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ANALYSIS OF LSD IN HUMAN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, FLUORESCENCE SPECTROSCOPY AND RADIOIMMUNOASSAY

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SUMMARY

A scheme of analysis is described in which the particular advantages of high-performance liquid chromatography (HPLC), fluorescence spectroscopy and radioimmunoassay (RIA) are exploited to the greatest effect. RIA affords a rapid and sensitive preliminary screening method, while the subsequent HPLC analysis using fluorimetric detection yields quantitative chromatographic evidence together with characteristic fluorescence spectra. Fractionation of samples by HPLC followed by RIA of the fractions gives further confirmation of the presence of LSD and its metabolites. The combined methodology has been applied to the analysis of LSD in body fluids for forensic and clinical purposes. Levels down to 0.5 ng of LSD per ml can be detected using the minimum of sample.

INTRODUCTION

The detection and measurement of LSD in biological fluids has presented great difficulty because of the low dose of the drug (1 μg/kg orally) and its extensive metabolism. Measurement of the fluorescence of LSD in organic solvent extracts of plasma can be used to detect down to 1 ng/ml plasma[1–3], but the presence of interfering fluorescent compounds drastically reduces the usefulness of this method for urine[4,5]. Moreover, the fluorescence spectrum of LSD is indistinguishable from that of many other ergot alkaloids. An improved method recently developed, involves high-performance liquid chromatography (HPLC) of diethyl ether extracts of serum or urine followed by fluorimetric detection[6]. By this means interfering fluorescent compounds are separated from LSD and an additional characteristic, retention volume, can be measured. Both the direct fluorescence and chromatographic methods require quite large volumes of sample (perhaps as much as 40 ml) to achieve the required sensitivity of 1 ng/ml.

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Several research groups have developed radioimmunoassays (RIAs) that have far greater sensitivity for LSD detection[7–11]. A feature of these RIAs is that LSD metabolites, and compounds with a structure very similar to LSD, cross-react significantly. The value obtained from the assay is consequently a compound figure which includes both the LSD level and a contribution from metabolites. Furthermore, relatively high levels of ergotamine, ergometrine and methysergide, such as might be present in drug overdose cases, cross-react sufficiently to indicate the apparent presence of LSD. In general, however, RIA has shown great promise as a routine screening procedure for LSD, but must be followed by other methods for confirmation.

The work described in this paper is a complete analytical scheme for the detection and measurement of LSD in biological fluids. The method employs a rapid preliminary screening by RIA to reject negative samples, followed by a quantitative analysis using HPLC in combination with fluorimetry and RIA. The scheme is applicable to forensic analysis where sample volumes are limited and where conclusive proof of identity must be obtained.

Because of the potential dangers of the drug, it was not possible to obtain human volunteers to take LSD. The results described in this paper were obtained from samples submitted by the police for analysis; in these cases the dose and time since ingestion were not known.

EXPERIMENTAL

Samples and reagents

Samples of blood, urine and stomach washings from people suspected of having taken LSD were obtained from the police through operational forensic science laboratories, and in two cases from hospital laboratories. The dose and time since ingestion were not known.

LSD tartrate and many ergot alkaloids were supplied by Sandoz (Feltham, Middlesex, Great Britain).

Procedure

The complete scheme of analysis is illustrated in Fig. 1. Samples of serum, urine or stomach washings were screened for the presence of LSD and its metabolites by RIA. Negative samples were discarded and positive samples were then analysed by reversed-phase HPLC using fluorimetric detection. A peak at the correct retention volume indicated the presence of LSD: this was confirmed by measurement of the fluorescence excitation and emission spectra of the component trapped in the fluorimeter flow-cell. The trapped material was then irradiated with UV light (320 nm) and the fluorescence spectra were re-scanned. Further confirmation of identity was obtained by chromatography of the sample on a silica column and by fractionation of an unextracted portion of the sample using the reversed-phase HPLC column. The fractions collected were examined individually by RIA.

