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Stereospecific Radioimmunoassays for *l*-Ephedrine and *d*-Ephedrine in Human Plasma

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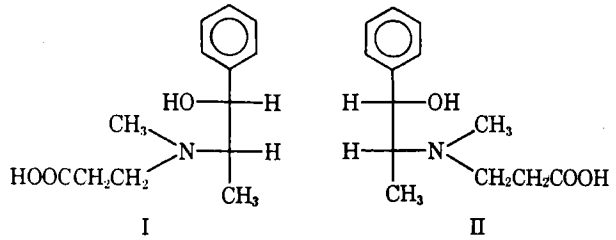
Abstract □ Haptens were prepared by the reaction of *d*-ephedrine or *l*-ephedrine with methyl acrylate and subsequent alkaline hydrolysis of the methyl ester groups. The haptens were coupled to bovine serum albumin by a mixed anhydride method, and the resulting drug–protein conjugates were used to immunize rabbits. Antiserum raised to these conjugates were highly stereospecific. Neither antiserum cross-reacted with the optical antipode of its substrate nor with racemic pseudoephedrine. Separate radioimmunoassays (RIAs), developed for *d*-ephedrine and *l*-ephedrine, were used to measure the concentrations of the enantiomers of ephedrine in the blood of two volunteers dosed with racemic ephedrine. The RIAs were validated by comparing the sum of the concentrations of the enantiomers, determined by RIA, with total ephedrine concentrations determined by a nonstereoselective GLC–ECD method.

Keyphrases □ Ephedrine—stereospecific radioimmunoassay using (*d*) and (*l*) antisera □ Radioimmunoassay—for *d*- and *l*-ephedrine, stereospecific □ Antisera—for *d*- and *l*-ephedrine, cross-reactivity, use in stereospecific radioimmunoassays

2-Methylamino-1-phenylpropanol has two chiral centers which give rise to four optical isomers. Racemic ephedrine is a mixture of the erythro pair of diastereomers (*l*), (1*R*,2*S*) and (*d*), (1*S*,2*R*), while the threo pair of diastereomers, (*l*), (1*S*,2*S*) and (*d*), (1*R*,2*R*) is called pseudoephedrine (1, 2). The four optical isomers differ from each other in their pharmacological activities (3–7) and rates of metabolism (8–13).

Preparations containing *l*- or *dl*-ephedrine are used for a wide variety of therapeutic applications, such as the treatment of nasal congestion in colds and allergic rhinitis, the treatment of orthostatic hypotension, as mydriatics, and as prophylactics against asthma attacks, urinary incontinence, and motion sickness (14). Of the published analytical methods for ephedrine (15–21), only GLC–ECD (22, 23) and GLC–MS (24) are applicable to the measurement of therapeutic concentrations in plasma; none of the methods distinguish between the enantiomers of ephedrine. Recently however, Findlay *et al.* (25) developed a stereospecific radioimmunoassay (RIA) for *d*-pseudoephedrine. This paper describes the development of separate RIAs for *l*-ephedrine and *d*-ephedrine and their validation by comparison with a GLC–ECD method.



I II

Figure 1—*Haptens for* l-*ephedrine (I) and* d-*ephedrine (II).*

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Table I—Cross-Reactions of Ephedrine Antisera

Compound	Cross-Reaction, % *d*-Ephedrine Antiserum	*l*-Ephedrine Antiserum
l-Ephedrine	<2	100
d-Ephedrine	100	<2
dl-Pseudoephedrine	<1	<1
dl-Norephedrine	0	<1
dl-*p*-Hydroxyephedrine	0	0

EXPERIMENTAL

Preparation of Drug–Protein Conjugates—*N*-(2-carboxyethyl) derivatives of both *d*- and *l*-ephedrine[1] were prepared by the treatment of *d*- and *l*-ephedrine with methyl acrylate[2], followed by the subsequent mild alkaline hydrolysis of the resulting methyl esters as described previously (26, 27). The purity of the haptens was established by GLC–FID[3] examination of the appropriate methyl esters prepared by reaction of the carboxylic acids with diazomethane[4]. Each chromatogram contained a single peak which had the same retention time (3.2 min) as the methyl esters obtained directly by the reaction of the ephedrine enantiomers with methyl acrylate. Thus, it was concluded that the haptens were free from reaction side products or other extraneous materials which might couple to carrier-antigen and subsequently compromise the specificity of the antisera. The chemical structures of the haptens (Fig. 1) were confirmed by direct probe mass spectrometry[5].

