Journal of Chromatography, 529 (1990) 103–112
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5287

Gas chromatographic–electron-impact mass fragmentometric determination of lysergic acid diethylamide in urine[a]

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(First received October 24th, 1989; revised manuscript received February 23rd, 1990)

ABSTRACT

A sensitive method for the detection and quantitation of lysergic acid diethylamide (LSD) in urine was developed. After initial solvent extraction, the compound was further purified by liquid–liquid extraction or by solid-phase extraction. The trimethylsilyl derivative of LSD was detected by gas chromatography–mass spectrometry (GC–MS) operated in the electron-impact mode with selected-ion monitoring. The presence of LSD was confirmed by comparing retention times and relative abundances of ions of unknowns with that of a standard. The recovery of this procedure was >89%. The intra-run and inter-run coefficients of variation were <5% and <7%, respectively. This procedure allows detection of LSD concentrations as low as 29 pg/ml. Quantitation of LSD was linear over the concentration range 50–2000 pg/ml.

INTRODUCTION

Lysergic acid diethylamide (LSD) is a potent psychoactive drug that has been extensively abused. Due to the very low dose consumed (usually 40–120 μg) and to rapid metabolism with less than 1% of the LSD excreted unchanged in urine [1,2], identification of LSD in biological samples is difficult. Furthermore, the instability of LSD in acid, heat [3], and light has made the identification even more challenging.

Several methodologies including immunoassay [4–7], high-performance liquid chromatography (HPLC) [8], thin-layer chromatography [2,8], and

[a]Presented at the 39th National Meeting of the American Association of Clinical Chemistry, San Francisco, CA, July 19–24, 1987.

0378-4347/90/$03.50

fluorometry [1,2], have been developed to detect LSD in biological samples. These procedures were utilized primarily to study metabolism and distribution of LSD in animals and humans. These procedures, however, have limited application in the positive identification of LSD. Gas chromatographic (GC) separation coupled with mass spectrometric (MS) identification is considered the method of choice in forensic urine testing for detection of a compound. A one-step extraction procedure followed by GC–MS confirmation has been reported for LSD [9], but we have repeatedly observed interfering impurities coeluting with LSD. Moreover, accumulation of polar impurities at the injection port frequently result in instrument failure.

In this report, we describe a procedure that can be applied to the analysis of a large number of specimens. For quantitation, lysergic acid N-methyl-N-propylamide (LAMPA) was utilized as an internal standard.

EXPERIMENTAL

Reagents

All solvents and reagents were of analytical- or HPLC-grade quality. Solid-phase extraction (SPE) cartridges containing silica-based propylamine sorbent (LC-NH_2, 0.5 g in a 3-ml tube) and LSD were purchased from Supelco (Bellefonte, PA, U.S.A.). LAMPA and 6-N-[$^2H_3$]LSD ([$^2H_3$]LSD) were purchased from Alltech–Applied Science (State College, PA, U.S.A.) and Research Triangle Institute (Research Triangle Park, NC, U.S.A.), respectively. Urine, screened negative by radioimmunoassay, preserved with sodium azide (0.1%, w/v) and adjusted to pH 7.3 with 2 *M* sodium hydroxide in water, was utilized to prepare standards and controls.

Equipment

A GC–MS system consisting of an HP 5890 gas chromatograph, HP 5970 mass-selective detector, HP 9153C computer, HP 9153B disc drive, and software, all from Hewlett-Packard (Palo Alto, CA, U.S.A.), was used. For derivatization of LSD, the reacti-vials and the metal heating blocks were purchased from Pierce (Rockford, IL, U.S.A.).

GC instrument conditions

The flow-rate of the carrier gas (helium) through the 15 m×0.25 mm I.D. capillary column (DB-5, J&W Scientific, Rancho Cordova, CA, U.S.A.) was 1.26 ml/min. The head pressure on the column was maintained at 140 g/cm^2. The samples were injected into the GC column in the splitless mode; temperature program: initial temperature of 190°C, increasing to 290°C at 20°C/min and maintaining the final temperature for 3.0 min. The temperature at the injection port was maintained at 235–245°C. Approximately 0.5 min after the

splitless injection, the instrument was turned on to split mode (1:30) to purge any solvent that could cause solvent tailing.