Radioimmunoassay

The RIA used in this work was a modification of that previously reported[11]. The antiserum used was produced in a sheep immunised with LSD coupled to bovine serum albumin via the LSD indole nitrogen. Assay conditions were the same except

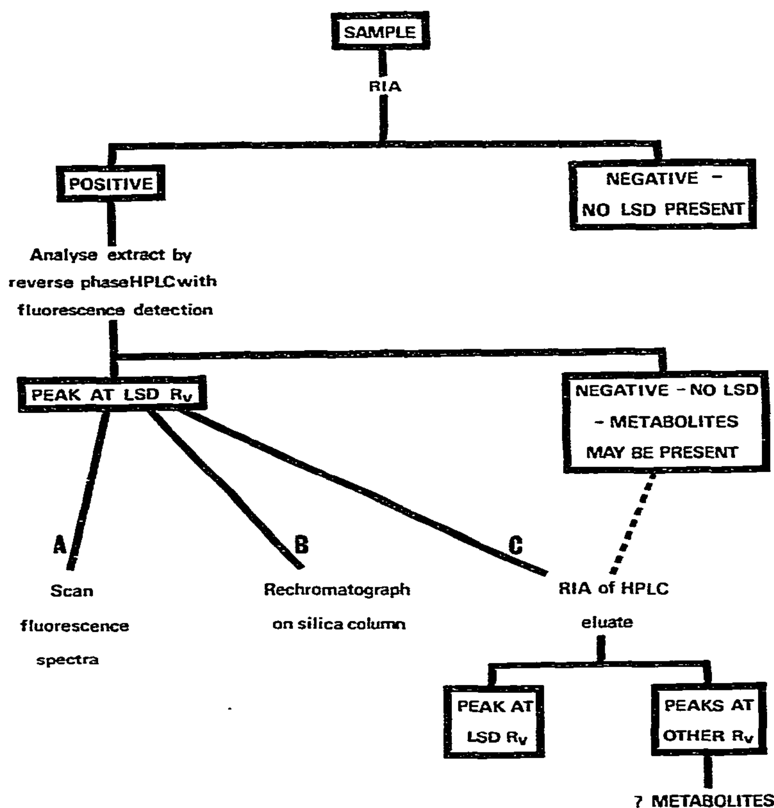

Fig. 1. Sequence of analyses for the detection of LSD in biological fluids. A, B and C are confirmatory procedures used when sufficient sample is available.

for the addition of ammonium sulphate solution to a final concentration of 1.2 *M* in the assay tube, which increased the efficiency of separation of free and bound LSD.

Extraction

Serum or urine was extracted by a method derived from that of Axelrod *et al.*[12]. A measured volume of body fluid was adjusted to pH 8.5 with sodium hydroxide solution (0.1 *M* aqueous) and saturated with sodium chloride. The mixture was then extracted with three volumes of *n*-heptane containing 2% isopentanol by gentle rolling in the dark in a stoppered test tube. A known volume of the organic layer was separated, evaporated to about 5 ml and back-extracted into a small known volume (*e.g.*, 200 μl) of hydrochloric acid (0.01 *M*).

High-performance liquid chromatography

A constant-flow pump (M-6000, Waters Assoc., Northwich, Great Britain) was used to deliver eluent to a column 10 cm $\times$ 4.6 mm I.D. slurry-packed with Spherisorb 5-ODS (Phase Separations, Clywd, Great Britain) or 15 cm $\times$ 4.6 mm I.D. slurry-packed with Spherisorb S5W (Phase Separations).

Eluent for the reversed-phase column (Spherisorb 5-ODS) consisted of

methanol (65%) and aqueous (0.025 *M*) disodium hydrogen phosphate (35%). The mixture was adjusted to pH 8.0 with 10% orthophosphoric acid. For the silica column (Spherisorb S5W) a mixture of methanol (60%) and aqueous (0.2 *M*) ammonium nitrate (40%) was employed. The eluent flow-rate was 1.0 ml/min which generated a back-pressure of 5.5 MPa and 4.1 MPa for the reversed-phase and silica columns, respectively.

Samples were introduced using a valve injector (Model U6K, Waters Assoc.), and eluted substances were detected using a spectro-fluorimeter (MPF-2A, Perkin-Elmer, Beaconsfield, Great Britain) equipped with a low-volume flow-cell (2 mm I.D.). The excitation and emission monochromators were set at 320 and 400 nm, respectively, each with a slit bandpass of 10 nm. The retention volumes of the ergot alkaloids and LSD derivatives were determined using fluorimetric detection, or for the non-fluorescent dihydro derivatives using a UV monitor set at 280 nm (CE 212, Cecil Instr., Cambridge, Great Britain). Eluent fractions were collected at 0.5-min intervals using an Ultrorac 7000 fraction collector (LKB, Croydon, Great Britain). Quantitation of LSD was performed by peak height measurement, using a calibration graph prepared by injection of known volumes of standard LSD solutions.

