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Stereospecific Radioimmunoassays for *d*-Pseudoephedrine in Human Plasma and Their Application to Bioequivalency Studies

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Abstract □ Antiserum to *d*-pseudoephedrine was raised in New Zealand White rabbits in response to immunization with a conjugate of bovine serum albumin and *d*-pseudoephedrine-*N*-3-propionic acid. The hapten was prepared by reaction of methyl acrylate with *d*-pseudoephedrine, followed by ester hydrolysis. Sodium boro[^{3}H]hydride reduction of *dl*-ephedrone gave [α-^{3}H]-*dl*-ephedrine, and a Welsh rearrangement with acetic anhydride followed by deacetylation gave [α-^{3}H]-*dl*-pseudoephedrine, which was used as a radioligand in radioimmunoassay procedures for direct plasma analyses. Three sensitive radioimmunoassay procedures were developed, two using [^{3}H]pseudoephedrine as the radioligand and either adsorption on coated charcoal or polyethylene glycol precipitation for separation of antibody-bound from free radioligand. The third method used an [^{125}I]tyrosine methyl ester analog of pseudoephedrine and charcoal separation, preceded by extraction and derivatization of pseudoephedrine with methyl acrylate. All three assays could detect ≤2.5 ng of pseudoephedrine/ml. The antiserum was stereospecific, showing low cross-reactivities with *l*-pseudoephedrine and *d*- and *l*-ephedrines. *d*-Norpseudoephedrine and some other related compounds also had low cross-reactivity in these radioimmunoassay procedures. Excellent agreement was found between pseudoephedrine concentrations in human plasma determined by radioimmunoassay and by a standard GLC method. The utility of radioimmunoassay was illustrated by application of one of these procedures to an assessment of the bioequivalence of immediate- and sustained-release pseudoephedrine formulations in normal volunteers. A sustained-release preparation containing 120 mg of pseudoephedrine hydrochloride given every 12 hr was shown by *AUC* comparisons to be bioequivalent to an immediate-release tablet (containing 60 mg of pseudoephedrine hydrochloride) given every 6 hr.

Keyphrases □ Radioimmunoassay—*d*-pseudoephedrine, human plasma, bioequivalency studies, immediate- and sustained-release tablets □ *d*-Pseudoephedrine—radioimmunoassay, bioequivalency studies, immediate- and sustained-release tablets □ Bioequivalency studies—radioimmunoassays of *d*-pseudoephedrine in human plasma, bioequivalency studies, immediate- and sustained-release tablets □ Adrenergics—radioimmunoassays for *d*-pseudoephedrine in human plasma, bioequivalency studies, immediate- and sustained-release tablets

d-Pseudoephedrine was first isolated from the Chinese plant *Ma Huang* by Chou and Read (1) in 1926, and studies of its pharmacology started soon thereafter (2, 3). Although pseudoephedrine is in widespread use as a proven, clinically effective nasal decongestant (4–6), little published information is available on its disposition and pharmacokinetics in human plasma following administration of therapeutic doses. Studies have examined kinetics chiefly from the viewpoint of urinary excretion (7–9), which is liable to be influenced by alterations in urinary pH (8). Such indirect studies were necessitated by the lack of methods sufficiently sensitive to detect the relatively low circulating concentrations of pseudoephedrine in plasma following therapeutic doses.

Reported GLC procedures (7, 10, and 11) were capable of detecting only 0.3–0.5 μg of pseudoephedrine/ml. Even with nitrogen detection, Bye *et al.* (12) were able to lower the sensitivity limit only to 25 ng/ml. Kuntzman *et al.* (8) reported a procedure that involved esterification of isolated pseudoephedrine with tritiated acetic anhydride followed by separation and quantitation of the resulting radioactive product. Although capable of detecting 50 ng of pseudoephedrine/ml of plasma, this method is still relatively insensitive and time consuming. A study (13) of plasma pseudoephedrine levels relative to efficacy, using a GLC method for drug determination, was able to determine plasma concentrations up to 6 hr following a 60-mg oral dose of pseudoephedrine hydrochloride.

This report describes the development of a specific antiserum to pseudoephedrine and its application to three specific radioimmunoassay procedures for the drug. Two procedures are direct and employ a tritium-labeled radioligand; the third requires extraction and derivatization of the drug prior to assay but employs a γ-labeled radioligand. The sensitivity limits of these procedures are <2.5 ng of pseudoephedrine/ml in all cases. Their use for drug disposition studies following oral administration of both immediate- and sustained-release pseudoephedrine preparations is illustrated.

EXPERIMENTAL

Melting points[1] are uncorrected. ^{1}H-NMR spectra[2] were obtained in deuterochloroform with tetramethylsilane as the internal standard unless otherwise indicated. Low-resolution mass spectra[3] were obtained by

[1] Thomas-Hoover apparatus, Arthur H. Thomas Co., Philadelphia, Pa.
[2] Model R-24A, Perkin-Elmer Corp., Norwalk, Conn.
[3] Model MAT 731 or CH5DF, Varian Associates, Palo Alto, Calif.

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electron-ionization methods. TLC plates were precoated silica gel[4] with a layer thickness of 0.25 mm. Radiochemical purities were determined by scanning the TLC plates using a radiochromatogram scanner[5]. Bovine serum albumin (Cohn Fraction V), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, tyrosine methyl ester, and dicyclohexylcarbodiimide were purchased from a commercial supplier[6].

Radioimmunoassay grade charcoal[7] was used in the radioimmunoassay separation system. Sodium [^{125}I]iodide (carrier free) and sodium boro[^{3}H]hydride (specific activity 28 Ci/mmole) were obtained commercially[8]. Methyl acrylate[9], chloramine-T[9], Freund's complete adjuvant[10], and polyethylene glycol 6000[11] were commercial products. Quantitation of radioactivity was in liquid scintillation fluid[12]. Radioactivity was determined in a liquid scintillation counter[13] or γ-counter[14], as appropriate. Ephedrine, pseudoephedrine, and related compounds were commercially supplied[15].

d-Pseudoephedrine-*N*-3-propionic Acid Methyl Ester (II)—To *d*-pseudoephedrine free base (I, 1.65 g, 0.01 mole) in absolute ethanol (25 ml) containing triethylamine (1.03 g, 0.012 mole) was added methyl acrylate (1.03 g, 0.012 mole), and the sealed mixture was stirred at room temperature overnight. The volatile components were evaporated under reduced pressure, leaving an oily residue that slowly crystallized from *n*-pentane to give a colorless solid (2 g), mp 37°; NMR: δ 7.3 (s, 5H, aromatic H), 4.7 (d, 1H, J = 1.5 Hz, α-H), 3.6 (s, 3H, $COOCH_3$), 3.3 (m, 5H), 2.8 (s, 3H, N–CH_3), and 1.0 (s, 3H, C–CH_3) ppm; mass spectrum: m/z 220 (M − CH_3), 178 (M − CH_2COOCH_3), and 144.

