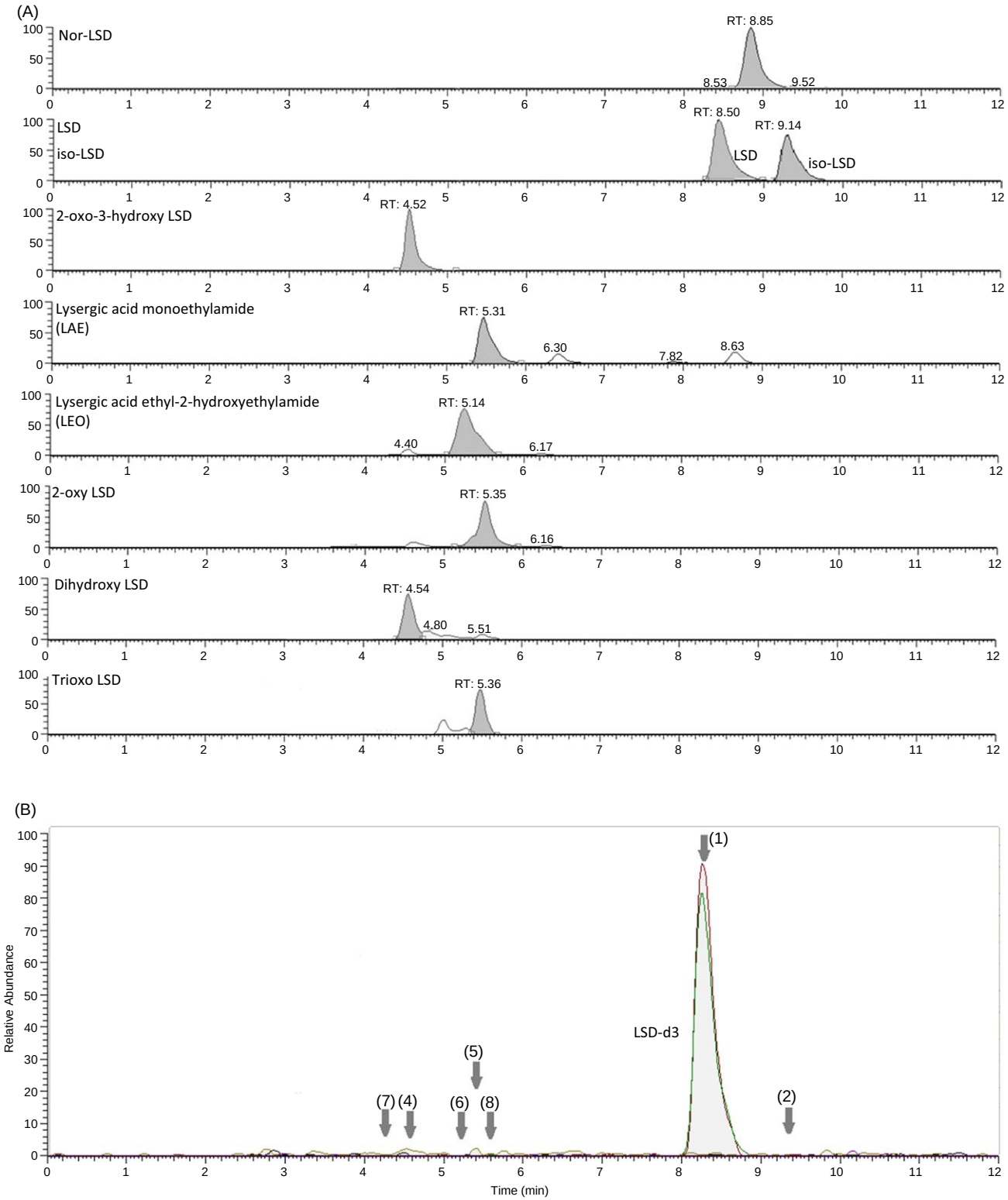

FIGURE 2 (A) Chromatogram of selected metabolites. Lysergic acid diethylamide (LSD), iso-LSD, nor-LSD, and 2-oxo-3-hydroxy-LSD are spiked at 1 ng/mL in plasma; the concentration of lysergic-acid monoethylamide, lysergic-acid-ethyl-2-hydroxyethylamide, 13/14-hydroxy-LSD, and 2-oxo-LSD is unknown. (B) Chromatogram of a healthy volunteer 4 h 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), 13/14-hydroxy-LSD (7), and 2-oxo-LSD (8)

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 five times greater than the blank. In addition, 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 five 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, and 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

Lysergic acid diethylamide, 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 minutes. The chromatographic separation of spiked samples and selected metabolites is depicted in Figure 2A, and the chromatogram of a subject's sample 4 h after LSD administration is presented in Figure 2B.