Fluorescence spectroscopy

A four-port switching valve (Phase Separations) was used to trap eluted material in the flow-cell and divert column eluent to waste. In this way, fluorescence excitation and emission spectra were measured on substances eluting from the column. To minimize photo-decomposition, a narrow excitation slit width and a rapid scan speed were used. The excitation shutter was kept closed except whilst scanning. The sample was then irradiated in the flow-cell (λ, 320 nm; slit, 40 nm) for a few minutes to destroy any LSD present and the excitation and emission spectra were re-scanned. The spectra were compared with those obtained for authentic LSD and for column eluent.

Fractionation of samples for RIA

Urine samples were adjusted to pH 8, centrifuged to remove particulate matter and up to 2.0 ml of urine was then injected directly onto the reversed-phase column. The fluorimeter was disconnected and twenty fractions of eluent were collected at 0.5-min intervals and evaporated to dryness under reduced pressure at room temperature. The fractions were reconstituted in 0.5 ml assay buffer for RIA.

Blood samples were fractionated in a similar fashion, but were first deproteinized to prevent protein from blocking the column. Serum or plasma was shaken with five volumes of methanol and centrifuged. The supernatant was decanted, evaporated to about 500 μl under a stream of nitrogen, diluted to 2.0 ml with water, centrifuged again and a measured volume injected on to the HPLC column.

RESULTS AND DISCUSSION

Radioimmunoassay

The characteristics of the RIA are not reported here in detail since they correspond closely to those previously described for this antiserum[11] (Antiserum I). The assay range was 0.5–64 ng of LSD per ml and only 0.2 ml of sample was required per

assay. In urine samples, levels of RIA cross-reactivity found were 2–175 ng/ml, plasma or serum levels were 0.5–4 ng/ml and stomach wash levels were 3–72 ng/ml.

LSD is extensively metabolized[12,13] and, from results obtained in the Rhesus monkey[14], it is likely that many LSD metabolites are present in the urine of LSD users. The identity of these metabolites has not been established, but it is probable that they cross-react in the RIA to some extent. This is consistent with the finding that RIA results for urine were often higher than the LSD level measured by HPLC. The cross-reactivities of some lysergic acid derivatives are given in Table I. Only lumi-LSD, lysergic acid monoethylamide and 2-oxo-LSD cross-reacted to a significant extent, although several other suspected LSD metabolites were not available to us.

TABLE I

COMPARISON OF CROSS-REACTIVITIES OF LSD AND RELATED COMPOUNDS

Compound	*Amount required for 50% depression of binding (pg)*
D-LSD	$2 \cdot 10^2$
L-LSD	10^7
Iso-LSD	10^7
D-Lysergamide	10^7
D-Lysergic acid	10^7
Iso-lysergic acid	10^7
Lumi-LSD	$9 \cdot 10^2$
2-Oxo-LSD	10^4
D-Lysergic acid monoethylamide	$3 \cdot 10^4$
Ergometrine	10^7
Methylergometrine	10^7
Dihydroergotamine	10^7
Ergocornine	10^7
Ergocristine	10^7
Ergocryptine	10^7
Ergotoxine	10^7
Methysergide	10^7

Extraction

The extraction procedure described gave recoveries of 60 and 70%, respectively, from urine samples spiked with 10 and 20 ng LSD per ml. Spiked serum extracted in a similar fashion gave recoveries of up to 90%. However, at levels of LSD below 10 ng/ml, a low and variable extraction efficiency was observed. The use of silanised glassware has not reduced this variability.

Blood samples with high LSD levels may be simply deproteinized (as for HPLC–RIA fractionation) and injected directly. Recovery of LSD by this procedure was up to 80%. In general, however, extraction is preferable since this removes much interfering fluorescent material.

The extraction method used here has advantages over that used by Christie *et al.*[6] who found iso-LSD as well as LSD in body fluid samples. Using the present method, no iso-LSD has been detected and it is concluded that iso-LSD is produced as an artifact of the previous extraction procedure.