MS instrument conditions

The mass-selective detector was operated in electron-impact mode at 70 eV with an ion source temperature of 200–250 °C; the m/z range was 50–400 a.m.u. The instrument was autotuned according to manufacture-recommended specifications. For sample analysis, the electron multiplier voltage of the detector was set at 800–1000 V above the autotune. The selected-ion monitoring (SIM) window and the dwell times of the ions were set at 0.2 a.m.u. and 25 ms, respectively. To avoid condensation, the GC–MS interface temperature was kept 5–10 °C higher than the final temperature of the column, which was operated with temperature programming.

Preparation of standards

LSD (10 mg) was dissolved in 0.001 *M* triethylamine in ethanol (100 ml) in an amber glass volumetric flask: subsequent dilution (1:200) with the same solvent gave a stock solution of concentration 500 ng/ml. An adequate volume of this solution was used to make LSD concentrations in urine in the range 25–2000 pg/ml. The solutions were aliquoted in 10-ml portions into polypropylene tubes and kept frozen at −20 °C until use. A stock solution of the internal standard, LAMPA, was prepared in triethylamine and ethanol (500 ng/ml) and used as described for LSD.

Extraction

LAMPA (40 μl, 500 ng/ml) and ammonium hydroxide (500 μl, 14.8 *M*), were added to urine samples (10 ml) in polypropylene tubes and the solutions were transferred to Teflon-lined screw-capped glass centrifuge tubes.

Sodium chloride (approximately 2 g) and *n*-butyl chloride (5 ml) were added to each tube. The tubes were capped and shaken horizontally (60 excursions per min) for 15 min. The solution was centrifuged at 1000 *g* for 5 min. The lower urine layer was frozen by immersing the tube into a dry ice–isopropanol mixture. The tubes were uncapped and the organic layer decanted into another Teflon-lined screw-capped glass centrifuge tube. The crude extract was purified by either of the two following methods.

Acid–base purification. Cyclohexane (5 ml) and phosphate buffer (10 ml, 0.1 *M*, pH 4.5) were added to the crude extract dissolved in *n*-butyl chloride. The tubes were capped and shaken horizontally (100 excursions per min) for 30 min. The solution was centrifuged at 1000 *g* for 2 min. The tubes were uncapped and the upper organic layer was removed and discarded. The aqueous acidic layer was washed with a solution of cyclohexane–*n*-butyl chloride (1:1, v/v, 5 ml), and the washed solution was removed and discarded. The acidic solution containing LSD was treated with ammonium hydroxide (500 μl, 14.8

M) and sodium chloride (approximately 2 g). *n*-Butyl chloride (5 ml) was added to the tubes. The tubes were capped and shaken horizontally (60 excursions per min) for 15 min. The aqueous layer was frozen by immersing the tube into a dry ice–isopropanol mixture. The tubes were uncapped and the upper organic phase was decanted into a Teflon-lined screw-capped reacti-vial. The solution was evaporated to dryness using a heated metal block at 70 °C under a stream of nitrogen. The residue was immediately dissolved in 0.001 *M* triethylamine in ethanol (100 μl) and stored at 0–5 °C until derivatization.

Solid-phase purification. The crude extract in *n*-butyl chloride was evaporated to dryness at 70 °C under a stream of nitrogen. It was reconstituted in 1 ml of 0.1% triethylamine in a cyclohexane–chloroform mixture (1:1, v/v) and transferred to an LC-NH_2 SPE cartridge which was pre-conditioned with 2 ml of the same solvent mixture. The cartridge was suspended from the neck of a 15-ml test tube and centrifuged at 250 *g* for 2 min. To remove impurities of low polarity, 0.1% triethylamine in chloroform (2 ml) was added to the cartridge and centrifuged at 250 *g* for 2 min. The collected solvent was discarded. The cartridge was placed in another test tube, and 3 ml of 3% methanol in chloroform containing 0.1% triethylamine were added. The tube containing the cartridge was initially centrifuged at 250 *g* for 2 min and then at 750 *g* for 5 min. The cartridge was discarded and the eluent transferred to a reacti-vial and evaporated to dryness using a heated metal block at 70 °C under a stream of nitrogen. The residue was immediately dissolved in 0.001 *M* triethylamine in ethanol (100 μl) and stored at 0–5 °C until derivatization.