Anal.—Calc. for $C_{14}H_{21}NO_3$: C, 66.90; H, 8.42; N, 5.57. Found: C, 67.78; H, 8.57; N, 5.61.

d-Pseudoephedrine-*N*-3-propionic Acid Sodium Salt (III)—The crystalline ester II (2 g) was dissolved in warm 2 *N* NaOH (10 ml) and stirred for 2 hr. Upon cooling, the sodium salt of the acid derivative separated and was filtered. The yield was 1 g of amorphous powder, mp >200° dec.; NMR (deuterated dimethyl sulfoxide): δ 7.25 (s, 5H, aromatic H), 5.5 (s, 1H), 4.25 (d, 1H, J = 1.5 Hz, α-H), 2.5 (m, 6H), 2.2 (s, 3H, N–CH_3), and 0.6 (d, 3H, J = 1.5 Hz, C–CH_3) ppm; mass spectrum: m/z 282, (free acid anion^{-1} · Na^{2+})$^+$, 541 (L, N–CH_3), (anion^{2-} · Na^{3+})$^+$, and 800, (anion^{3-} · Na^{4+})$^+$.

d-Pseudoephedrine-*N*-3-propionic Acid–Bovine Serum Albumin Amide (IV)—The pH of a solution of the salt of acid III (0.1 g, 0.4 m*M*) and bovine serum albumin (0.1 g) in water (15 ml) was adjusted to 5.5 with dilute hydrochloric acid. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.1 g) was added, and the solution was stirred at room temperature for 20 hr. The immunogen was purified by ultrafiltration[16]. The solution was lyophilized, and the powdery protein residue was stored over desiccant at −80°.

d-Pseudoephedrine-*N*-3-propionic Acid–Tyrosine Methyl Ester Amide (IV*a*)—A solution of *d*-pseudoephedrine-*N*-3-propionic acid (0.6 g, 1.8 m*M*) and tyrosine methyl ester free base (0.35 g, 1.8 m*M*) was prepared in acetonitrile (3 ml) and dimethylformamide (1 ml). Sufficient water and dilute hydrochloric acid were added to achieve a homogeneous solution (pH 6). The solution was cooled to 0°, a solution of dicyclohexylcarbodiimide (0.49 g, 2.2 m*M*) in cold dimethylformamide (0.8 ml) was added, and the reaction mixture was stirred at 0° overnight. After filtration, the solution was added to a mixture of tetrahydrofuran (100 ml), acetic acid (15 drops), ethanol (15 ml), and triethylamine (1 ml) to destroy excess dicyclohexylcarbodiimide. Stirring was continued for 1 hr at room temperature. The solution was filtered, and the filtrate was evaporated under reduced pressure.

The residue was dissolved in chloroform (100 ml) and filtered, and the solution was extracted with 5% sodium bicarbonate (2 × 25 ml) and then with 0.1 *N* HCl (2 × 25 ml). The aqueous acid extract was made basic with excess sodium carbonate and extracted into chloroform (3 × 50 ml), and the solvent was dried with magnesium sulfate and clarified with charcoal prior to being evaporated. A methanol solution of the residue was applied to silica gel plates (0.5 mm, 20 × 20 cm) and developed in ethylene dichloride–methanol–ammonium hydroxide (100:30:4). The product band was visualized with fast blue B salt, scraped off, and eluted from the silica gel with methanol (2 × 5 ml).

The methanol solution was concentrated to 0.5 ml, and the preparative TLC purification was repeated. The amorphous residue (0.1 g), mp 162° dec., was stored over desiccant at −80°; NMR: δ 7.5 (s, 5H, aromatic H), 7.0 (q, 4H, J = 1.0 Hz, aromatic H), 5.0 (d, 1H, J = 1.0 Hz), 4.4 (d, 1H, J = 1.5 Hz, α-H), 3.8 (s, 3H, $COOCH_3$), 3.3–2.4 (m, 8H), 2.3 (s, 3H), and 0.7 (d, 3H, J = 1.0 Hz, C–CH_3) ppm; mass spectrum: m/z 307.1648, $C_{16}H_{23}N_2O_4$ (M − $CH_2C_6H_4OH$).

[^{125}I]-*d*-Pseudoephedrine-*N*-3-propionic Acid–Tyrosine Methyl Ester Amide (V)—Into a small test tube containing a glass bead was pipetted 70 μl of a solution of IV*a* (1.54 μg, 3.7 n*M*) in 0.5 *M* phosphate buffer, pH 7.7, followed by sodium [^{125}I]iodide (1 mCi, 0.56 n*M*) in 10 μl of dilute sodium hydroxide, pH 8.0. A freshly prepared solution of chloramine-T (25.2 μg, 89.6 n*M*) in 25 μl of 0.05 *M* phosphate buffer, pH 7.7, was added, and the mixture was agitated at room temperature for 1 min. A freshly prepared solution of sodium metabisulfite (341 μg, 1790 n*M*) in 100 μl of 0.05 *M* phosphate buffer, pH 7.7, was added immediately with mixing.

The mixture was set aside for 10 min at room temperature and then was applied to a silica gel plate (20 × 20 cm), which was developed in the solvent system described for IV*a*. X-ray film was exposed to the TLC plate, and the band (R_f 0.6) corresponding to the main dark band on the X-ray film was scraped off, extracted with methanol (4 × 5 ml), and stored at −80° until used.

[α-^{3}H]-*dl*-Pseudoephedrine Hydrochloride (VI)—dl-α-*Bromopropiophenone*—Bromine (20.0 g, 0.125 mole) in methylene chloride (50 ml) was added dropwise to a stirred solution of propiophenone (13.42 g, 0.10 mole) in methylene chloride (80 ml) at 30–35°, and the mixture was heated under reflux for 60 min. Evaporation to dryness gave a pale-yellow oil, which was used in the next reaction without further purification.

dl-*Ephedrone Hydrochloride*—A solution of the crude propiophenone in ethanol (10 ml) was added dropwise under nitrogen to a stirred ice-cold solution of methylamine gas (10.0 g, 0.32 mole) in ethanol (130 ml) during 50 min, and the mixture was stirred at 0° for an additional 40 min. A mixture of 12 *N* HCl (30 ml) and ice was added to pH < 7, and the aqueous solution was extracted with ether and evaporated to dryness. The yellow solid was extracted with chloroform (4 × 50 ml), and the residual methylamine hydrochloride was discarded. Evaporation of the chloroform filtrates gave an off-white solid, which was recrystallized twice from ethanol–acetone to give 3.41 g (17.1%) of *dl*-ephedrone hydrochloride as off-white crystals, mp 176–179° [lit. (21) mp 176–177°]; NMR (dimethyl sulfoxide-d_6 + D_2O): δ 1.5 (d, 3H, C–CH_3), 2.7 (s, 3H, N–CH_3), 5.2 (q, 1H, α-H), and 7.4–8.2 (m, 5H, aromatic H) ppm. TLC in chloroform–2-propanol–triethylamine (62:35:3) showed a single spot, R_f 0.53.