Chromatography and fluorescence spectroscopy

Retention volumes for 24 ergot alkaloids and lysergic acid derivatives are given in Table II for both silica and reversed-phase columns. Fig. 2 illustrates the degree of correlation between retention on the two columns. Although LSD is unequivocally resolved on either system, only the ODS column adequately separates ergot alkaloids from each other. This was necessary in a case involving ergotamine tartrate which was submitted as a suspected LSD poisoning. The reversed-phase column was therefore used routinely. The LSD levels found in samples from suspected LSD users were: in serum or plasma 1.6–5 ng/ml, in urine 0.8–54 ng/ml and in stomach wash 5–11 ng/ml. A chromatogram of a plasma extract containing LSD is shown in Fig. 3.

TABLE II

CHROMATOGRAPHIC RETENTION OF ERGOT ALKALOIDS AND LSD

Compound	*Retention (relative to LSD)*	
	Spherisorb 5-ODS	*Spherisorb S5W*
D-LSD	1.00 (4.9 ml)	1.00 (3.4 ml)
Iso-LSD	2.04	1.28
D-Lysergamide	0.38	2.76
D-Lysergic acid	0.23	0.65
Lysergol	0.61	0.76
Lumi-LSD*	0.53	0.85
	1.15	
	1.68	
2-Oxo-LSD*	0.65	0.59
D-Lysergic acid monoethylamide	0.52	0.81
Dihydroergocornine	2.60	0.74
Dihydroergocristine	3.68	0.76
Dihydroergocryptine	2.63	0.71
Dihydroergotamine	2.48	0.81
Ergocornine	1.39	0.79
Ergocristine	2.31	0.81
Ergocryptine	1.86	0.78
Ergocryptinine	2.08	0.72
Ergometrine	0.26	0.79
Ergometrinine	0.47	0.79
Ergosine	1.22	0.78
Ergosinine	1.22	0.75
Ergotamine	1.57	0.81
Ergothioneine	>6	>2.35
Methysergide	0.65	0.85
Methylergometrine	0.39	0.76

* Retention volumes were corrected for sample size when large volumes were injected.

The limit of detection of LSD by this method depends mainly on the amount of interfering substance in the sample. Less than 100 pg of LSD can be measured in buffer solutions, but in biological fluids levels below 1 ng/ml presented difficulties, particularly in the case of urine.

A number of fluorescent compounds, including ergot and other alkaloids have been considered for use as internal standards. Ergosine in particular had good chro-

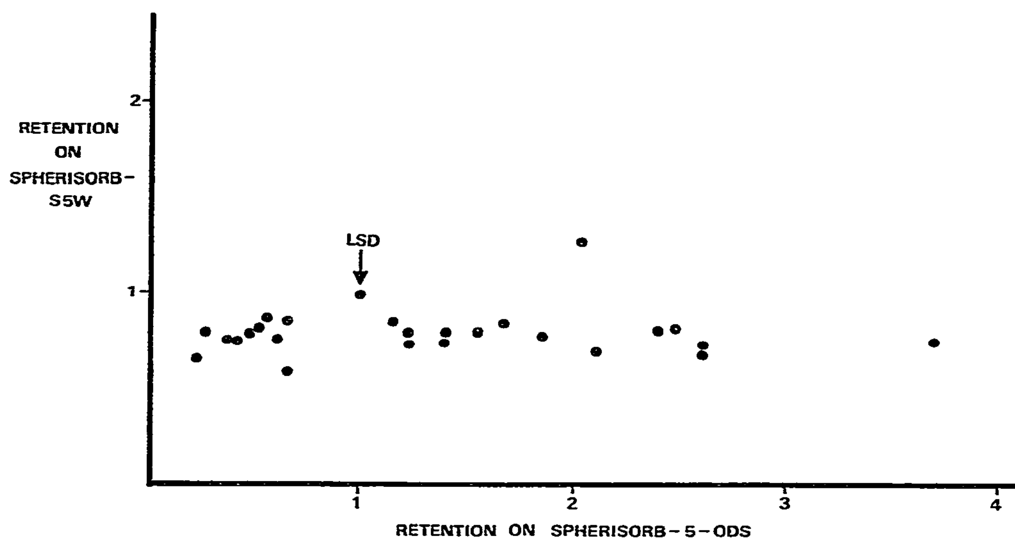

Fig. 2. Correlation of retention (relative to LSD) for ergot alkaloids on Spherisorb 5-ODS and Spherisorb S5W columns.

