TECHNICAL NOTE

John de Kanel,[1] *M.Sc.; William E. Vickery,*[1] *BettiAnn Waldner,*[1] *AS; Reva M. Monahan,*[1] *and Francis X. Diamond,*[1] *B.Sc.*

Automated Extraction of Lysergic Acid Diethylamide (LSD) and N-demethyl-LSD from Blood, Serum, Plasma, and Urine Samples Using the Zymark RapidTrace™ with LC/MS/MS Confirmation

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ABSTRACT: A forensic procedure for the quantitative confirmation of lysergic acid diethylamide (LSD) and the qualitative confirmation of its metabolite, N-demethyl-LSD, in blood, serum, plasma, and urine samples is presented. The Zymark RapidTrace™ was used to perform fully automated solid-phase extractions of all specimen types. After extract evaporation, confirmations were performed using liquid chromatography (LC) followed by positive electrospray ionization (ESI+) mass spectrometry/mass spectrometry (MS/MS) without derivatization. Quantitation of LSD was accomplished using LSD-d_3 as an internal standard. The limit of quantitation (LOQ) for LSD was 0.05 ng/mL. The limit of detection (LOD) for both LSD and N-demethyl-LSD was 0.025 ng/mL. The recovery of LSD was greater than 95% at levels of 0.1 ng/mL and 2.0 ng/mL. For LSD at 1.0 ng/mL, the within-run and between-run (different day) relative standard deviation (RSD) was 2.2% and 4.4%, respectively.

KEYWORDS: forensic science, lysergic acid diethylamide, LSD, N-demethyl-LSD, blood, serum, plasma, urine, HPLC-MS-MS, electrospray ionization, RapidTrace, solid-phase extraction

Increased rates of lysergic acid diethylamide (LSD) use continue to be reported in the literature (1–3). Statistics indicating that LSD use among 12th graders has increased from 5.6% in 1992 to 7% in 1993 (4) are alarming considering the potential adverse consequences of use (5–7), including one case of murder (8) which has been reported. The analysis of LSD in biological fluids has still not become routine because of the difficulty of developing a forensic assay capable of reliably detecting the low levels of drug expected. From a typical 20 to 100 μg dose, only 1% is excreted unchanged within 24 h (9). This low initial dose and LSD's rapid elimination (half-life = 3.6 h) (9) cause concentrations in blood and urine to rapidly decrease below 1 ng/mL (10). The 10% to 30% confirmation rate, with gas chromatography/mass spectrometry (GC/MS) detection limits above 1 ng/mL, of radioimmunoassay presumptive positives within the military (11) is further indication that methods with substantially better sensitivity are needed.

Many analytical methods for LSD in biological fluids have been reviewed (12,13). Confirmation methods have recently been described using GC/MS (9,14–16), GC/MS/MS (10), LC/MS (17–19), and LC/MS/MS (11,20,21). Most of these methods fail to achieve substantial improvements in sensitivity. Those that do succeed make use of complex multistep extraction procedures, derivatization of analytes, or large sample volumes of up to 5 mL which make them difficult to apply to routine analysis of blood.

The automated solid-phase extraction procedure and LC/MS/MS method presented here make use of commercially available materials and instrumentation without modification. The same basic method using only 1 mL of sample has been applied to blood, serum, plasma, and urine with a quantitative range from 0.05 to 5.0 ng/mL so that all sample types may be combined into a single analytical run.

Materials and Methods

Reagents, Standards, Controls, and Supplies

d-Lysergic acid diethylamide (LSD), [N^6-methyl-$^2H_3$] lysergic acid diethylamide (LSD-d_3), lysergic acid methylpropylamide (LAMPA), and N^6-demethyllysergic acid diethylamide (N-demethyl-LSD, nor-LSD) standards were purchased from Radian Corp., Austin, TX. LSD in urine control material was purchased from Roche Diagnostic Systems, Somerville, NJ, and Diagnostic Products Corp., Los Angeles. LSD in serum, plasma, and blood control material was prepared in-house. The solvents acetonitrile, methylene chloride, n-propanol, and methanol, used for solid-phase extraction or LC/MS/MS analysis, were high-performance liquid chromatography (HPLC) grade purchased from Fisher Scientific Co., Fairlawn, NJ. Water was prepared with a Solution 2000 cartridge deionization unit (Solution Consultants, Jasper, GA). All other chemicals were reagent grade. Varian Bond Elut Certify solid-phase extraction cartridges (3 mL/300 mg) were obtained from Varian Sample Preparation Products, Harbor City, CA.

