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ACKNOWLEDGMENTS AND ADDRESSES

Radioimmunoassay for Lysergide (LSD) in Illicit Drugs and Biological Fluids

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Abstract ☐ A simple and convenient radioimmunoassay system was developed for the qualitative detection and quantitative determination of lysergide (LSD) in illicit drugs, human plasma, serum, or urine. The method utilizes a commercially available tritiated lysergide and specific antiserum, which can be obtained in large quantities from sheep after immunization with a lysergic acid–human serum albumin conjugate. Quantities of lysergide as low as 1 ng./ml. can be determined in plasma, serum, or urine, and lower levels can be detected qualitatively. The sensitivity of the assay is limited by the specific activity of the commercially available tritiated compound. Antibody cross-reactivity and, thus, interference in the assay were observed with other highly similar ergot alkaloids, such as ergonovine, methylergonovine, and ergotamine, but not with simpler indole structures.

Keyphrases ☐ Lysergide—radioimmunoassay of illicit drugs and biological fluids ☐ LSD—radioimmunoassay of illicit drugs and biological fluids ☐ Radioimmunoassay—lysergide (LSD) in illicit drugs and biological fluids ☐ Hallucinogens—radioimmunoassay of lysergide (LSD) in illicit drugs and biological fluids

The qualitative detection and quantitative determination of lysergide (LSD) in illicit drugs and in biological fluids have been a major interest in recent years because of the medical and social implications attendant its use. Chemical, spectral, and chromatographic methods for the determination of lysergide (IV*b*, Scheme I) were reviewed previously (1, 2), and it has been generally agreed that certain fundamental advances in micro-

analysis are required to detect and determine with confidence the nanogram or picogram levels, for example, of IV*b* or metabolites fleetingly present in urine or plasma after ingestion of the drug (1). A radioimmunoassay system (3) for IV*b* appears promising in this regard and was reported by one research group (4). This method (4) utilized rabbit antiserum to a lysergic acid–polylysine–hemocyanin conjugate, along with a specially synthesized lysergic acid–amino acid copolymer containing ^{125}I, and a double-antibody technique for the separation of free and bound IV*b* in the assay. Although details have not yet been reported, a commercial kit appears to be based upon similar methodology[1].

This paper reports the development and evaluation of a workable radioimmunoassay system for IV*b*. The system promises to be useful in the assay of illicit drug products or samples of biological fluids. The assay utilizes a commercially available tritiated IV*b* and specific antiserum which was obtained in large amounts from sheep after immunization with a direct covalent conjugate of lysergic acid (I) and human serum albumin (II). Separation of free and bound forms of IV*b* is achieved by the adsorption of unbound drug on dex-
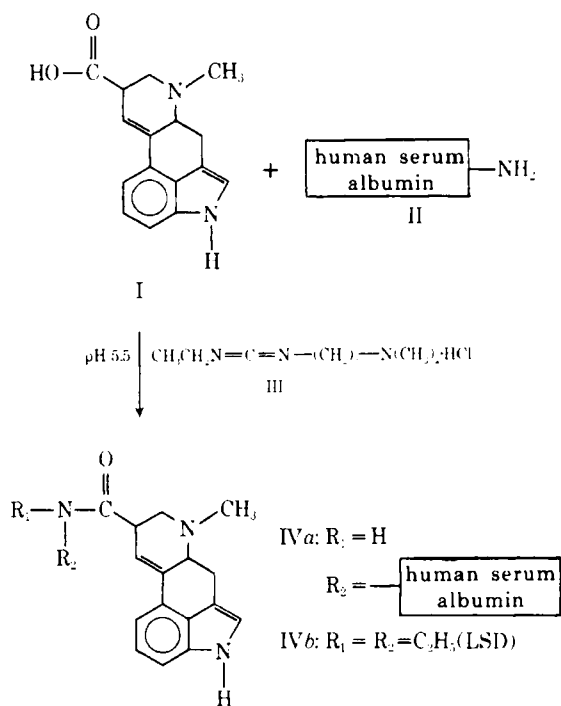
Scheme I—*Covalent coupling of lysergic acid (I) to human serum albumin (II) by a water-soluble carbodiimide reagent (III), producing antigen (IVa)*

tran-coated charcoal. To date, a sensitivity in the quantitative sense of about 1 ng. has been achieved using the commercially available tritiated IVb. Qualitatively, picogram levels may be readily detected.