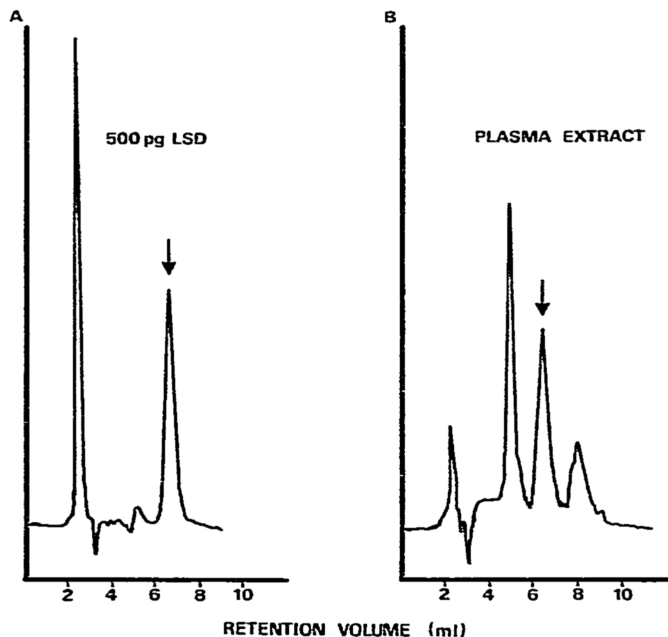

Fig. 3. Chromatogram (reversed-phase column) of an extract of plasma containing LSD (Table III, case 3). A, Authentic LSD (500 pg); B, plasma extract (equivalent to 0.1 ml plasma). Arrows indicate the peaks due to LSD.

matographic and extraction characteristics but was found to be unstable in acid solution. No satisfactory internal standard was found and quantitation was achieved by peak height measurement and reference to a calibration graph.

The measurement of fluorescence spectra of eluted components adds greatly to the specificity of the method. Other ergot alkaloids that have similar fluorescence spectra are separated from LSD on the HPLC column and thus do not interfere, while non-ergot fluorescing substances are unlikely to have the same fluorescence decay characteristics upon irradiation with UV light to produce the non-fluorescent lumi-LSD[15–17]. In practice, however, it was difficult to obtain spectra on less than 5 ng LSD (injected on-column) due to the background fluorescence of the fused silica used to construct the flowcell. Fig. 4 shows fluorescence excitation and emission spectra measured in this way on a component of an extract of urine from a suspected LSD user. The non-fluorescent dihydro derivatives, which could have interfered in the RIA, were excluded by the HPLC procedure.