[α-^{3}H]-dl-*Ephedrine Hydrochloride*—To a stirred solution of ephedrone hydrochloride (90.1 mg, 0.45 mmole) in ethanol (12 ml) under dry argon was added dropwise a solution of sodium boro[^{3}H]hydride (8 Ci at 28 Ci/mmole, ~0.34 mmole) in ethanol (19 ml) during 2 min. After a further 60 min at room temperature, a solution of 12 *N* HCl (1.6 ml) in water (2.9 ml) was added dropwise. After stirring for 20 min, the colorless solution was diluted with water (90 ml) and ether (70 ml), and 1 *N* NaOH (30 ml) was added to pH > 10. The aqueous solution was extracted with ether (4 × 50 ml), and the combined ether solutions were washed with water (100 ml), evaporated to near dryness *in vacuo* at 0°, and treated with methanol (20 ml) and 12 *N* HCl (2.0 ml). Evaporation to dryness *in vacuo* yielded 89.9 mg (98.8%) of crude ephedrine as an off-white solid. TLC in chloroform–2-propanol–triethylamine (62:35:3) showed ephedrines (R_f ~0.19) containing a trace of residual ephedrone (R_f 0.53), and TLC in 2-butanone–2-propanol–ammonium hydroxide (6:3:1) showed mainly ephedrine (R_f 0.49) containing a trace of pseudoephedrine (R_f 0.82).

[α-^{3}H]-dl-*Pseudoephedrine Hydrochloride*—A stirred mixture of crude ephedrine (89.9 mg) and acetic anhydride (3.0 ml) under dry argon was heated under reflux for 30 min. The cooled solution was evaporated to dryness *in vacuo*, water (2.0 ml) and 12 *N* HCl (0.6 ml) were added, and the mixture was distilled for 2 hr while fresh water was added continuously to the flask to replace the distillate, volume for volume. Evaporation to dryness *in vacuo* was followed by the addition of hot water (1.5 ml) and then the addition of 50% NaOH solution to the stirred ice-cold solution to pH > 13. The precipitate of [α-^{3}H]-*dl*-pseudoephedrine was collected by filtration, washed with ice-cold water (1.0 ml), dissolved in methanol (10 ml), and treated with 12 *N* HCl (1.0 ml).

TLC in 2-butanone–2-propanol–ammonium hydroxide (6:3:1) showed

[4] Silica gel 60 F-254, E. Merck & Co., Darmstadt, West Germany.
[5] Berthold LB 2760, Beta Analytical Inc., Coraopolis, Pa.
[6] Sigma Chemical Co., St. Louis, Mo.
[7] Schwarz-Mann, Orangeburg, N.Y.
[8] Amersham Corp., Arlington Heights, Ill.
[9] Eastman Organic Chemicals, Rochester, N.Y.
[10] Difco Laboratories, Detroit, Mich.
[11] Fisher Scientific Co., Raleigh, N.C.
[12] Aquasol-2, New England Nuclear Corp., Boston, Mass.
[13] Model 3320 or 2650, Packard Instrument Co., Downers Grove, Ill.
[14] Model 5260 Autogamma, Packard Instrument Co., Downers Grove, Ill.
[15] Knoll Fine Chemicals, New York, N.Y.
[16] PM-10 membrane, Amicon Corp., Lexington, Mass.

almost entirely pseudoephedrine (R_f 0.82), no ephedrone and no ephedrine, and a small amount of unknown material (R_f 0.0). Evaporation to dryness *in vacuo* yielded 58.2 mg (64.0% from ephedrone) of crude [^{3}H]pseudoephedrine hydrochloride as an off-white solid. Two recrystallizations from 3-methyl-1-butanol–tetrahydrofuran afforded 3.8 mg of pure [α-^{3}H]-*dl*-pseudoephedrine hydrochloride as a colorless crystalline solid. TLC in 2-butanone–2-propanol–ammonium hydroxide (6:3:1) showed almost single-spot [^{3}H]pseudoephedrine (R_f 0.82) with a trace of material at the origin. Radiochromatogram scanning showed ~0.4% [^{3}H]ephedrine in otherwise radiochemically pure [^{3}H]pseudoephedrine. TLC in chloroform–2-propanol–triethylamine (62:35:3) again showed almost pure [^{3}H]pseudoephedrine (R_f 0.16) with a trace of material at the origin. Radioactive chromatogram scanning showed ~2.6% of the activity in the origin material in otherwise 97.4% radiochemically pure [^{3}H]pseudoephedrine. Autoradiograms showed the same distribution pattern of radioactivity as shown by plate scanning. The specific activity was determined by scintillation counting to be 5.0 Ci/mmole.

[α-^{3}H]-*dl*-Pseudoephedrine-*N*-3-propionic Acid Methyl Ester (VII)—A methanol solution of [α-^{3}H]-*dl*-pseudoephedrine (2.82 μg, 69 μCi, 300 μl) containing triethylamine (36.3 mg, 358 μM, 10 μl) and methyl acrylate (47.8 mg, 555 μM, 50 μl) was sealed and mixed at room temperature overnight. The solution was gently evaporated (dry nitrogen stream), and the residue was dissolved in 300 μl of methanol. A TLC radiochromatogram in ethylene dichloride–methanol–ammonium hydroxide (100:20:4) showed a single band that migrated with the unlabeled compound.

[α-^{3}H]-*dl*-Pseudoephedrine-*N*-3-propionitrile (VIII)—A methanol solution of [α-^{3}H]-*dl*-pseudoephedrine (2.82 μg, 69 μCi, 300 μl) containing triethylamine (36.3 mg, 358 μM, 10 μl) and acrylonitrile (40.3 mg, 760 μM, 50 μl) was sealed and mixed at room temperature overnight. The solution was gently evaporated (dry nitrogen stream), and the residue was dissolved in 300 μl of methanol. A radiochromatogram in the TLC system used for VII showed a single band that migrated with the unlabeled compound.

[α-^{3}H]-*dl*-Pseudoephedrine-*N*-acetyl Amide (IX)—A methanol solution of [α-^{3}H]-*dl*-pseudoephedrine (2.82 μg, 69 μCi, 300 μl) containing triethylamine (36.3 mg, 358 μM, 10 μl) and acetyl chloride (55.2 mg, 703 μM, 50 μl) was sealed and mixed at room temperature overnight. The solution was gently evaporated (dry nitrogen stream), and the residue was dissolved in 300 μl of methanol. A radiochromatogram in the system used for VII showed a single band that migrated with the unlabeled compound.

Immunization Procedures—Male New Zealand White rabbits received a primary immunization of 1 mg of bovine serum albumin–drug conjugate in 1 ml of 0.9% saline emulsified with 1 ml of Freund's complete adjuvant as two intramuscular (*vastus lateralis*) and eight subcutaneous (along each side of the dorsal column) injections of 0.2 ml each. At intervals of 2, 4, and 6 weeks following the primary immunization and at monthly intervals thereafter, booster immunizations of 0.5 mg of immunogen in 0.5 ml of saline, emulsified with 0.5 ml of Freund's complete adjuvant, were administered at multiple subcutaneous sites. Following the second and all subsequent booster immunizations, blood samples were collected from the central ear artery and the serum was separated and stored at −80°.