The haptens were coupled to bovine serum albumin by a modified mixed anhydride method (26). The solutions were dialyzed[6] against bicarbonate buffer (0.042 *M*, pH 8.0, 6 × 500 ml) and then against acetate buffer (0.012 *M*, pH 4.0, 6 × 500 ml). Both buffer systems contained 0.2% sodium azide[2]. After lyophilization[7], the *d*- and *l*-ephedrine–protein conjugates were obtained as white crystalline solids. The numbers of hapten residues per mole of bovine serum albumin, calculated by the isotope dilution technique, were 17 for *d*-ephedrine–protein conjugate and 21 for *l*-ephedrine–protein conjugate.

Immunization—The hapten–protein conjugates (1 mg) were dissolved in 0.25 ml of normal saline and emulsified with 0.25 ml of Freund's complete adjuvant[8]. The emulsion was administered by intradermal injection (0.5 ml) to eight, 4-month-old, female New Zealand White rabbits. At 2-week intervals thereafter, the injections (0.5 ml) were repeated with a similar emulsion prepared with Freund's incomplete adjuvant. Serum samples were obtained from the marginal ear vein at weekly intervals after the third injection. Titers of the antisera were checked by evaluating the binding characteristics of tritiated *dl*-ephedrine[9]. The procedure was essentially as described in *Radioimmunoassay Procedures*, except that various dilutions of the antisera in distilled water were evaluated. Selectivities of the antisera were checked periodically by measuring the ability of *d*- or *l*-ephedrine to displace the radiotracer from its binding sites on the antibodies. The antisera were harvested by cardiac puncture when their selectivities were optimal, lyophilized[7], and stored at −70° until required.

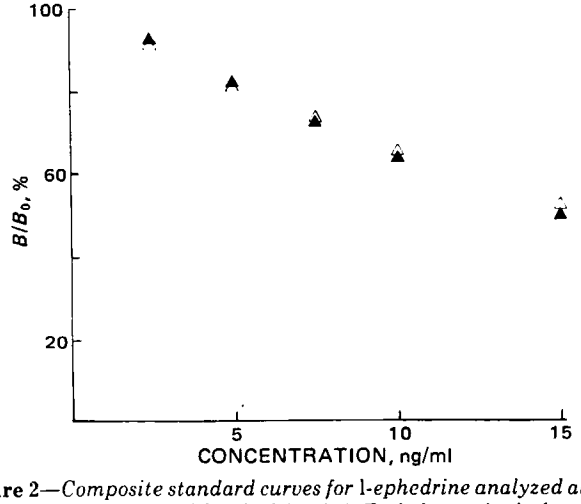

Figure 2—*Composite standard curves for* l-*ephedrine analyzed alone (△) and in the presence of* d-*ephedrine (▲). Each data point is the mean of five determinations.*

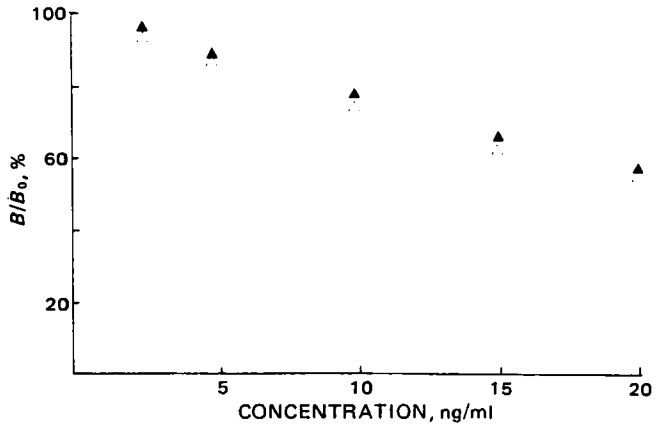

Figure 3—*Composite standard curves for* d-*ephedrine analyzed alone (△) and in the presence of* l-*ephedrine (▲). Each data point is the mean of five determinations.*

Radioimmunoassay Procedures—A 50–200-μl aliquot of plasma, spiked or from volunteers dosed with *dl*-ephedrine, was transferred to the bottom of a 2 × 75-mm tube[10]. [^{3}H]*dl*-Ephedrine (~2000 cpm) in 300 μl of phosphate buffer (0.2 *M*, pH 6.5) was added, and the solutions were mixed[11]. Antiserum (200 μl of a 10% dilution in distilled water) was added, and the solutions were mixed[11] again and incubated at 4° for 30 min. A suspension (1.0 ml) of dextran-coated charcoal[12], previously chilled to 4°, was added and, after mixing[11], each tube was incubated at 4° for an additional 10 min. The resulting suspension was then centrifuged[13] (1720 × *g*) at 4° for 10 min. The supernatant solution was decanted into a scintillation vial containing 18.0 ml of scintillation cocktail[14] and counted[15].