Fractionation of samples for RIA

Urine samples in which LSD had been detected by HPLC–fluorescence were

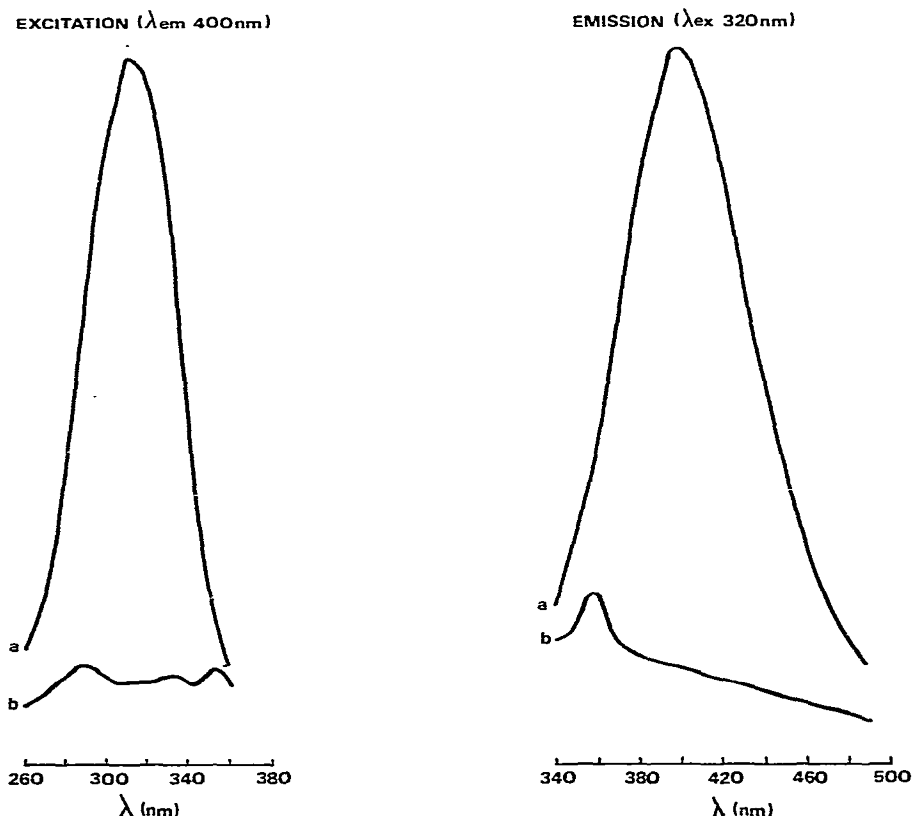

Fig. 4. Fluorescence spectra of a trapped chromatographic peak from an extract of urine from a suspected LSD user. a, Component with the retention volume of LSD (Spherisorb 5-ODS); b, the same component after irradiation for 5 min (λ = 320 nm; slit, 40 nm).

fractionated (without extraction) and the fractions assayed by RIA. In each case a peak of reactive material was found at the retention volume of LSD. Each sample also contained reacting material which was eluted before LSD in one or more peaks. A typical radioimmunochromatogram is shown in Fig. 5. Samples from subjects known not to have taken LSD did not show appreciable cross-reactivity. Deproteinized blood samples and stomach washes were fractionated in the same way. These all gave a single peak of reactivity at the retention volume of LSD: the earlier peaks seen in the radioimmunochromatograms of urine were absent.

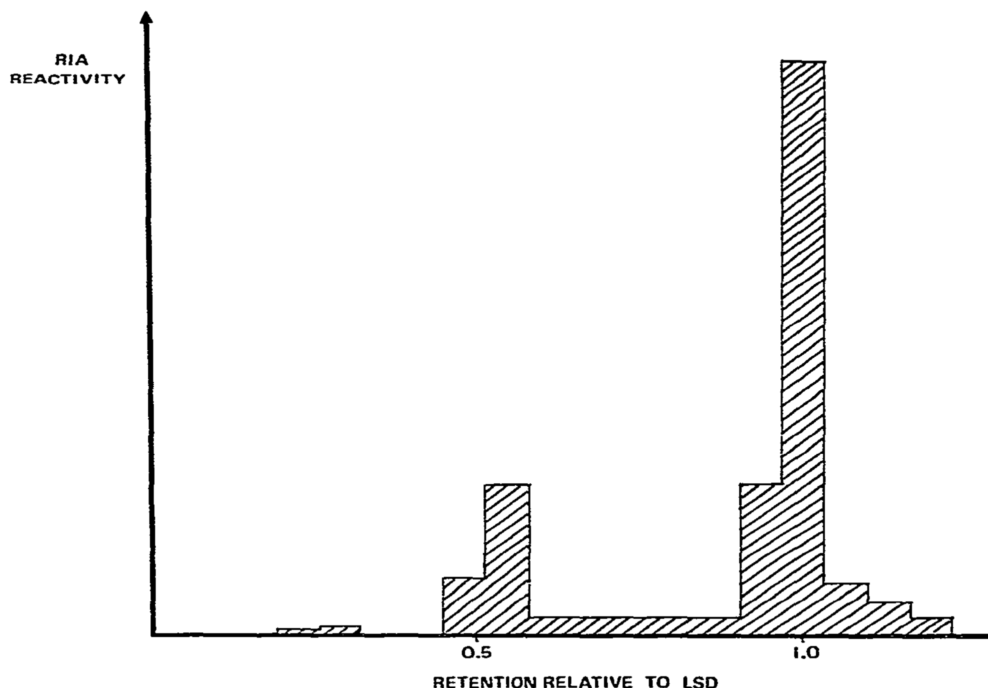

Fig. 5. Radioimmunochromatogram of urine from a person suspected to have taken LSD.