EXPERIMENTAL

Materials—The following were used: human serum albumin[2] (crystalline, B grade), a coupling reagent N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride[3], Freund's complete adjuvant[4], lysergide[5] (0.1 mg./ml. in ethanol), dextran[6] (radioimmunoassay grade), charcoal[6] (radioimmunoassay grade), 5-hydroxytryptamine (serotonin) creatinine sulfate complex[7], 5-hydroxy-L-tryptophan[7], tryptamine hydrochloride[7], N-methyltryptamine[7], 5-methoxy-N,N-dimethyltryptamine[7], octoxynol[8], 2,5-diphenyloxazole[9], and 2,2-phenylenebis(5-phenyloxazole)[9].

The D-lysergic acid[7] (I) employed in the conjugation to albumin was found to be homogeneous by TLC [silica gel, R_f 0.5, butanol–acetic acid–water (3:1:1)]. The radiolabeled lysergide[10] was generally labeled with tritium and had a specific activity of 1.9 c./mmole. General labeling refers to a random, *i.e.*, nonuniform and undetermined, distribution of tritium at various nonexchangeable positions.

Coupling of Lysergic Acid to Human Serum Albumin—To a solution of 24 mg. of lysergic acid (I) in 20.0 ml. of water containing 10 % by volume ethanol was added 3.0 ml. of water containing 30 mg. human serum albumin (II) (Scheme I). To this was added in one portion a solution of 34 mg. N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (III) in 2.0 ml. water. The pH of the solution was adjusted from 6.01 to 5.50 with 1 N HCl, and the mixture was stirred magnetically in a dark, stoppered vial at 25° for 42 hr. The reaction mixture was then placed in a dialysis bag and exhaustively dialyzed at about 5° for 1 week against distilled

water, 4 M KCl, a buffer of pH 7.4 (0.15 M NaCl and 0.01 M phosphate), and finally water. All dialysates were measured in volume and absorbances were measured at 308 nm. (pH 7.4 buffer), the λ_{max} for lysergic acid. From absorbance measurements, it was calculated that about 5–6 mg. of lysergic acid was coupled to 30 mg. of human serum albumin. This corresponds to a content of about 40–50 moles of lysergic acid/mole of albumin in the conjugate. This value may be subject to a rather large error due to the rather low solubility of lysergic acid in the dialysate mixtures, with the possibility of precipitation, although this could not be visually observed. After dialysis, the contents of the bag were lyophilized to a fluffy off-white powder (IVa).

Preparation of Lysergic Acid–Human Serum Albumin Conjugate for Injection into Sheep—A solution of 4.0 mg. conjugate in 0.5 ml. pH 7.4 buffer (0.15 M NaCl and 0.01 M phosphate) was absorbed slowly into 0.8 g. partially dehydrated 7 % polyacrylamide gel, which had been shredded by passing through a No. 25-gauge hypodermic needle from a syringe. The final product was desiccated to approximately 0.8 g. and placed in a 5-ml. disposable plastic syringe. Then Freund's complete adjuvant was drawn in through the needle to a volume of 4.0 ml. Immediately after vigorous shaking to resuspend the shredded gel, the mixture was injected into a sheep, using approximately 1 ml. at each of four intramuscular injection sites in both shoulders and flanks. A second sheep was injected similarly with a thoroughly emulsified mixture of 4.0 mg. of the conjugate in 1.8 ml. buffer and 2.2 ml. Freund's complete adjuvant.

Bleeding and Booster Immunization Schedule—Blood samples were collected from each animal initially, immediately prior to each booster immunization, and approximately 7 10 days after the booster. Boosters were composed of 1.0 mg. of conjugate in 4.0 ml. adjuvant–gel or emulsified adjuvant buffer and were given at 4, 6, 10, and 36 weeks after the initial injection of conjugate. Clotted blood was centrifuged after clot retraction, and the serum was decanted and kept frozen (-10) after the addition of 0.05 % sodium azide.

Radioimmunoassay Procedure: Determination of Standard Radioimmunoassay Curve for IVb—All samples each day were run in duplicate to obtain two consistent figures. In a typical experiment, 0.8 ml. pH 7.4 buffer (0.15 M NaCl and 0.01 M sodium phosphate) was added to each of 22 5-ml. polystyrene test tubes (12 $\times$ 75 mm.); that is, 11 sets of duplicates were run. Then 0.2 ml. buffer (or buffer containing test compound) or plasma, serum, or urine was added to make a final volume of 1.0 ml. To tubes containing plasma, 50 μl. of 0.2 N sodium citrate solution was also added. Next was added 10 μl. of 95 % ethanol containing 1.0 ng. of randomly (nonuniformly) labeled ^{3}H-IVb (specific activity 1.9 c./mmole). This corresponds to about 13,000 d.p.m./tube or about 3000–4000 c.p.m. at the normal observed counting efficiency of 25 30 %.