Silylation

The extracts dissolved in 0.001 *M* triethylamine in ethanol were placed in a reacti-vial and evaporated to dryness in a heated metal block at 70 °C under a stream of nitrogen. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (20 μl) was added to the dry residue. The vial was capped and heated on a metal block at 70 °C for 15 min. After cooling to room temperature, the vial was uncapped, and approximately 2 μl were injected onto the GC–MS system. The derivatized compound was stable for at least seven days when stored at 0–5 °C in a tightly closed vial. Because the trimethylsilyl (TMS) derivative of LSD is sensitive to moisture, the cold vial was allowed to come to room temperature before it was uncapped and used for GC–MS analysis.

GC column conditioning

Since LSD is sensitive to silicic acid, any amount of this compound present in the GC column will adversely effect the sensitivity of detection. Therefore, conditioning of column prior to sample injection is important. Although conditioning can be accomplished by injecting 2–3 μl of BSTFA or hexamethyldisilazane (HMDS) five times into the column and operating the instrument in the temperature program mode, conditioning by injecting 2–3 μl of deriva-

tized urine extract four times was found to be more effective. The basic components in the urine appeared to be responsible for the conditioning. When the instrument was not in use for more than 24 h it was necessary to recondition the column again prior to injecting samples.

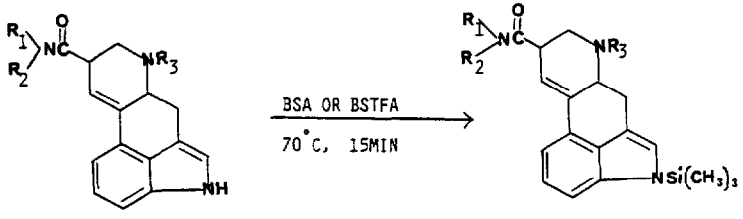
Extraction efficiency

Six replicates of LSD in drug-free urine (10 ml, 400 pg/ml) without internal standard were extracted and purified according to the described procedure.

Steps involving transfer of solution were carefully done to avoid loss of compound. After purification, internal standard (40 μl, 500 ng/ml) was added. The dried extracts were derivatized as described. At the same time, a control group containing LSD (0.1 ml, 40 ng/ml) and internal standard (LAMPA, 40 μl, 500 ng/ml) in six tubes were evaporated and derivatized with BSTFA. Both groups were analyzed by GC–MS. The percentage recovery was determined by comparing the results of LSD in urine with those of controls. The overall yield of solvent extraction and acid–base purification varied from 92 to 95%, whereas the overall yield of solvent extraction and solid-phase purification varied from 89 to 92%.

RESULTS AND DISCUSSION

The SIM technique was utilized to identify and quantitate LSD in urine. To select the ions, a known amount of LSD (10 ng) was derivatized with BSTFA (20 μl), and approximately 800 pg were injected onto the GC–MS system in the scan mode. Similarly, LAMPA and [$^2H_3$]LSD were also derivatized and injected separately onto the GC–MS system, in the scan mode. The structures of the compounds and their derivatives are illustrated in Fig. 1. These three derivatives, LSD-1-N-TMS (LSD-TMS), LAMPA-1-N-TMS (LAMPA-TMS), and [$^2H_3$]LSD-1-N-TMS ([$^2H_3$]LSD-TMS), displayed similar mass fragmentation (Fig. 2). The proposed mechanism of mass fragmentation and



LSD $R_1 = R_2 = C_2H_5$, $R_3 = CH_3$

$^2H_3$ - LSD $R_1 = R_2 = C_2H_5$, $R_3 = C^2H_3$

LAMPA $R_1 = CH_3$, $R_2 = 1\text{-}C_3H_7$, $R_3 = CH_3$

Fig. 1. Structures of LSD, [$^2H_3$]LSD, and LAMPA, and their trimethylsilyl derivatives.