[1]Director, Research and Development, scientist, analysts, and senior scientist, respectively, National Medical Services, Inc., Willow Grove, PA.

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A number of dilute standards and reagent solutions were used. Standards were diluted with methanol prior to use in spiking biological materials to the desired concentration. Acetate buffer, pH = 4.0, was prepared by adding 100 mL of a 0.2 M ammonium acetate solution to 400 mL of a 0.2 M acetic acid solution and diluting to 1 L. The acetonitrile/n-propanol solvent was prepared by mixing 954 mL of acetonitrile with 46 mL of n-propanol. The LC mobile phase was prepared by mixing 35% ammonium acetate buffer solution with 65% acetonitrile/n-propanol solvent. Acetic acid (1 M) was prepared by dilution of 28.6 mL of glacial acetic acid to 500 mL total volume with water. Phosphate buffer, pH = 6.0 ± 0.1, was prepared by diluting 1.70 g of Na_2HPO_4 and 10.6 g of NaH_2PO_4 in 1 L of water. The mixed solvent was prepared by combining 780 mL of methylene chloride, 200 mL n-propanol, and 20 mL of concentrated ammonium hydroxide.

Instrumentation

Solid-phase extractions were accomplished using a set of ten Zymark (Hopkinton, MA) RapidTrace™ modules. Samples were evaporated, after extraction, with a Zymark TurboVap LV evaporator. Analyte separations were accomplished using a Hewlett Packard (Santa Clara, CA) 1100 quaternary gradient liquid chromatograph with a Zorbax SB-phenyl 4.6 mm × 7.5 cm column (Mac-Mod Analytical, Chadds Ford, PA) of 3.5 μm particle size. Samples were injected using a Hewlett Packard G1313A autosampler. Quantitation and identification were accomplished using a Micromass (Altrincham, Cheshire, UK) Quattro II LC/MS/MS system operated in the positive electrospray mode (ESI+).

Extraction

The extraction procedure was based upon that described in the Varian Bond Elut Certify applications manual (Feb. 1994) for serum, plasma, or whole blood with some modification. To 1 mL of serum, plasma, urine, or whole blood was added 100 μL of LSD-d_3 (0.01 ng/μL) internal standard solution in methanol, 1 mL of water, and 2 mL of phosphate buffer. Samples were then mixed. Whole blood samples were centrifuged at approximately 3000 rpm for 5 min and the supernatant solution transferred to a new test tube. The mixed solvent used to elute analytes was prepared fresh each week. Other solvents used in the extraction were prepared after they were exhausted. Buffered samples, extraction cartridges, and collection tubes were then loaded onto each RapidTrace module to be used. The procedure presented in Fig. 1 was then loaded from the personal computer controller and the extraction started.

After completion of the RapidTrace procedure, extracts were evaporated to dryness under a stream of nitrogen at 55°C in a TurboVap LV evaporator. As a backup method, the use of a heating block at temperatures up to 70°C was determined not to adversely affect results. The higher temperature was used in the heating block to increase the speed of evaporation to that achieved by the TurboVap at 55°C. 150μL of LC mobile phase was then added to each tube, which was vortexed briefly before transfer to brown autosampler vials with glass micro inserts.

LC/MS/MS Method

Prior to sample analysis, ESI + MS/MS parameters were optimized by infusion of LSD dissolved in mobile phase. The electrospray probe position, collision cell gas pressure, source, MS1, and MS2 tuning parameters were adjusted to maximize sensitivity of product ions produced by argon collision induced dissociation (CID) of protonated LSD. A multiple reaction monitoring (MRM) method shown in Fig. 2 was constructed for LSD, LSD-d_3, and N-demethyl-LSD. The LC column was connected to the electrospray

Procedure Name LSDMIXED.SPE
Created 6/6/96 1:57:28 PM
Last Modified 4/7/97 8:39:59 PM
Extracts LSD from serum, plasma, blood and urine using mixed solv. on Varian Certify 3 ml/300mg col.