To obtain a standard calibration curve, 0–25-ng. quantities of cold IVb in a maximum of 100 μl. of 95 % ethanol were added to selected tubes. Again to desired tubes was added 10 μl. of diluted or undiluted antiserum. After vigorous stirring for 5 sec. on a mixer (Vortex), the tubes were allowed to stand at room temperature for exactly 2 hr. After chilling to 0 for 15 min. in ice, 0.5 ml. of cold and well-mixed dextran-coated charcoal suspension[11] was added to each tube and the tube contents were mixed thoroughly once again. The tubes were then incubated for 15 min. at 0 and centrifuged in the cold (4) for exactly 5 min. (5000 r.p.m.). Then the supernate was decanted carefully into 10 ml. of scintillation counting solution containing toluene (2 l.), octoxynol[8] (1 l.), 2,5-diphenyloxazole (12 g.), and 2,2-phenylenebis(5-phenyloxazole) (300 mg.). The samples were then counted[12].

Typical data on the composition of incubation mixtures, observed counts for a 10-min. period, and percent bound IVb are summarized in Table I. The channels ratio method was employed to determine counting efficiency, disintegrations per minute, and final percent binding figures. Figure 2 shows typical standard immunoassay curves obtained by plotting data of Table I or similar data. Aside from counting time, the entire procedure can be completed in about 4 hr. and involves only about 2 hr. of actual working time.

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[2] Calbiochem Corp.
[3] Aldrich Chemical Co.
[4] Difco Labs.
[5] Sandoz Corp., obtained from Dr. M. Braude, Center for the Studies of Narcotics and Drug Abuse, National Institute of Mental Health.
[6] Schwarz/Mann.
[7] Sigma Chemical Co.
[8] Triton X-100, Fisher Chemical.
[9] Fisher Chemical.
[10] New England Nuclear Corp.

[11] The charcoal suspension was prepared immediately prior to use by the dilution of 2 ml. of a stock suspension with 18 ml. buffer, the stock suspension containing 2.5 g. charcoal and 62.5 mg. dextran in 25 ml. buffer.
[12] Packard model 3320 scintillation spectrometer.

Table I—Data for Typical Determinations of Standard Lysergide Radioimmunoassay Curves at Two Antiserum Dilutions[a]

Tube Number	Lysergide (IVb) Standard, ng.	Net Activity (counts/10 min., Corrected for Quenching and Background)	Percent Bound Lysergide (IVb)
		Final Dilution of Antiserum = 1:100	
1	0	1,260	3.8
2	0	28,780; 28,730	86.8; 86.8
3	1.0	27,558; 28,181	84.2; 83.8
4	2.0	26,434; 25.088	79.5; 72.5
5	3.0	22,029; 23,982	65.9; 69.4
6	5.0	19,032; 19,763	54.8; 58.1
7	7.0	16,419	45.7
8	10.0	12,921; 12,718	38.1; 35.8
9	15.0	10,409; 10,524	30.2; 30.0
10	25.0	6,689; 6,161	21.8; 18.2
11	0	39,763; 38,225	100.0; 99.3
		Final Dilution of Antiserum = 1:400	
1	0	1,123; 1,150	3.7; 3.7
2	0	23,990; 24,370	67.8; 67.5
3	1.0	18,747; 18,427	53.5; 53.2
4	2.0	12,580; 12,110	36.2; 34.4
5	3.0	9,723; 9,403	27.8; 25.8
6	4.0	7,711; 7,799	22.8; 22.4
7	5.0	7,153; 6,023	19.6; 19.8
8	6.0	5,956; 5,973	17.0; 16.0
9	8.0	4,744; 4,783	13.8; 13.4
10	10.0	4,142	11.3
11	0	35,219; 35,468	100.0; 97.2

[a] The data are for samples containing pooled normal human serum (see text). Samples designated 1 contained no antiserum. All samples contained 0.8 ml. buffer, 0.2 ml. serum, and 10 μl. ethanol containing 1.0 ng. ³H-IVb. Duplicate samples were assayed as indicated by the two figures for counts of radioactivity. The counting channel data only are shown. No dextran-coated charcoal was added to Tube 11; this, therefore, represents a standard of total counts added.