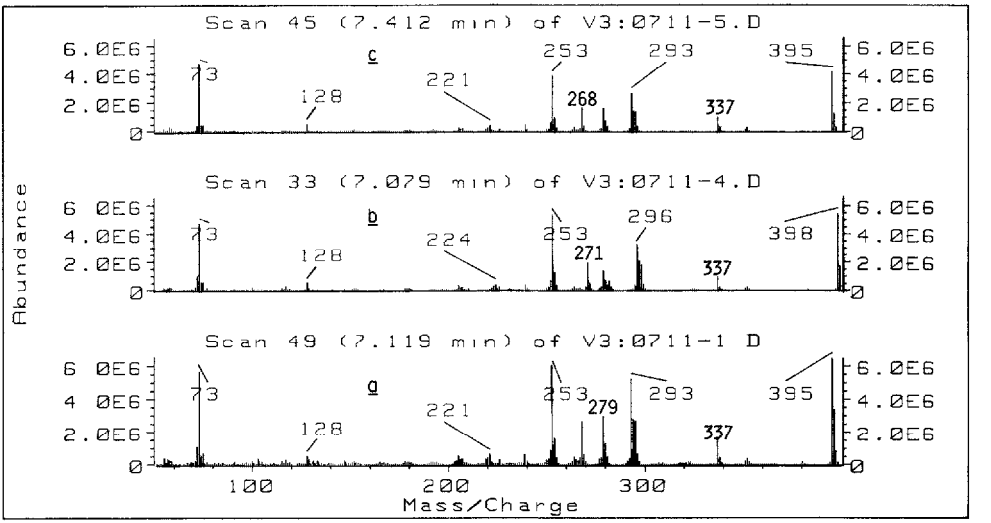


Fig. 2. Mass fragmentation spectra of LSD-TMS (a), [$^2H_3$]LSD-TMS (b), and LAMPA-TMS (c).

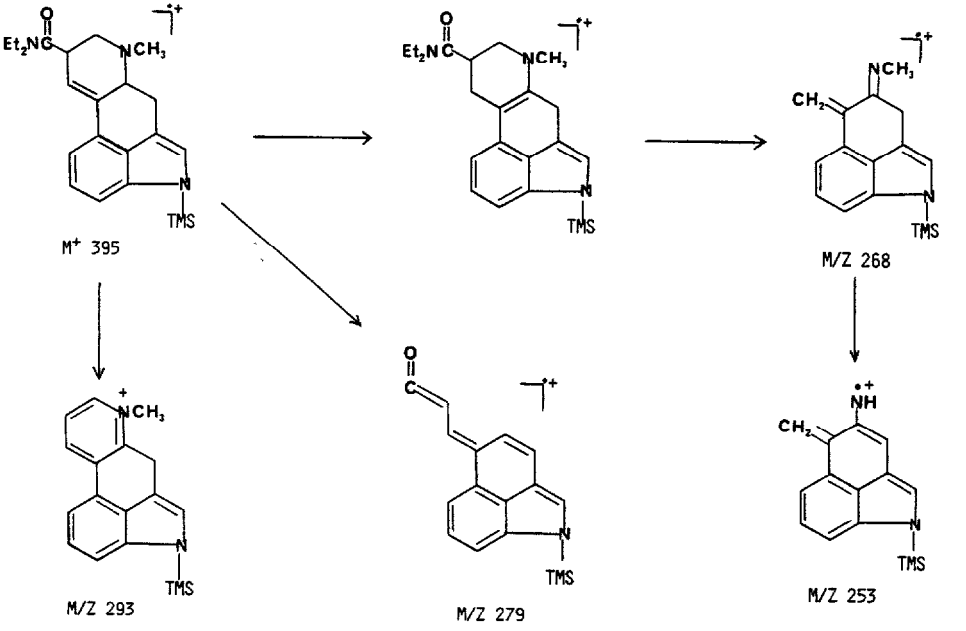


Fig. 3. Mechanism of mass fragmentation of LSD-TMS.

the fragment structures of LSD-TMS are shown in Fig. 3. These structures help to gain insight into the molecule and, therefore, are useful in selecting ions for characterization of the compound. Since the relative abundance of ions

in a compound is an intrinsic property of the compound and remains unchanged under the same instrument conditions, this characteristic was utilized in this study to identify the compound. Therefore, when the retention time and relative abundances of ions of an unknown compound are the same as that of a known standard compound, the unknown and the standard should be considered identical. In this study, the molecular ion and two additional ions that retain the major portion of the molecule are considered as SIM ions. For the internal standard, the molecular ion and a second ion were monitored. Since a deuterated congener is considered to be the most suitable internal standard, [$^2H_3$]LSD was initially chosen for quantitation of LSD in urine. However, this compound was unsuitable as an internal standard because both [$^2H_3$]LSD-TMS and LSD-TMS have the same retention time and produced common ions (*m/z* 337, 279, and 253) (Fig. 2). When the LSD concentration was greater than 5000 pg/ml, isotopic spillover from LSD-TMS to [$^2H_3$]LSD-TMS made the ions *m/z* 398 (M^+) and 271 unsuitable for use as internal standard ions.