Human Study—Seventeen healthy male subjects received, for 5 days, either an immediate-release tablet[17] containing 2.5 mg of triprolidine hydrochloride and 60 mg of pseudoephedrine hydrochloride every 6 hr or a sustained-release capsule[18] containing 5 mg of triprolidine hydrochloride and 120 mg of pseudoephedrine hydrochloride every 12 hr. The study followed a double-blind crossover design so that all subjects received both treatments with a 1-week period intervening to allow for removal of residual drugs from the plasma. At each drug administration, subjects received a tablet and a capsule, one of which contained active drug and the other contained placebo materials according to the planned treatment schedule. The sustained-release preparation was administered at 8 am and 8 pm, while the immediate-release tablets were given at 8 am, 2 pm, 8 pm, and 2 am.

In each phase of the study, blood samples were collected into glass tubes[19] containing the disodium salt of ethylenediaminetetraacetic acid at 8 am, 10 am, 8 pm, and 10 pm on Days 1–4, at 8 am, 9 am, 10 am, 12 noon, 2 pm, 3 pm, 4 pm, 6 pm, 8 pm, and 10 pm on Day 5, and at 8 am and 2 pm on Day 6. Plasma was separated by centrifugation and stored at −20° until analyzed for pseudoephedrine content. The participants had not ingested any drugs for at least 1 week prior to entering the study.

General Radioimmunoassay Procedures—The assay buffer contained 0.05 M NaH_2PO_4–Na_2HPO_4 (pH 7.4), 0.15 M NaCl, 0.01 M ethylenediaminetetraacetic acid disodium salt, and 1% bovine serum albumin, except where otherwise indicated. The pH of this buffer, hereafter referred to as 1% bovine serum albumin–phosphate-buffered saline, was adjusted to 7.4. For separation of antibody-bound radiolabel from free radiolabel, a bovine serum albumin-coated charcoal suspension was prepared by dissolving 0.6 g of charcoal in 100 ml of ice-cold 2% bovine serum albumin–phosphate-buffered saline. The suspension was stirred for at least 30 min at 0° prior to use.

The alternative separation system consisted of 350 g of polyethylene glycol 6000 dissolved in 1 liter of phosphate-buffered saline. For use with this system, radioligand and rabbit antibody solutions were prepared in buffer containing 0.1% bovine serum albumin (0.1% bovine serum albumin–phosphate-buffered saline); bovine serum albumin was not added to phosphate-buffered saline for any other assay applications using the polyethylene glycol 6000 immunoglobulin precipitation.

All assay points were established in duplicate, and most unknown samples were analyzed at two dilutions.

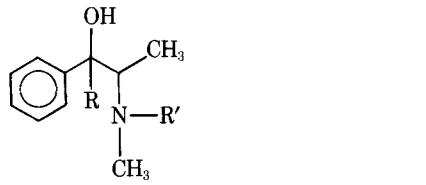
Antiserum Titration—*Charcoal Separation*—[^{125}I]-*d*-Pseudoephedrine-*N*-3-propionic acid–tyrosine methyl ester amide (100,000 dpm/assay), [^{3}H]-*dl*-pseudoephedrine-*N*-3-propionic acid methyl ester (16,000 dpm/assay), [^{3}H]-*dl*-pseudoephedrine-*N*-3-propionitrile (16,000 dpm/assay), [^{3}H]-*dl*-pseudoephedrine-*N*-acetyl amide (16,000 dpm/assay), or [^{3}H]-*dl*-pseudoephedrine (16,000 dpm/assay) in buffer (0.1 ml) was incubated with varying dilutions of rabbit antiserum in buffer (0.1 ml) and additional assay buffer (0.8 ml) at 4° overnight. Tubes containing only radiolabeled solution and buffer added to obtain the same total incubation volume served as total count tubes and background tubes.

Ice-cold 2% bovine serum albumin–charcoal suspension (0.5 ml) was added to all tubes except the total count tubes; the contents of the tubes were mixed, incubated at 4° for 5 min, and centrifuged (≥1000×g, 10 min). The tritium-containing supernates were decanted into scintillant (10 ml) and mixed, and the antibody-bound radioactivity was quantitated. The radioiodine-containing supernates were decanted into plastic tubes (12 × 75 cm) and capped, and their radioactivity contents were quantitated. The dilution that bound 50% of the amount bound in the presence of excess antiserum was defined as the working titer of the antiserum.

Polyethylene Glycol Separation—To ensure maximal precipitation of the antibody–radiolabel complex in the radioimmunoassay procedure using polyethylene glycol 6000 as an immunoglobulin precipitant, carrier immunoglobulin protein in the form of 0.1 ml of filtered (0.22 μm) pooled normal human plasma was added to the assay mixture and the amount of added buffer was reduced by 0.1 ml. After the assay mixture was incubated overnight at 4°, 1 ml of the polyethylene glycol 6000 solution was added to each assay tube (polypropylene, 12 × 55 mm) at 0° in an ice bath. The tubes were thoroughly mixed, incubated at 0° for 30 min, and centrifuged at ≥1000×g for 30 min. Supernates were decanted from the immunoglobulin pellet containing antibody-bound radiolabel, the inverted tubes were allowed to drain for 15 min, and then the assay tubes were pounded on paper towels to remove the remaining supernates quantitatively. The pellets were then resolubilized in phosphate-buffered saline (0.3 ml) with mixing over 30 min. Total count tubes received 0.2 ml of phosphate-buffered saline plus 0.1 ml of labeled solution. When a homogeneous solution was achieved, scintillation fluid (2 ml) was added to the assay tubes, which were capped and mixed. These tubes were placed in specially designed scintillation vials that kept them correctly positioned for scintillation counting.

Radioimmunoassay of Standard, Control, and Unknown Plasma Samples—*Direct Radioimmunoassay with [α-^{3}H]-*dl*-Pseudoephedrine Label*—A series of *d*-pseudoephedrine hydrochloride standard solutions in filtered (0.22 μm), pooled, normal human plasma (0.1 ml) ranging in concentration from 1 to 250 ng/ml (free base equivalents) was incubated as already described (for the polyethylene glycol separation) with [α-^{3}H]-*dl*-pseudoephedrine (16,000 dpm/assay, 0.1 ml), *d*-pseudoephedrine antiserum (1:100 dilution, 0.1 ml), and assay buffer (0.7 ml). A similar series of three control solutions of *d*-pseudoephedrine added to normal human plasma varying in concentration across the range of the standard curve served as controls of assay accuracy and reproducibility. Unknown human plasma samples (0.1 ml), diluted appropriately with normal human plasma to enter the range of the assay, were also incubated with [α-^{3}H]-*dl*-pseudoephedrine, *d*-pseudoephedrine antiserum, and assay buffer as described. Total counts and background tubes were as already described, each containing 0.1 ml of normal human plasma and a total final volume of 1 ml.

[17] Actifed, Burroughs Wellcome Co., Research Triangle Park, N.C.
[18] Actifed SA, Burroughs Wellcome Co., Research Triangle Park, N.C.
[19] Vacutainer, Becton-Dickinson and Co., Rutherford, N.J.