3.1 | Selectivity

None of the six plasma samples showed any interference within the measured mass range and time frame (Figure 3). 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 hours at ambient temperature (20-23°C). The concentrations of the extracted and sealed plasma samples that

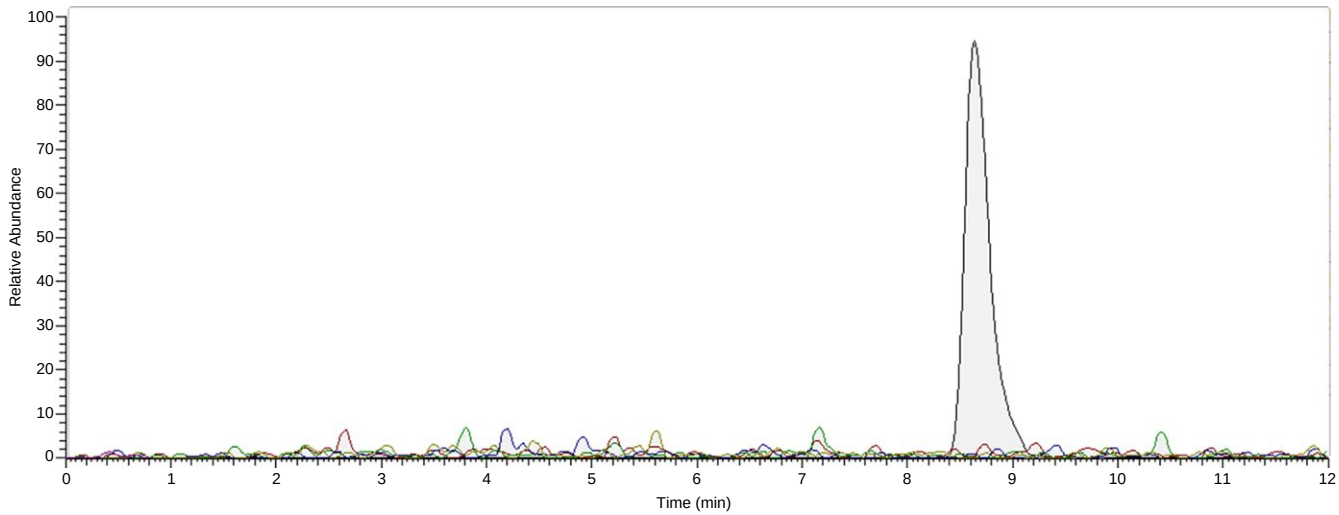

FIGURE 3 Chromatogram of 6 blank plasma samples from six different subjects, and a blank sample containing lysergic acid diethylamide-d3

were stored within the closed autosampler at 4°C were stable up to 24 hours.

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 Figure 4.