To investigate LAMPA as an internal standard, fragmentation spectra of LSD-TMS and LAMPA-TMS were compared. The fragment ions and their relative abundances were found to be similar to each other. However, LAMPA-TMS has a different GC retention time from that of LSD-TMS. Therefore, the three major ions *m/z* 395 (M^+), 293, and 253 that are utilized to detect LSD-TMS could also be utilized to detect LAMPA-TMS. Due to very small amounts of LSD present in urine, monitoring three ions instead of five ions for both LSD-TMS and LAMPA-TMS also increased sensitivity.

When large numbers of samples are to be tested, purification of the crude extract was important. Without purification, a considerable amount of nonvolatile material was deposited in the glass liner and on the column. Loss of sensitivity of the instrument became a major problem. In several experiments, instrument failure was observed after only five injections. Sometimes the column's performance deteriorated sufficiently that it had to be replaced.

During assay development, two methods of purification were investigated. The solid-phase method was relatively simple and time-saving, however, variation was considerable between cartridge lots and between cartridge suppliers. Variation was also high when the triethylamine was not added to the cartridge solvents; the yield varied from 40 to 92%. Interaction of LSD and LAMPA with silicic acid may have led to the breakdown of these compounds in the cartridge.

Another major problem observed in solid-phase purification was interference from compounds eluted from the plastic cartridge. Presence of molding material used in shaping the tube and the frit may be the reason for this interference.

A second procedure utilizing acid–base separation and solvent extraction was developed to purify LSD from the crude urine extract. Aqueous acid was used first to separate the basic components from the extract. Since LSD is

sensitive to acid, its stability in this media was also examined. The compound was dissolved in a mixture of *n*-butyl chloride–cyclohexane (1:1, v/v) and was extracted with phosphate buffer over a pH range of 4.0–8.0. The acid extract was treated with base and the solution was extracted with organic solvent. Subsequent evaporation and derivatization produced LSD-TMS and LAMPA-TMS. Maximum recovery of LSD was found at pH 4.5 (Fig. 4). Below pH 4.5, the compound breaks down and above this pH the extraction is relatively inefficient. The effect of molarity of phosphate buffer on extraction efficiency was also examined. The experiment was performed with phosphate buffer of pH 4.5 in triplicate at each molarity (0.5, 0.25, 0.1, 0.05, and 0.01 *M*). The recovery varied from 80 to 95% with optimum yield at 0.1 *M* phosphate buffer.

LSD was also found to be sensitive to chlorinated solvent, especially when the solvent is basic. Reaction with the solvent may be the reason for the loss of the compound. The problem was obviated in the present assay by limiting the time of exposure of LSD, hence, no loss of compound was observed.

When quantitation was performed with LAMPA as internal standard, results of acid–base and solid-phase purifications were similar. A calibration curve was obtained from urine containing known concentrations of LSD (25, 50, 100, 250, 500, 1000, and 2000 pg/ml) and a fixed concentration of internal standard (2000 pg/ml). When the analysis was performed with three replicates at each concentration and the area ratios of molecular ion (M^+ 395) of drug versus internal standard were plotted as a function of LSD concentration, excellent linearity was obtained over the concentration range 50–2000 pg/ml. The slope, intercept, and correlation coefficient were 0.00066, −0.02052, and 0.9992, re-

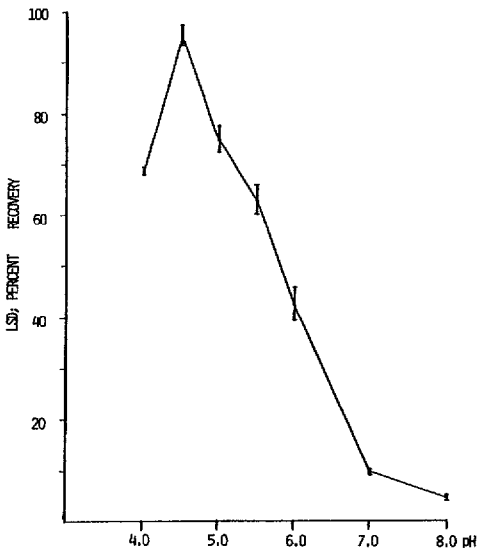


Fig. 4. Recovery of LSD at different pH values of phosphate buffer.

spectively. To determine the limit of detectable concentration (C_L) [10], drug-free urine specimen from fifteen individuals were tested by this procedure. The C_L was found to be 29 pg/ml. Over the concentration range 50–2000 pg/ml, the intra-run and inter-run coefficients of variation were found to be less than 5% and less than 7%, respectively.