V: R = H, R′ = $(CH_2)_2CONHCH(CO_2CH_3)CH_2$–(p-^{125}I-phenyl)–OH

VI: R = ^{3}H, R′ = H

VII: R = ^{3}H, R′ = $(CH_2)_2CO_2CH_3$

VIII: R = ^{3}H, R′ = $(CH_2)_2CN$

IX: R = ^{3}H, R′ = $COCH_3$

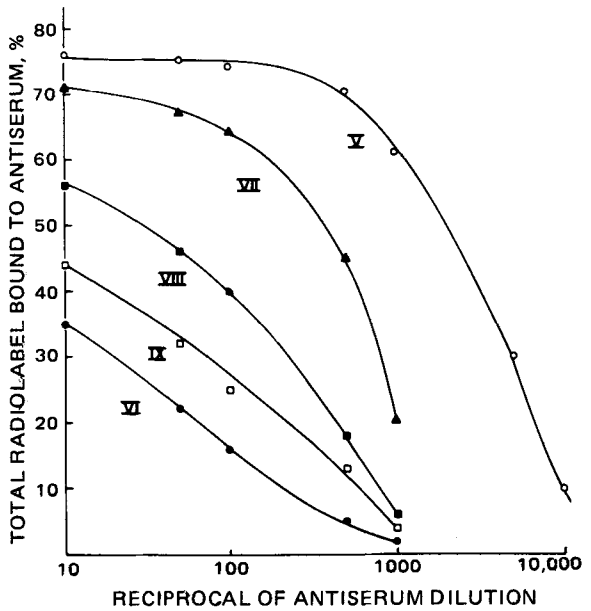


Figure 1—*Binding of various* d-*pseudoephedrine radioligands to increasing dilutions of* d-*pseudoephedrine antiserum.*

Separation of antibody-bound radiolabel from free radiolabel was achieved by charcoal adsorption of free radiolabel or polyethylene glycol 6000 precipitation of antibody-bound radiolabel as already described. Standard curves were expressed as % B/B_0 *versus* log *d*-pseudoephedrine free base concentration, where B_0 represents the amount (counts per minute) of [α-^{3}H]-*dl*-pseudoephedrine bound in the absence of any unlabeled *d*-pseudoephedrine and B is the amount bound at a given *d*-pseudoephedrine standard concentration (corrected for nonspecific binding).

*Indirect (Extracted and Derivatized) Assay Using [^{125}I]-*d*-Pseudoephedrine-*N*-3-propionic Acid–Tyrosine Methyl Ester Amide Label*—A similar series of *d*-pseudoephedrine standard solutions, control solutions, and appropriately diluted unknowns in filtered normal human plasma ranging in concentration from 0.2 to 50 ng/ml was prepared. One-milliliter aliquots of standards, controls, or unknown dilutions were pipetted into 15-ml glass centrifuge tubes with polytef-lined screw caps, followed by 2 *N* NaOH (0.5 ml, in saturated sodium sulfate). The basic plasma samples were shaken for 15 min with 7 ml of a mixture of cyclopentane–ether (1:1). After centrifugation, the lower aqueous layer was frozen in a dry ice–acetone bath and the solvent layer was decanted into a clean centrifuge tube.

The process was repeated with an additional 7 ml of solvent, and the combined extracts were evaporated under nitrogen at room temperature. The residue was taken up in methanol (2 ml) containing methyl acrylate (10 μl, 9.56 mg, 0.11 m*M*). The tubes were shaken gently overnight at room temperature, the contents were made acidic by the addition of 1 drop of a saturated solution of hydrogen chloride gas in methanol, and the tubes were shaken for 1 min. After evaporation under nitrogen at room temperature, the residues were dissolved in assay buffer (1 ml), and the tubes were shaken for 1 min.

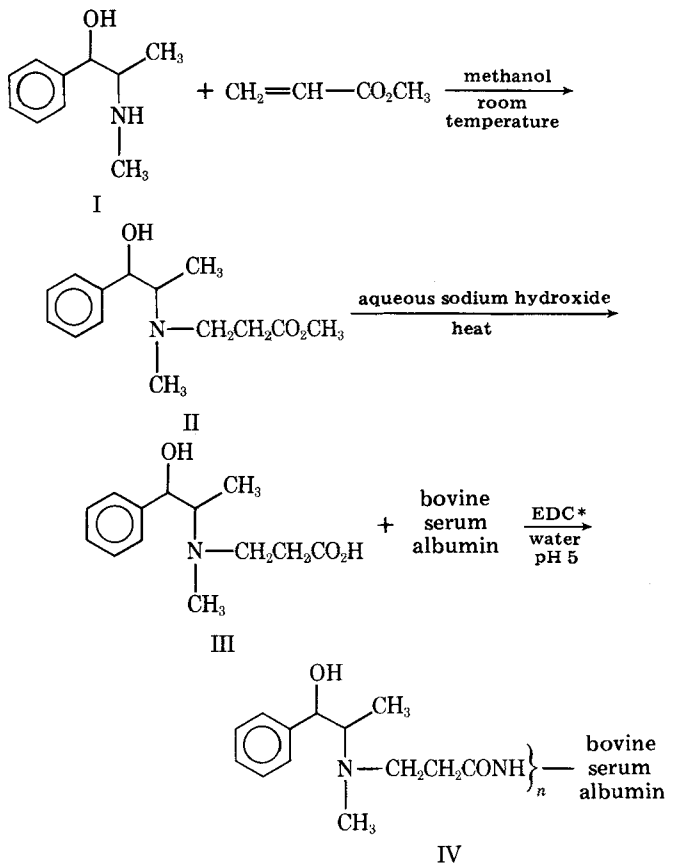
The radioimmunoassay incubation mixture was similar to that employing the tritium label and consisted of standards, controls, or unknown plasma dilutions, after extraction and derivatization, in assay buffer (0.5 ml), [^{125}I]-*d*-pseudoephedrine-*N*-3-propionic acid–tyrosine methyl ester amide (100,000 dpm) in assay buffer (0.1 ml), anti-*d*-pseudoephedrine serum (1:1500 dilution) in assay buffer (0.1 ml), and assay buffer (0.3 ml). After incubation for 2 hr at 0°, free radiolabel was adsorbed onto bovine serum albumin-coated charcoal as already described; the antibody-bound radiolabel contained in the supernates was quantitated. Data were reduced to standard curves, and control and unknown dilution concentrations were determined as already described.

Cross-Reactivity Studies—The procedure was similar to that described for the direct radioimmunoassay. A series of standard solutions of *d*-pseudoephedrine hydrochloride (0.2–100 ng of free base equivalents/ml) and some metabolites and structurally related compounds (1–100,000 ng/ml) in normal human plasma were incubated directly, or after extraction and derivatization, with antiserum and radiolabel and processed as described. Standard curves were drawn for each compound. Cross-reactivities were expressed as the percentage ratio of the *d*-pseudoephedrine concentration required to inhibit binding of the radiolabel to antiserum by 50% (I_{50}) to that of each respective compound (14).

RESULTS

Immunogen Synthesis—The hapten used for the immunogen preparation, *d*-pseudoephedrine-*N*-3-propionic acid (III), was prepared by aqueous base hydrolysis of the ester product of a Michael addition of methyl acrylate to *d*-pseudoephedrine (Scheme I). The product was characterized by NMR and mass spectral analyses prior to coupling to bovine serum albumin *via* a carbodiimide-mediated amide bond formation using reaction conditions similar to those described by Cheng *et al.* (15).