RESULTS AND DISCUSSION

As shown in Scheme I and as outlined in detail in the *Experimental* section, lysergic acid (I) was covalently coupled directly to human serum albumin (II) by the water-soluble carbodiimide derivative N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (III). The conjugate (IVa), which presumably contains lysergic acid residues coupled primarily to side-chain amino groups of lysine, thus contains all of the structural elements of IVb except for the two amide ethyl groupings. Calculations based upon the UV absorption maximum at 308 nm. for lysergic acid indicated binding of 40-50 moles of lysergic acid/mole of human serum albumin. Since the lysine content of this protein is about 58 residues/mole, this figure seems rather high. An abnormally high value may result from the rather low solubility of lysergic acid in aqueous buffers (filtrates from the coupling reaction), since the quantity coupled was calculated by difference.

Injection of the purified conjugate (4 mg.) in polyacrylamide gel and adjuvant at each of four injection sites in one sheep, followed by a booster injection with 1 mg. of conjugate after 4 weeks, resulted in an antiserum even after only 6 weeks which was capable of binding 35-45% of 1 ng. of radioactive IVb in 1 ml. of incubation mixture at a final dilution of 1:1000 and 75-85% at dilutions of 1:100. Figures 1 and 2 show percentage binding figures at various dilutions for serum obtained after the first booster immunization (6 weeks total). The binding capacity of antiserum taken from this sheep after the third and fourth boosters at 10 and 36 weeks appeared to be only slightly higher than that obtained at the 6-week period. The binding capacities of antiserum obtained from the second sheep (injected with antigen in adjuvant only, with no polyacrylamide gel) were slightly lower than those of the first sheep but were equally adequate for use in assays at the same dilutions (Figs. 1 and 2). The antiserums appear to have completely retained binding capacity after 18 months storage at - 10°. Three typical standard calibration curves are reproduced in Fig. 2. Displacement of the labeled IVb (1.0 ng./ml.) by nonradioactive IVb from antiserum of final dilution 1:100 began at a concentration of about 1 ng./ml. and dropped off most dramatically between 2 and about 30 ng./ml. Final serum dilutions of 1:400 resulted in a reproducible standard
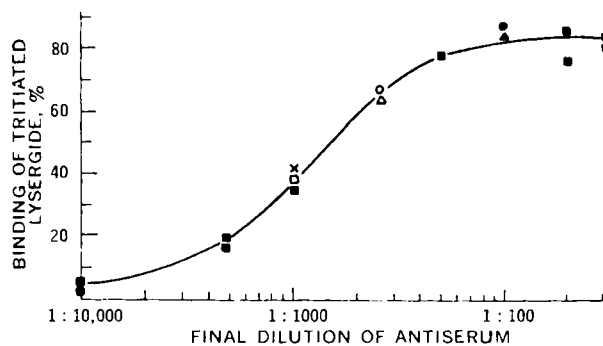

Figure 1—*Binding of tritiated lysergide (1.0 ng./ml.) by antiserum to a lysergic acid-human serum albumin conjugate. Key:* ●▲○△□ X, *data as given in Table I and Fig. 2; and* ■, *additional experimental results.*

curve between 1 and 10 ng./ml. If conditions of the assay were adhered to rigidly, the standard curve varied only very slightly; however, a series of standards was always incorporated into each day's work.

The addition of up to 20% (0.2 ml./ml.) normal sheep serum, pooled normal human serum or normal human plasma, or human urine to the assay tubes did not appear to affect the shape or position of the immunoassay curves significantly. Blanks in the absence of antiserum amounted to 3-5% in all cases, and counts corresponding to this amount would have to be subtracted from unknowns. However, in all tubes containing human plasma, 50 μl. of 0.2 N sodium citrate was added to prevent clotting, which appeared to be triggered by the addition of the sheep antiserum. Observed binding appeared to be independent of time between 0.5 and 3 hr.; however, 2.0 hr. was employed routinely. Temperature and incubation time proved to be quite critical during adsorption of the free IVb to dextran-coated charcoal, and a 15-min. incubation time at 0° followed by 5 min. of centrifugation at 4° was optimum.

When using the assay procedure as described, the practical lower limit for quantitative determination of IVb appears to be about 1 ng. This limit is determined by the relatively low specific activity (1.9 c./mmole) of the labeled IVb available commercially; material of higher specific activity is too unstable for storage[13]. The use of smaller amounts of labeled IVb enabled detection and rough estimation of much smaller quantities of IVb, but the uncertainty arising from the decreased count measured increased dramatically.
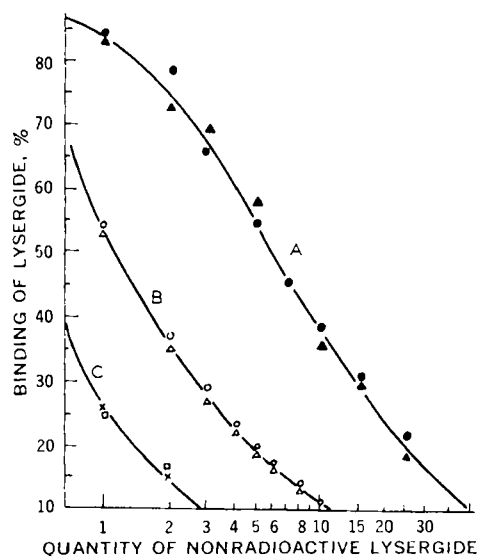