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

Lysergic acid diethylamide 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

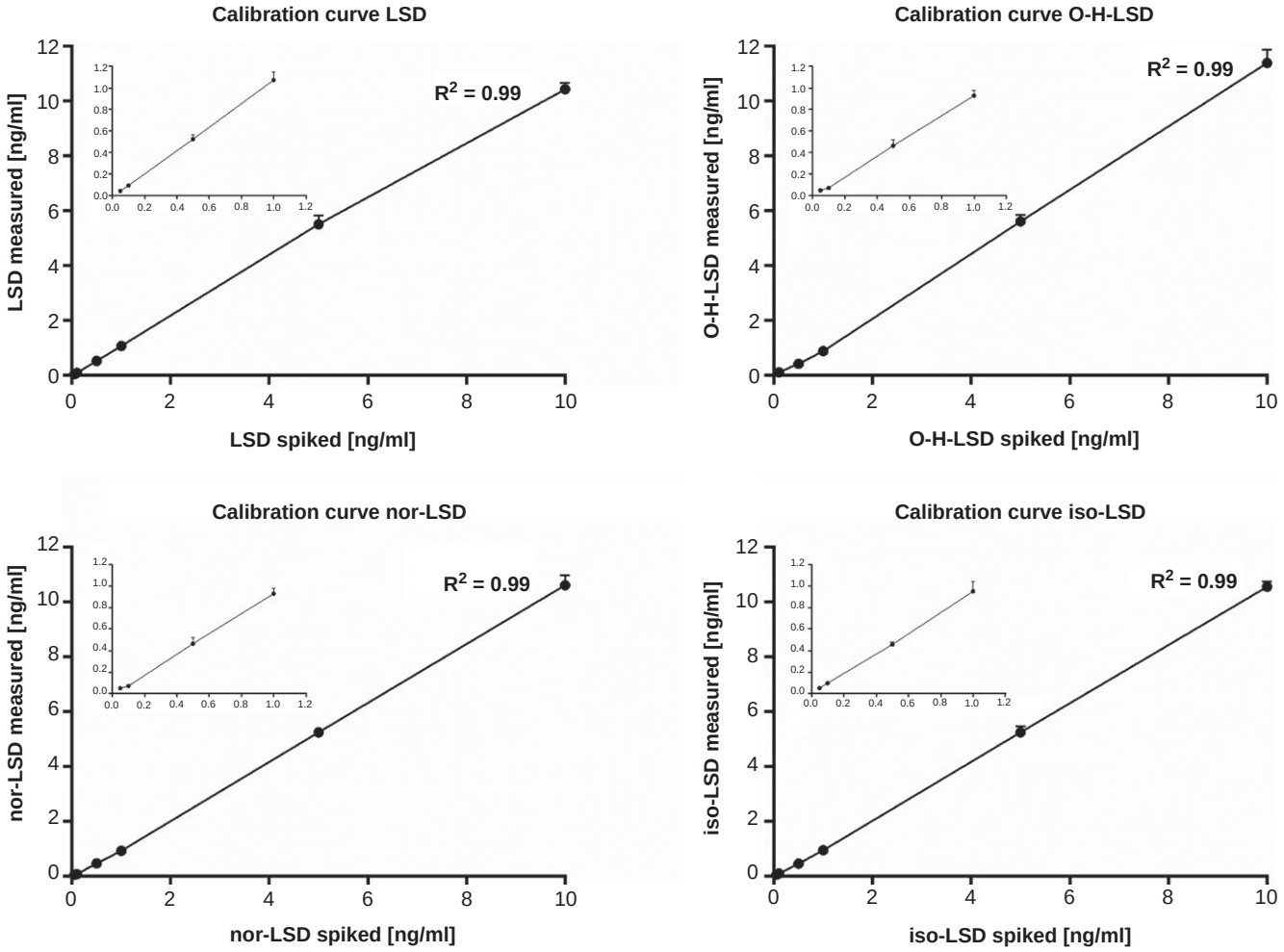

FIGURE 4 Calibration curves of lysergic acid diethylamide (LSD), iso-LSD, nor-LSD, and 2-oxo-3-hydroxy-LSD in human plasma

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 (Table 1). Nor-LSD could only be quantified in two subjects (3 and 4 hours post-administration), and LAE, LEO, 2-oxy LSD, and 13/14-hydroxy-LSD were detected in some of the samples. 13/14-hydroxy-LSD glucuronides were undetectable because they were cleaved during ionization. Detailed study descriptions, pharmacokinetic data, and pharmacokinetic-pharmacodynamic analyses will be published elsewhere.

4 | DISCUSSION AND 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 (eg, liquid-liquid extraction using chlorobutane and tert-butylmethylether) have been performed but have not led to significant changes in the LOQ. Considering the light and air sensitivity 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 hours 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

TABLE 1 Measured plasma concentrations (C_{max}) and the corresponding time points (T_{max}) following oral administration of 100 μg lysergic acid diethylamide in 24 healthy 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
C_{max} (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
T_{max} (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

after the administration of 200 μg LSD. Steuer et al.[20] additionally identified O-H-LSD and 13/14-hydroxy-LSD (glucuronides). 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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