Urine solutions of LSD stored at $-18°C$ in a polypropylene container were found to be stable for at least six months. Storage of specimen in glass containers was avoided because it may interact with the glass container when the urine pH is less than 7. Triethylamine in ethanol was found to be suitable for the preparation of standard solutions of LSD in glass containers. Since glass containers have no detrimental effect when the LSD is kept in a basic media, silylation of glass was not necessary. Due to impurities extracted from plastic containers, the solvent extraction of LSD was performed in glass tubes. In this extraction, the urine was treated with base in a plastic tube and transferred to a glass tube for solvent extraction.

Using the acid–base purification procedure, interfering peaks from urine were occasionally a problem. Use of solid-phase followed by acid–base purifications eliminated these interferences. The ion chromatograms of a urine specimen and a standard after extraction, acid–base purification, derivatization, and analysis by GC–MS are illustrated in Fig.5.

For confirmation of LSD, the relative area abundances of the ions at m/z 293/395 and 253/395 of the unknown are compared with that of a standard. These two ion ratios were established by injecting an extracted standard (400 pg/ml) into the GC–MS system. In this experiment the LSD ratios for 293/395 and 253/395 were found to be 0.44 and 0.67, respectively. With a urine extract, the area ratio 253/395 of LSD of the specimen is approximately 27% higher (0.85) than that of the standard, indicating an impurity with m/z 253 coeluting with LSD. Re-extraction of the same specimen by solid–phase followed by acid–base purification provided ion ratios of the specimen within $\pm 10\%$ of that of the standard (Fig. 5). Fortunately, we have found the frequency of this interference to be no more than 6% of samples analyzed ($n=168$) with concentrations >400 pg/ml.

The procedure described herein is useful when applied to an exceedingly large number of samples. In routine analysis, liquid–liquid extraction was the method of choice. However, if interference observed, the extraction was repeated with solid-phase extraction followed by the liquid–liquid procedure. Most of the times, the interference was observed only in the m/z 253 ion chromatogram. For quantitation of LSD in urine, LAMPA was used as the internal standard. Due to isotopic spill over, [$^2H_3$]LSD was found unsuitable as the internal standard. Although the limit of detection is 29 pg/ml, considerable interferences were observed when the LSD concentration was less than 100 pg/ml. These interferences may be due to the presence of impurities in illicit

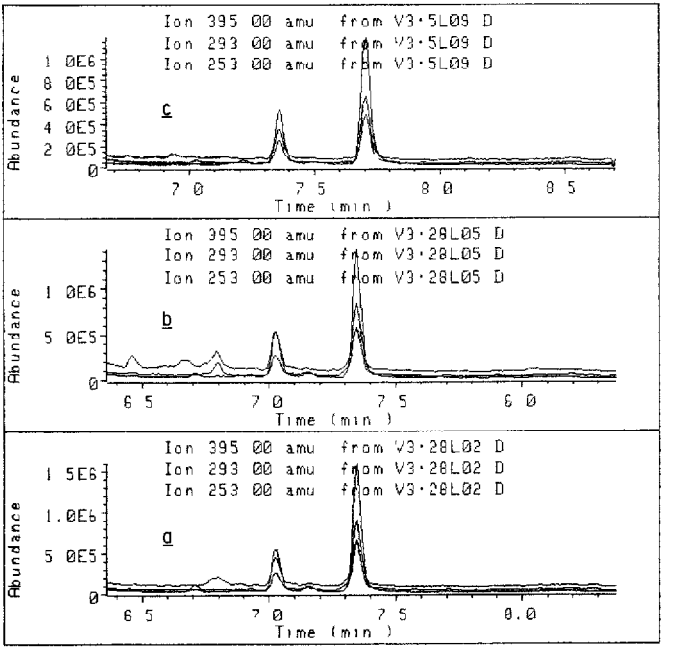


Fig. 5. Ion chromatogram of an extracted standard (400 pg/ml) and a specimen (501 pg/ml). Standard (a) and specimen (b) after acid–base purification, and specimen (c) after solid-phase and acid–base purification.

LSD preparation. In GC analysis, column conditioning was found to be important; otherwise considerable loss of sensitivity was observed.

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