Radioligand Syntheses—*[α-^{3}H]-*dl*-Pseudoephedrine Hydrochloride (VI)*—This compound was prepared by a modification of the methods described previously (16–19) for the unlabeled compound. Bromination of propiophenone with bromine in refluxing methylene



Scheme I—Synthesis of d-*pseudoephedrine–bovine serum albumin conjugate (*EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride).*

Table I—Interassay Accuracy and Precision of Pseudoephedrine Radioimmunoassay Procedures [a]

Iodine 125–Extraction–Derivatization Method [b]			
Concentration [c] added	0.3	0.7	3.0
Concentration measured ± *CV*, % (*n* = 4)	0.3 ± 3	0.69 ± 3	2.98 ± 8
Tritium–Direct–Charcoal Separation Method			
Concentration added	3	7	30	60
Concentration measured ± *CV*, % (*n* = 4)	2.8 ± 4	6.4 ± 2	27.7 ± 2	61.0 ± 1
Tritium–Direct–Polyethylene Glycol 6000 Separation Method			
Concentration added	3	30	60
Concentration measured ± *CV*, % (*n* = 7)	3.1 ± 5	28.3 ± 2	60.3 ± 1

[a] Pseudoephedrine was added to blank human plasma for all recovery studies. [b] Plasma samples were carried through the entire extraction–derivatization procedures. [c] All concentration values are nanograms per milliliter.

chloride solution gave *dl*-α-bromopropiophenone, which was condensed with methylamine gas in ethanol at 0° essentially by the method of Hyde *et al.* (20) to give, after purification, *dl*-ephedrone hydrochloride. Treatment of this compound with sodium boro[^{3}H]hydride in ethanol gave a quantitative yield of predominantly the *erythro* compound, [^{3}H]-*dl*-ephedrine hydrochloride. In the Welsh procedure (18, 19), this *erythro* compound undergoes an N → O acyl migration with inversion of configuration upon reaction with refluxing acetic anhydride while the *threo* isomer undergoes the same migration with retention of configuration.

Thus, acetylation of the crude ephedrine, followed by hydrolysis in aqueous hydrochloric acid, gave the free base [^{3}H]pseudoephedrine, which was converted to VI (64% yield from ephedrine). Two recrystallizations from isoamyl alcohol–tetrahydrofuran gave pure [^{3}H]pseudoephedrine hydrochloride for use in radioimmunoassay studies. Two useful methods for determination of the enantiomeric purity of the final product were developed during the synthetic work. TLC on silica gel layers in 2-butanone–2-propanol–ammonium hydroxide (6:3:1) clearly separated ephedrine (R_f 0.49) and pseudoephedrine (R_f 0.82). Also, the chemical shift of the α-proton doublet in the ^{1}H-NMR spectrum could be used to detect ephedrine (δ 5.24, d, J = 3 Hz) in the presence of pseudoephedrine (δ 4.62, d, J = 10 Hz) and vice versa. The specific radioactivity of VI was estimated to be 5 Ci/mmole.

N-*Functionalized Radioligands*—Reaction of acetyl chloride, acrylonitrile, or methyl acrylate with VI, as described under *Experimental*, gave the *N*-acetyl amide (IX), *N*-propionitrile (VIII), or *N*-propionic acid methyl ester (VII) derivatives, respectively (Fig. 1). These radioligands were used to examine the affinity of the antiserum for radioligands bearing differing components of the bridge between hapten and bovine serum albumin in the immunizing conjugate.

^{125}*I-Labeled Radioligand*—A radioiodine-substituted ligand (V, Fig. 1) was prepared from *d*-pseudoephedrine-*N*-3-propionic acid–tyrosine methyl ester amide by reaction with sodium [^{125}I]iodide and chloramine-T by the general method of Hunter and Greenwood (21). The specific radioactivity of the ligand was estimated to be 270 Ci/m*M*.

Comparative Binding of Radioligands by Antibody—Figure 1 compares the ability of increasing dilutions of antiserum 515-1 to bind a constant amount of tritiated radioligands under identical incubation conditions. Since the specific activities of the tritiated radioligands were the same, the figure indicates the relative affinity of the antibody for pseudoephedrine and its various side-chain analogs. As the series progressed from underivatized [^{3}H]pseudoephedrine to *N*-acetyl-[^{3}H]-pseudoephedrine to the *N*-propionitrile analog and, finally, to the *N*-propionic acid methyl ester, the immediate precursor of the hapten derivative, higher dilutions of antiserum were capable of binding similar amounts of the radioligand. Clearly, the presence of the side chain was well recognized by this antiserum. The antiserum appeared to recognize the ^{125}I-labeled radioligand well, although affinity comparisons with the ^{3}H-labeled radioligands are difficult due to the higher specific activity of the iodinated compound.

Assay Precision and Sensitivity—Typical mean standard curves for the three individual radioimmunoassays are shown in Fig. 2. Each mean curve was constructed from four individual standard curves. The standard deviations on the curves indicate the excellent interassay precision. Further confirmation of interassay precision is shown by the assay data (means and standard deviations) for *d*-pseudoephedrine-spiked plasma controls that varied across the range of standard curve sensitivity for the separate radioimmunoassay procedures (Table I). The coefficient

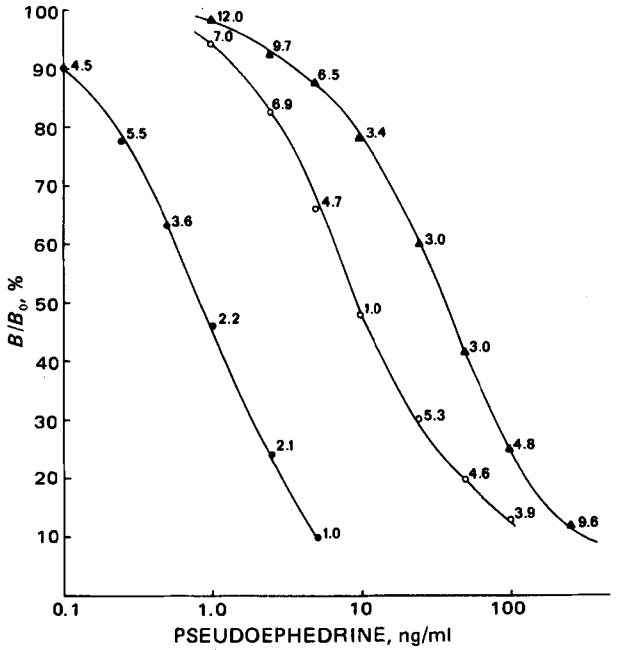


Figure 2—*Typical standard curves for* d*-pseudoephedrine radioimmunoassay procedures. Key:* ●, *iodine 125–derivatization–charcoal;* ○, *tritium–direct–charcoal; and* ▲, *tritium–direct–polyethylene glycol.*

of variation values did not exceed 8% for any control sample; for most samples, the coefficient of variation was <3%.