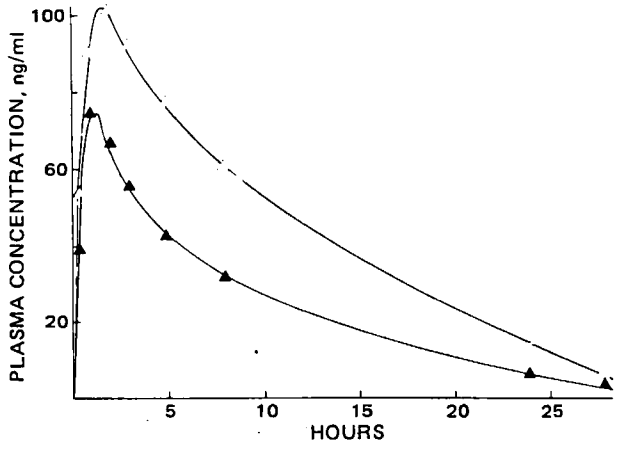

Figure 4—d-*Ephedrine (△) and* l-*ephedrine (▲) concentrations in the plasma of a healthy human volunteer following a single 50-mg oral dose of* dl-*ephedrine hydrochloride.*

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] British Drug Houses, Toronto, Canada.
[3] Model 3920, Perkin-Elmer Co., Montreal, Quebec, Canada. 5% OV-7 (Chromatographic Specialties, Brockville, Ontario, Canada) on acid-washed dimethylchlorosilane-treated high-performance chromosorb W. Operating temperatures: injection port and detector 300°, column oven 275°. Nitrogen flow rate: 60 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum detector response.
[4] Prepared from Diazald, Aldrich Chemical Co., Milwaukee, Wis.
[5] V. G. Micromass MM 16F.
[6] Fisher dialyzer tubing (size C), Fisher Scientific Co., Pittsburgh, Pa.
[7] Bench top freeze-dryer Model 75034, Labconco Corp., Kansas City, Mo.
[8] Grand Island Biological Co., Grand Island, N.Y.
[9] Generally labeled; specific activity 1.7 Ci/mmole; Nuclear Research Centre, Negev, Israel. Radiochemical purity was checked by TLC and HPLC.
[10] Falcon, Calif.
[11] Vortex Genie, Fisher Scientific Co., Canada.
[12] Bio-RIA, Montreal, Canada; used without modification.
[13] Model TJ-6 refrigerated centrifuge, Beckman Instruments Inc., Fullerton, Calif.
[14] PCS II TM, Amersham Corp., Arlington Heights, Colo.
[15] LKB Rackbeta Liquid Scintillation Counter, Model 1215 equipped with automatic quench compensation, Fisher Scientific Co., Canada.

Table II—Concentrations of Ephedrine as Determined by GLC and as Calculated from the Sum of Enantiomer Concentrations Determined by RIA

Hours	Analysis by RIA		Total Concentration, ng/ml	
	l-Ephedrine, ng/ml	*d*-Ephedrine, ng/ml	RIA	GLC
0.5	77	80	157	164
1	103	125	228	256
1.5	100	119	219	223
2	95	106	201	210
3	82	93	175	190
5	61	68	129	117
7.5	43	42	85	82
24	3.7	3.4	7.1	7.4

Tracer Solutions—Appropriate aliquots of the methanolic solution of tritiated *dl*-ephedrine were diluted daily with phosphate buffer (0.2 *M*, pH 6.5) so that 300 μl of the diluted tracer solution gave ~8000 cpm.

Calibration Curves—*l*-Ephedrine hydrochloride (5 mg) was dissolved in 400 ml of distilled water. Serial dilutions in plasma were made to provide working standards of 2.5, 5.0, 7.5, 10, and 15 ng/ml. Similarly, serial dilutions of *d*-ephedrine in plasma were made to provide working standards of 2.5, 5.0, 10, 15, and 20 ng/ml. Calibration curves for *l*-ephedrine, both with and without the presence of *d*-ephedrine, were obtained by using the spiked plasma samples in the aforementioned RIA procedure (antiserum for *l*-ephedrine). The calibration curves for *l*-ephedrine in the presence of *d*-ephedrine were constructed by pairing a specified concentration of *l*-ephedrine with *d*-ephedrine. The paired *l*-ephedrine–*d*-ephedrine concentrations were 2.5:20.0, 5.0:15.0, 7.5:10.0, 10.0:5.0, and 15.0:2.5 ng/ml of plasma.