	Step	Source	Destination	ml	ml/sec	liq. chk
1	Condition	MIXED	w-2	1.5	.10	No
2	Condition	MEOH	w-2	3.0	.10	No
3	Condition	diH2O	w-1	1.0	.10	No
4	Condition	pH6.0	w-1	3.0	.10	No
5	Load	Sample	w-1	5.1	.05	No
6	Purge-Cannula	diH2O	Cannula	5.5	.50	No
7	Rinse	pH6.0	w-1	3.0	.10	No
8	Rinse	AcOH	w-1	2.0	.10	No
9	Rinse	MEOH	w-2	3.0	.10	No
10	Rinse	MIXED	w-3	.50	.10	No
11	Collect	MIXED	Fracti	1.8	.10	No
12	Purge-Cannula	MEOH	Cannula	2.5	.40	No
13	Purge-Cannula	diH2O	Cannula	2.5	.40	No

LoadCannulaDepth 0
MixCannulaDepth 0
MixCycles 2
MixVolume .5
MixSpeed .5
ReagentMixCycles 2

Reagent Setup

No.	ReagentName	Abbrev	SipSpeed
0	Vent	Vent	.5
1	DI H2O	diH2O	.4
2	pH6 PHOS BUFF	pH6.0	.4
3	1M ACETIC ACID	AcOH	.4
4	Not used		
5	METHANOL	MEOH	.3
6	MIXED SOLVENT	MIXED	.3
7	Not used		
8	Not used		
9	Mixing Vessel	Mixer	.25
10	Sample	Sample	.25

Column air push volume 1.8
Column air push volume speed multiplier 2

No.	Waste Name	Abbrev
1	aqueous	w-1
2	org./water soluble	w-2
3	org./non-water sol.	w-3

FIG. 1—*Automated extraction procedure loaded from the PC into the Zymark RapidTrace modules.*

Micromass MRM method of 8 mass pairs (ESP+)

Inter Channel Delay (Secs):	0.02
Span (Daltons):	0.00
Start Time (Mins):	1.00
End Time (Mins):	4.00
Repeats:	1.00
Dwell (Secs):	0.10
Collision Energy (eV):	24
Cone (Volts)	40

Analyte	Channel	Parent MH^+	Product ion	Relative Response %	Retention Time (min)
N-demethyl-LSD	1	310.20	209.10	100	2.73
	2	310.20	237.10	92.6	2.73
LSD	3	324.20	197.10	16.3	2.83
	4	324.20	223.10	100	2.83
	5	324.10	208.10	22.2	2.83
LSD-d_3	6	327.20	200.10	22.3	2.85
	7	327.20	211.10	5.4	2.85
	8	327.20	226.10	100	2.85

FIG. 2—*MS/MS method used to monitor analyte ions.*

probe, the flow rate increased to 250 μL/min, the MRM method loaded into the MS/MS data system, and the automatic injection of 50 μL of each specimen, standard, and control was begun.

Results and Discussion

Optimization of the solid-phase extraction method was simplified by using the Zymark RapidTrace. Its ability to mix reagents and collect multiple fractions from a single column was used to improve the basic procedure published by Varian. The excellent precision obtained by the method was in part due to the reproducible flow rates which were delivered to each cartridge by positive liquid pressure irrespective of sample viscosity differences and variability in column packing density. Another advantage of using the RapidTrace is the ability to separate waste liquids. In this way the cost of disposal of waste organic solvents may be minimized. Perhaps the best feature of the RapidTrace is the ease of transferring methods between labs. A validated method will be carried out the same way, every time, with minimal training of different technicians.

A number of different solvent systems containing volatile buffers and LC column combinations were compared to produce retention times, for all analytes, less than 5 min and optimum MS sensitivity. Buffers containing triethylamine produced good LC separations but were found to reduce MS sensitivity below desired levels. An LC mobile phase containing 35% pH 4.0 ammonium acetate buffer with the balance made up of acetonitrile/n-propanol was found to produce total LC cycle times less than 6 min with a phenyl column and MS sensitivity down to 0.05 ng/mL.