The indirect radioimmunoassay procedure, utilizing a radioiodine ligand and extraction and derivatization of *d*-pseudoephedrine prior to assay, is the most sensitive and specific technique, clearly able to detect 0.2 ng of *d*-pseudoephedrine/ml (IC_{50} = 4.3 ng/ml). The direct radioimmunoassay procedure, utilizing a tritium radioligand and a bovine serum albumin-coated charcoal bound/free separation scheme, is the next most sensitive procedure with a detection limit of 1 ng of *d*-pseudoephedrine/ml (IC_{50} = 10 ng/ml). However, due to the observed time-dependent tendency of the bovine serum albumin-coated charcoal to remove [^{3}H]-*dl*-pseudoephedrine from its binding sites on the antibody, the capacity of the assay (standards, controls, and unknowns) is limited.

For this reason, an alternative direct radioimmunoassay procedure using [^{3}H]-*dl*-pseudoephedrine was developed. Polyethylene glycol 6000 precipitates immunoglobulins (22) and, thus, antibody-bound radiolabel from the assay mixture. This precipitation achieves a separation of antibody-bound from free radiolabel that is relatively independent of time. The lower detection limit of this assay is 2.5 ng/ml (IC_{50} = 35 ng/ml).

Assay Specificity—The relative specificities of the three radioimmunoassay procedures are shown in Table II. *d*-Norpseudoephedrine cross-reacted between 0.8 and 5.0%, with the indirect extraction–derivatization radioimmunoassay procedure being most specific and the direct–charcoal method being least specific. *l*-Ephedrine, the diastereomer of *d*-pseudoephedrine, was recognized by the antiserum 200–800 times less effectively than *d*-pseudoephedrine. In this case, while cross-reactivity was low in all three procedures, the direct–charcoal method was the most specific. *l*-Pseudoephedrine, the optical isomer of *d*-pseudoephedrine, had very low cross-reactivity in all assays (<0.003–0.01%).

More detailed cross-reaction studies were pursued using the polyethylene glycol 6000–direct procedure. The antibody was clearly able to distinguish the lack of a β-methyl group (*N*-methylphenylethanolamine, 0.26%), the lack of an α-hydroxy group (methamphetamine, 0.08%), oxidation of the α-hydroxy group (ephedrone, <0.003%), and hydroxylation of the aromatic ring (*p*-hydroxyephedrine, <0.003%). Endogenous phenylethanolamines, such as epinephrine, and drugs that may be coadministered with pseudoephedrine, such as triprolidine and codeine, cross-reacted <0.003%.

Validation of Radioimmunoassays (Correlation with Standard GLC Assay)—Final validation of the assays was achieved by comparative analysis by radioimmunoassay and GLC of human plasma samples containing *d*-pseudoephedrine (obtained from volunteers who had ingested a 60-mg tablet of *d*-pseudoephedrine hydrochloride). Figure 3 shows correlation diagrams relating plasma *d*-pseudoephedrine levels

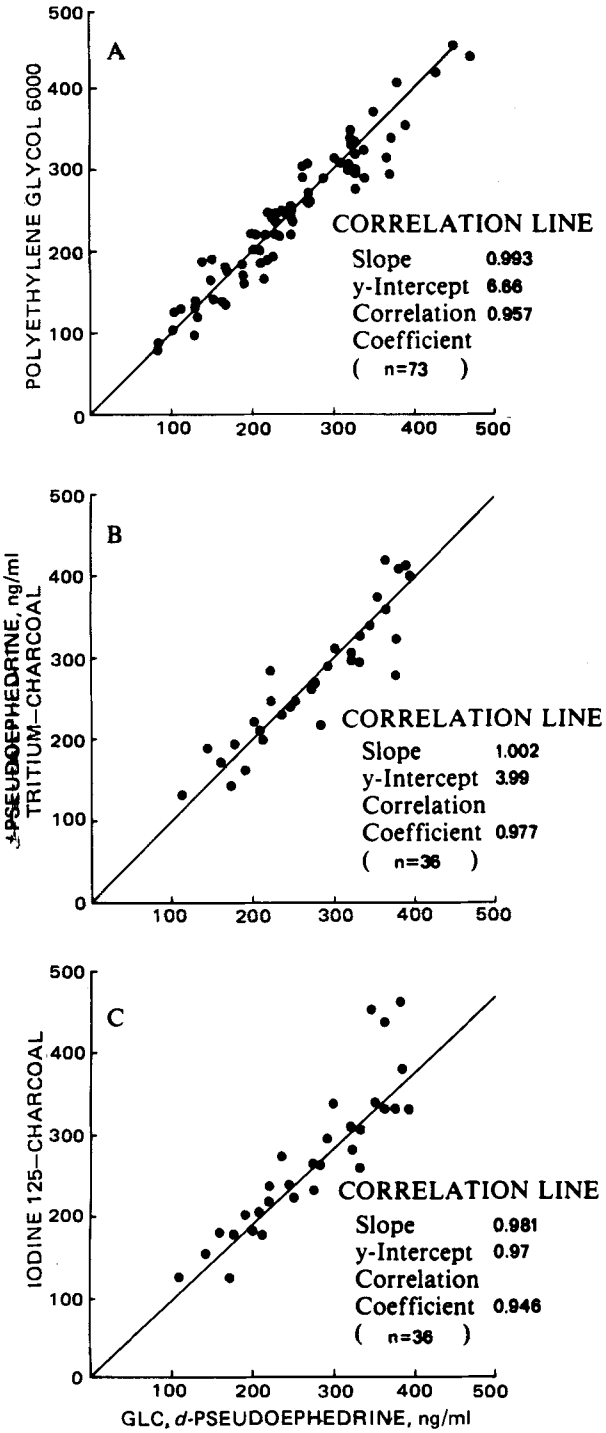


Figure 3—*Correlation of pseudoephedrine concentrations in human plasma samples as analyzed by a GLC procedure and tritium–direct–polyethylene glycol radioimmunoassay (A), tritium–direct–charcoal radioimmunoassay (B), or iodine 125–derivatization–charcoal radioimmunoassay (C).*

determined by a derivatization GLC procedure with electron-capture detection (10) to those determined by the three radioimmunoassays. In each case, linear regression analysis gave correlation coefficients between 0.946 and 0.977 and slopes of 0.981–1.002, indicating excellent agreement between all three radioimmunoassay procedures and the reference GLC assay.

Disposition Studies in Humans—For convenience and ease of operation, the direct radioimmunoassay utilizing [^{3}H]pseudoephedrine and the polyethylene glycol 6000 separation method was used in all human drug disposition studies. This assay was applied to a bioequivalence comparison of *d*-pseudoephedrine from a sustained-release preparation

Table II—Cross-Reactivities (%) of Related Compounds in Pseudoephedrine Radioimmunoassay Procedures

Compound	Tritium–Direct–Charcoal	Tritium–Direct–Polyethylene Glycol 6000	Iodine 125–Extraction–Derivatization
d-Pseudoephedrine	100	100	100
d-Norpseudoephedrine	5.0	1.75	0.8
l-Pseudoephedrine	0.01	0.05	<0.003
l-Ephedrine	0.13	0.44	0.32
l-Norephedrine	—	0.02	—
d-Ephedrine	—	0.35	—
N-Methylphenylethanolamine	—	0.26	—
Methamphetamine	—	0.08	—
dl-*p*-Hydroxyephedrine	—	<0.003	—
Ephedrone	—	<0.003	—
Metaraminol	—	<0.003	—
Methoxamine	—	<0.003	—
Phenylethylamine	—	<0.003	—
l-Phenylephrine	—	<0.003	—
Epinephrine	—	<0.003	—
Triprolidine	—	<0.003	—
Codeine	—	<0.003	—

(containing 120 and 5 mg, respectively) and an immediate-release preparation (containing 2.5 and 60 mg, respectively) of *d*-pseudoephedrine hydrochloride and triprolidine hydrochloride in 17 healthy male subjects. The study extended over 126 hr in each of two separate periods, during which all subjects received both formulations (*Experimental*). Doses of sustained-release medication were administered at 12-hr intervals (8 am and 8 pm), while immediate-release tablets were given at 6-hr intervals (8 am, 2 pm, 8 pm, and 2 am) throughout the first 120 hr. Mean plasma pseudoephedrine levels in these subjects over the entire 126-hr study period as determined by the direct radioimmunoassay are shown in Fig. 4.