Calibration curves for *d*-ephedrine, both with and without the presence of *l*-ephedrine, were obtained in a similar manner except that the antiserum for *d*-ephedrine was used in the RIA. All calibration curves were constructed by plotting percent bound/percent bound at zero concentration (B/B_0) *versus* concentration (in ng/ml) of substrate in the sample.

Specificities—Cross-reactivities of the optical isomers and metabolites of ephedrine were determined according to the criteria of Abraham (28).

Human Studies—Single doses of *dl*-ephedrine hydrochloride (50 mg) were administered to each of two healthy male volunteers. Blood samples were collected in evacuated heparinized collection tubes[16], without allowing the blood to contact the rubber stopper. Samples collected over a 24-hr period were centrifuged, and separated plasma was stored at −15° until analyzed.

Validation Studies—The aforementioned plasma samples were each analyzed by the RIA for *d*-ephedrine, the RIA for *l*-ephedrine, and a published GLC–ECD method (23) for *dl*-ephedrine. Plasma concentration *versus* time plots were constructed for *dl*-ephedrine from the GLC–ECD data and compared with similar plots obtained by adding together the concentrations of the individual enantiomers determined by the RIA procedures.

RESULTS AND DISCUSSION

The cross-reactivities of the antisera for *d*- and *l*-ephedrine are shown in Table I. Both antisera were highly stereospecific. Neither antiserum cross-reacted with the optical antipode of its substrate, nor with racemic pseudoephedrine. Neither antiserum recognized the ring-modified metabolite *dl*-*p*-hydroxyephedrine nor the side chain-modified metabolite *dl*-norephedrine. The latter is particularly significant because many antisera cross-react with compounds that differ in structure from the substrate only at the point of haptenic modification. For example, similar *N*-substituted haptens were used in the development of antisera for chlorpromazine, imipramine, amitriptyline, and *trans*-doxepin. Each antiserum cross-reacted (100%) with the *N*-desmethyl metabolite of its appropriate substrate (29, 30). In the present case, however, the antisera for the enantiomers of ephedrine appear to be specific for both ring system and side chain.

Standard curves for *l*-ephedrine (Fig. 2) and *d*-ephedrine (Fig. 3) were constructed after analysis of spiked plasma which contained the substrate alone or the substrate plus varying quantities of its enantiomer. The concentrations of the contaminating enantiomer were varied from 16.7 to 800% of the substrate concentration. This allowed for the possibility that plasma concentration ratios of the enantiomers differed from unity after dosing with the racemic drug.

The standard curve obtained using the *l*-ephedrine antisera in the presence of only *l*-ephedrine fit the equation $y = -3.18x + 98.31$, with a correlation coefficient of 0.9956 (Fig. 2). When the standard curve for *l*-ephedrine was analyzed in the presence of *d*-ephedrine the resultant standard curve fit the equation $y = -3.42x + 99.57$, with a correlation coefficient of 0.9974. These two curves are virtually superimposable, indicating that the *l*-ephedrine antisera was not sensitive to the presence of *d*-ephedrine (Fig. 2).

When the *d*-ephedrine antiserum was used to construct a standard curve in the presence of only *d*-ephedrine a straight line was obtained which fit the equation $y = -2.30x + 98.58$ and had a correlation coefficient of 0.9952. The standard curve of *d*-ephedrine analyzed in the presence of *l*-ephedrine was also a straight line with the equation $y = -2.35x + 102.22$ and a correlation coefficient of 0.9983 (Fig. 3).

These experiments demonstrated that the presence of the optical antipode has little effect on the standard curves and that the RIA procedures can be used to measure the concentration of *d*-ephedrine or *l*-ephedrine in the plasma of a patient dosed with *dl*-ephedrine.

Figure 4 shows the plasma concentration–time curves obtained for *d*- and *l*-ephedrine after a single 50-mg oral dose of racemic ephedrine hydrochloride was administered to a healthy male volunteer. *d*-Ephedrine reached significantly higher plasma concentrations and was eliminated more slowly than *l*-ephedrine. Similar results (Table II) were obtained when the study was repeated in a second volunteer. Both enantiomers reached higher concentrations in the plasma of the second individual, although the peak concentration of the more active *l*-ephedrine was again significantly lower than that of *d*-ephedrine.