Quantitation of each analyte was accomplished using ion intensities of one product ion of protonated LSD (223) and N-demethyl-LSD (237) divided by the most intense product ion of protonated LSD-d_3 (226). The calibration curve [$a + b(1/x) + c(1/x)^2 = y$; x = concentration and y = ion area ratio] was fitted to these data to better estimate low concentrations. Use of this curve from the lower limit of quantitation, 0.05 ng/mL, to the upper limit of quantitation, 5.0 ng/mL, fit the calibration data with a coefficient of determination greater than 0.999 for all runs. Forensic identifications were accomplished using the LC retention times and product ion ratios. Identification was considered acceptable if the LC relative retention time compared with the LSD-d_3 internal standard was within 1% and all ion ratios measured were within 20% of that measured for known standards in the analytical run. Product ions resulting from protonated molecular ion CID were monitored. Three ions were monitored for LSD and LSD-d_3. N-demethyl-LSD produces only two product ions with sufficient intensity for reproducible measurement of ion ratios. These two product ions should be sufficient for identification considering that they must result from the protonated molecular ion at a specific LC relative retention time. Typical retention times and ion ratios are given in Fig. 2.

The possibility of incorrectly identifying the LSD isomer, LAMPA, as LSD was evaluated. The short retention times of all analytes in this method, while desirable for analytical efficiency, do result in relative retention times for LAMPA and LSD within 1%. Qualifying LSD product ion area ratios at 208/223 and 197/223 deviate from that expected for LSD by 25% and 50%, respectively. Such a large ion ratio deviation could not result in LAMPA being mistaken for LSD.

The LOD and LOQ of the method were determined by analysis of serum, urine, and blood fortified with the analytes. The instrument noise level may be set by the software. For this reason we defined the LOD as that level where the RSD of five replicate analyses of specimens spiked with analyte was between 30% and 50%. The LOQ was defined as that analyte level where the RSD ($n = 5$) was less than 20%. The LOD for both LSD and N-demethyl-LSD was found to be 0.025 ng/mL. The LOQ for LSD was found to be 0.05 ng/mL. The LOQ was not determined for the LSD metabolite because of large between-run variation observed for replicate analysis of controls, as discussed later.

The method efficiency, precision, and accuracy were determined for all specimen types. The recovery of LSD was determined by comparison of LC/MS/MS results obtained for neat analyte injections and results after extraction of samples fortified with LSD ($n = 5$). The recovery of LSD was greater than 95% for all sample types at 0.1, 0.2, 2.0, and 5.0 ng/mL. The within-run RSD ($n = 5$) was found to be 2.7% at 0.25 ng/mL and 2.2% at 1.0 ng/mL. The between-run RSD ($n = 5$) was found to be 9.1% at 0.25 ng/mL and 4.4% at 1.0 ng/mL. The accuracy of the method was determined through repeated analysis, with each analytical run, of purchased urine controls at levels of 0.25 ng/mL and 1.0 ng/mL. After 29 separate runs, during a three-month period, which included blood, serum, and urine from real patients, results were within 15% of the 0.25 ng/mL and 1.0 ng/mL control samples 83% and 93% of time, respectively. Results were within 20% of both control target values for all runs.

Results for N-demethyl-LSD were found to be more variable. The recovery, as determined by comparison with neat injections, was above 95%. The within-run RSD ($n = 5$) was 8% at 0.25 ng/mL and 3.1% at 1.0 ng/mL. Between-run RSD ($n = 5$) was 19.8% at 0.25 ng/mL and 13.8% at 1.0 ng/mL. The ion area ratio, 237/209, used for identification is very reproducible. Because of the quantitative variability of N-demethyl-LSD, the method is used for qualitative identification to provide additional evidence of LSD use.

The stability of LSD and N-demethyl-LSD was evaluated. Samples of each specimen type, spiked with both analytes at 0.25 and 1.0 ng/mL, were stored for two weeks at 4°C or −20°C in the dark. They showed no measurable decrease in concentration after storage. The concentration of the analytes in samples stored at room temperature in clear vials on a lab bench under normal fluorescent lights decreased to about 50% of the starting concentration over a 14-day period.

Conclusion

A forensic procedure for the confirmation of LSD and N-demethyl-LSD metabolite at levels down to 0.05 ng/mL in blood, plasma, serum, and urine has been developed. The automated solid-phase extraction procedure using the Zymark RapidTrace is reliable and simple for technicians to carry out successfully. While extractions are being carried out by the RapidTrace, technicians may perform other value-added tasks. The Micromass Quattro II LC/MS/MS system has the sensitivity for the demanding task of analysis of LSD in body fluids.

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Additional information and reprint requests:
John de Kanel, Director
Research and Development
National Medical Services, Inc.
3701 Welsh Road
Willow Grove, PA, 19090