Figure 2—*Typical standard radioimmunoassay curves for the determination of lysergide at three antiserum dilutions (A, 1:100; B, 1:400; and C, 1:1000). Key:* ●▲, OA, *and* XC *indicate duplicate assay results. The curves shown have not been mathematically fit.*

13 A. Petty, New England Nuclear Corp., personal communication.

Several compounds structurally related to IV*b* were examined for their ability to cross-react with the antiserum obtained, *i.e.*, their ability to displace radiolabeled IV*b* from antibody binding sites. Antibodies were unable to distinguish among IV*b*, I, and the structurally similar ergot alkaloids ergonovine and methylergonovine. Ergotamine also exhibited cross-reactivity, but at approximately 10-fold higher concentrations. The following indoles did not exhibit any ability to displace radiolabeled IV*b* from antibody binding sites at concentrations of at least 10 mcg./ml. of incubation mixture: tryptamine, *N*-methyltryptamine, 5-methoxydimethyltryptamine, 5-hydroxytryptophan, and 5-hydroxytryptamine (serotonin). No exhaustive study of cross-reactivity was completed; however, these results compare in general with those reported by Vunakis *et al.* (4). Judging from information supplied in a commercial kit[1], antiserum produced by other methods may exhibit somewhat greater specificity. One outstanding example appears to be that these authors report little cross-reactivity with I at concentration levels similar to IV*b*, while these two compounds appear essentially indistinguishable using our antiserum.

SUMMARY

A quick and convenient radioimmunoassay system was developed for the quantitative determination of lysergide (IV*b*) in drug products, human plasma, serum, or urine at levels as low as 1 ng. or for its qualitative detection at picogram levels. Liter quantities of antiserum were obtained since sheep were employed for the production of antibodies. An advantage of the method is in its utilization of commercially available tritiated IV*b*; however, the low specific activity of this material also places a limit upon the sensitivity. The method also has the advantage of not requiring a second antibody technique for the separation of free and bound IV*b* but is based upon adsorption of free IV*b* to dextran-coated charcoal. Current efforts are directed toward the acquisition of IV*b* of higher specific activity and the development of methods utilizing immobilized antibodies for efficiently concentrating IV*b* in biological samples so that the quantitative assay may be extended to the picogram levels required in the study of the absorption, distribution, metabolism, and excretion of IV*b*. However, even in its current state the method promises to be of considerable utility in the assay of illicit drug samples and in animal investigations where larger quantities of lysergide IV*b* are employed.

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ACKNOWLEDGMENTS AND ADDRESSES

Identification of a Blood Metabolite of Methsuximide by GLC-Mass Spectrometry

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Abstract ☐ The chemical structure of a prominent methsuximide metabolite was determined by combined GLC mass spectrometry. The experimental data indicate that the metabolite is an *N*-demethylated methsuximide. A simple GLC method is described for simultaneous estimation of methsuximide and its metabolite in blood samples.

Keyphrases ☐ Methsuximide—identification of *N*-demethylated metabolite in blood, man and rabbits, GLC-mass spectroscopy ☐ GLC-mass spectroscopy—identification, methsuximide *N*-demethylated metabolite in blood ☐ GLC—analysis, methsuximide and *N*-demethylated metabolite in blood

An important aspect in the treatment of patients with epilepsy is the determination of an effective and safe dosage schedule of anticonvulsant drugs. The estimation of these drugs in physiological fluids, particularly in blood, provides useful metabolic information for practitioners. Many drugs used are actively metabolized *in vivo* (1-3), and the identification and quantitation of these metabolites are of considerable clinical importance.

A general procedure for the determination of anticonvulsants in blood was recently described (4). When the method was applied to patients who had been receiving methsuximide (*N*,2-dimethyl-2-phenylsuccinimide), it became apparent that the drug had been rapidly metabolized both in humans and in rabbits and that the major metabolite had GLC characteristics different than the parent drug.

The purpose of this investigation was to determine the chemical structure of the methsuximide metabolite. The GLC properties of both the human and animal