The intraassay variances (31) of the RIA procedures, *i.e.*, the overall coefficients of variation for five replicates each at five concentrations were: *l*-ephedrine antiserum with *l*-ephedrine alone, 1.63 (2.67); *l*-ephedrine antiserum with *l*-ephedrine in the presence of *d*-ephedrine, 2.45 (3.30); *d*-ephedrine antiserum with *d*-ephedrine alone 2.90 (4.20); and *d*-ephedrine antiserum with *d*-ephedrine in the presence of *l*-ephedrine 3.76 (5.73). The value in parentheses is the highest coefficient of variation for each case. Interassay variances were of the same order. In each assay, the detection limit was <0.5 ng of the appropriate ephedrine enantiomer in a 200-μl sample.

To validate the RIA procedures, aliquots of the plasma samples from the two volunteers were examined also by a nonstereoselective, though sensitive, GLC–ECD method (23). This procedure was used to measure the total plasma concentration of ephedrine which was compared with the sum of the concentrations of the individual enantiomers determined by RIA (Table II). The results yielded a straight line when total plasma ephedrine as determined by GLC–ECD was plotted against the sum of *d*-ephedrine and *l*-ephedrine as determined by RIA. The slope of this line was 1.1, with a correlation coefficient of 0.99. These results show the plasma concentration–time curves generated by RIA and by GLC–ECD to be comparable. This indicates that the RIA procedures are not subject to interference from the metabolites of ephedrine or from endogenous plasma constituents.

[16] Vacutainer tubes, Beckton, Dickenson and Co., Toronto, Canada.

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A Highly Sensitive Pyrogen Test for Antibiotics I: Detection of Trace Amounts of Endotoxin in Injectable Sodium Ampicillin Preparations

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Received August 24, 1981, from the **Department of Antibiotics, National Institute of Health, Kamiosaki 2-chome, Shinagawa-ku, Tokyo 141, Japan* and the ‡*Department of Quality Control, Takeda Chemical Industries, Ltd., Doshomachi, Osaka 541, Japan.* Accepted for publication June 17, 1982.

Abstract □ The rabbit pyrogen test (specified in the pharmacopeia) and the Limulus amebocyte lysate (LAL) test are influenced by high concentrations of certain antibiotics. Therefore, it has not been possible to detect trace amounts of endotoxin which may contaminate these antibiotics. To detect trace amounts of endotoxin in injectable sodium ampicillin, an ultrafiltration technique was utilized which removed the antibiotic and left a solution which contained predominantly the endotoxin. After ultrafiltration, a trace amount of pyrogen (which otherwise could not be detected) was found using both the rabbit pyrogen and the LAL tests. The endotoxin was also determined quantitatively using a chromogenic endotoxin reagent which is made by combining the Limulus amebocyte lysate and a synthetic substrate with a suitable chromophore.

Keyphrases □ Ampicillin—detection of trace amounts of endotoxin using ultrafiltration with pyrogen tests, chromogenic assay □ Ultrafiltration—of ampicillin solutions, use with pyrogen tests to detect trace amounts of endotoxin in antibiotics □ Chromogenic assay—of ampicillin solution, detection of trace amounts of endotoxin in antibiotics

It is important to develop highly sensitive methods for detecting trace amounts of pyrogen to ensure that pharmaceutical preparations are completely pyrogen free. Several cases of fever have been reported after the injection of β-lactam antibiotics such as methicillin and cloxacillin (1–6). However, in only one case (methicillin) was the presence of a pyrogen detected (6). Fever is caused in most cases by lipopolysaccharide from the outer layer of the cell walls of Gram-negative bacteria. Pyrogens, a type of endotoxin, are often complex, high molecular weight substances containing lipid A (7).

Endotoxins are usually detected by the pyrogen test using rabbits, or by the Limulus amebocyte lysate (LAL) test. The former method is specified in the pharmaceutical compendia of both the U.S. (8) and Japan (9). The latter method is specified in the United States Pharmacopeia XX (10). In the pyrogen test, the rise in the body temperature of rabbits caused by the endotoxin is sometimes inhibited by the pharmacological activity of the coexistent drugs. The sensitivity of the LAL test also is affected by the presence of certain drugs (11). In such cases, it would be desirable to separate the endotoxin from the drug and concentrate the endotoxin.

Minami *et al.* used an ultrafiltration method to separate endotoxins from antipyretics (12). Sullivan *et al.* showed that β-lactam antibiotics such as sodium penicillin G do not combine with endotoxin using ultrafiltration (13).

It was reported that gel formation in the LAL test was induced by the amidase activity of a clotting enzyme in the lysate, which is activated by a bacterial endotoxin (14). Harada *et al.* applied this principle to the colorimetric determination of endotoxins using a synthetic substrate which activates the amidase activity of the enzyme and releases a chromophore (15).

This study reports the detection of trace amounts of

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