The reversed-phase system was particularly advantageous for these separations since elution is in order of lipid solubility[18] and LSD metabolites were expected to elute before the parent drug. Furthermore, large (2 ml) quantities of urine can be loaded onto the column with no deleterious effect on the separation.

Examination of the radioimmunochromatogram (Fig. 5) shows why the initial RIA results for urine samples were higher than the result obtained by HPLC for LSD alone. The peak eluting early in the chromatogram is thought to be due to LSD metabolites and is not observed in chromatograms of stomach washes or some serum samples, where metabolites are expected to be absent, or present only in low concentrations. Since the specificity of RIA is much greater than that of fluorescence, the detection of a peak of RIA-reactive material at the retention volume of LSD increases the certainty of identification.

Table III illustrates the application of the analytical method to samples from seven recent cases.

TABLE III

SUMMARY OF RESULTS FROM SEVEN CASES SUBMITTED FOR LSD ANALYSIS

R_v = retention volume; RR_v = retention volume relative to LSD.

Case	Origin	Sample	Direct RIA	HPLC–fluorescence	HPLC–RIA	Comment
1	A girl, thought to have taken an LSD tablet became unconscious and died approx. 44 h later	Stomach wash taken 12 h after ingestion	4 ng/ml	Direct injection: 5 ng/ml. Excitation and emission spectra indicated LSD		The initial RIA and HPLC analysis gave similar values since no LSD metabolites were present
2	Subject believed to have taken LSD at a drug party	Urine	15 ng/ml	Urine extract: 3 ng/ml	Major peak has R_v corresponding with LSD	The high level of LSD activity found by direct RIA was probably due to LSD metabolites
3	A man arrested for criminal damage claimed to be high on LSD	Blood taken 1 h after arrest	4 ng/ml	Plasma extract: 5 ng/ml	Single major peak with R_v corresponding to LSD	The absence of metabolite peaks in the HPLC–RIA and the close agreement of the initial RIA and HPLC–fluorescence results suggest that the LSD metabolite level was low
4	5-year-old child admitted to hospital with acute psychosis	Urine taken 4 days after suspected LSD ingestion	2 ng/ml	Urine extract: 0.8 ng/ml	Results inconclusive due to low level of LSD	

5	Man who admitted having taken LSD	Stomach wash (3.0 ml was submitted)	72 ng/ml	Direct injection: 11 ng/ml	Strong cross-reactivity at R_t of LSD	
6	12-year-old child admitted to hospital with symptoms of hyperactivity	Serum Urine Stomach wash.	2 ng/ml Negative Negative	No serum remained after RIA. Urine extract: peaks at RR_v 1.1 and 1.5; stomach wash extract: peak at RR_v 1.5		The peak at RR_v 1.5 corresponded to ergotamine and that at RR_v 1.1 was found to be a breakdown product of ergotamine in strong acid. No LSD was present, but ergotamine levels in urine and stomach wash were 10 ng/ml and 620 ng/ml, respectively. The child had access to a number of drugs including ergotamine tartrate
7	A baby (9 months) ingested a number of LSD microdots. Seven tablets were recovered from its vomit	Urine Blood (haemolysed)	175 ng/ml 0.5 ng/ml	Urine extract: 54 ng/ml; serum extract: 1.6 ng/ml. Fluorescence spectra were identical with LSD	Strong cross-reactivity at the R_v of LSD and at an earlier metabolite peak	The presence of LSD in urine was confirmed by mass spectrometry of the HPLC fraction (m/e 323, 221). The comparatively low serum level is difficult to explain.

CONCLUSIONS

The scheme of analysis described exploits the particular advantages of each method to the greatest effect. Thus, although RIA is not sufficiently specific to be used alone, the rapid initial screening by RIA allows negative samples to be rejected. The presence and quantity of LSD in RIA-positive samples can then be confirmed by HPLC with fluorimetric detection, followed by fluorescence spectroscopy, HPLC–RIA and HPLC on silica if sufficient sample is available, depending upon the degree of certainty of identification required.

The overall analytical methodology, using RIA and HPLC separately and in combination affords overwhelming evidence for the presence of LSD. In negative cases, however, the expenditure of effort is minimal, and only 0.2 ml of the sample is consumed.

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