Steady-state concentrations were approached with both drug formulations by the 3rd day of treatment, with mean trough concentrations of 280–350 ng/ml and mean peak concentrations of 420–560 ng/ml for the two preparations. Additional blood samples were taken from 8 am to 8 pm on Day 5 to define more closely the plasma concentration–time profiles at steady state for immediate- and sustained-release preparations. The mean plasma concentration–time curves for all subjects during this period are shown in Fig. 5. The mean observed peak concentration following the first (8 am) dose of the immediate-release tablet (containing 60 mg of pseudoephedrine hydrochloride) was 565.8 ng/ml and that following the second (2 pm) dose was 554.6 ng/ml. The maximum concentration observed following administration (at 8 am) of the sustained-release preparation (which contained 120 mg of pseudoephedrine hydrochloride) was 550.6 ng/ml. Although the mean peak plasma concentrations achieved with the two formulations were not significantly different, mean plasma concentrations of pseudoephedrine were maintained for a considerably longer period with the sustained-release preparation.

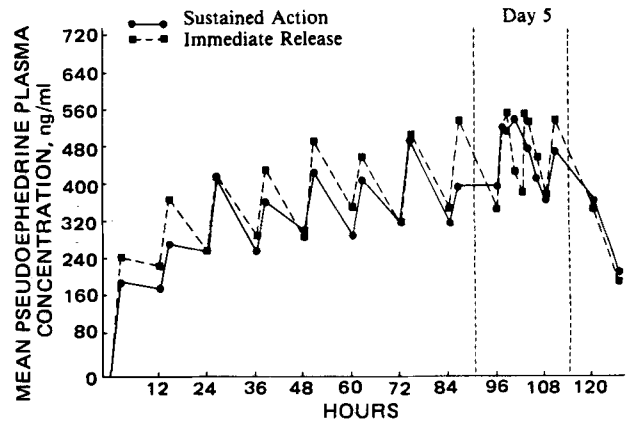


Figure 4—*Mean plasma pseudoephedrine concentrations over 126 hr in 17 healthy volunteers who received either a sustained-release preparation of pseudoephedrine every 12 hr or an immediate-release preparation every 6 hr for the first 5 days.*

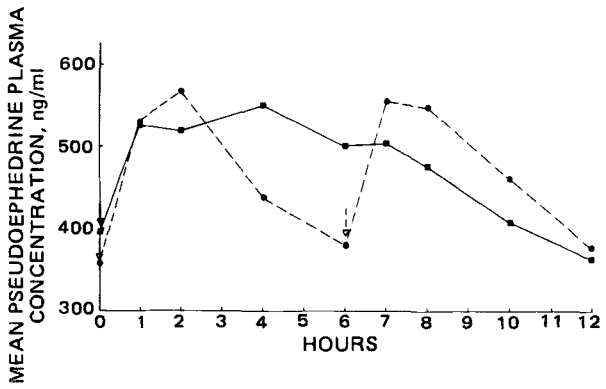


Figure 5—*Detailed presentation of the 8 am–8 pm period on Day 5 of the study presented in Fig. 4. Key:* ■, *sustained release; and* ●, *immediate release.*

DISCUSSION

Previous investigations demonstrated the need for conjugation of drugs to macromolecules to render them immunogenic (23–25). In the present study, *d*-pseudoephedrine was easily derivatized *via* the nitrogen atom by reaction with methyl acrylate followed by alkaline hydrolysis to give the *N*-3-propionic acid derivative for coupling to bovine serum albumin for immunization purposes. Antiserum raised in response to this immunization procedure was used to develop three radioimmunoassay procedures. In one, a tyrosine methyl ester derivative of the original hapten, prepared by carbodiimide-mediated reaction with tyrosine methyl ester followed by iodination, provided a radioligand with the convenience of a γ-emitting radioisotope. However, since this ligand could not be displaced by *d*-pseudoephedrine at the required sensitivity level, this assay modification required prior extraction of all pseudoephedrine-containing samples and derivatization with methyl acrylate before their addition to the radioimmunoassay system.

[α-^{3}H]-*dl*-Pseudoephedrine and unlabeled pseudoephedrine were used to demonstrate that the drug could be extracted and derivatized by this method with a mean overall recovery of >95% across the concentration range of 0.1–500 ng/ml when performed under the conditions specified under *Experimental*. This assay modification has better sensitivity and specificity than the other variations and may be useful in studies where maximal sensitivity is required, such as tissue distribution experiments. The assay variants employing [^{3}H]pseudoephedrine as the radioligand are somewhat less sensitive than the derivatization assay using the ^{125}I-labeled radioligand and use the antiserum at a much lower titer, but these variations have the distinct advantage of providing reliable drug analyses directly on plasma, *i.e.*, without prior extraction. The assay using the polyethylene glycol 6000 method for separation of antibody-bound from free radioligand had the advantage of being less time dependent when compared to the bovine serum albumin-coated charcoal separation method. Thus, large assay runs were more reliably performed with the former system.

All three assay systems are accurate and precise (Table I), and have sensitivity limits in the range of 0.2–2.5 ng/ml for iodine 125–derivatized and tritium–polyethylene glycol methods, respectively (0.2–0.25-ng actual mass of pseudoephedrine, see *Experimental*). This sensitivity far exceeds that of the earlier GLC procedures for pseudoephedrine (7, 10, 11) and is better than that of a more recent GLC method using nitrogen detection that was capable of detecting a lower limit of 25 ng/ml (12). Acetylation of pseudoephedrine with [^{3}H]acetic anhydride provides a method capable of detecting 50 ng/ml of the drug in plasma (8). Recently, a pseudoephedrine pharmacokinetic study (26) was reported in the rat using an electron-capture GLC microtechnique capable of detecting 2 ng of drug. This method is still less sensitive and far more cumbersome than direct radioimmunoassay with the [^{3}H]pseudoephedrine radioligand described here. All three radioimmunoassay variants were validated by comparison with an accepted GLC analytical method (10), giving an excellent correlation in each case (Fig. 3).

The ability of drug antiserum to discern changes in molecular architecture is normally greatest for structural changes at sites distant from the site of attachment of the drug to the macromolecular carrier. Thus, *d*-norpseudoephedrine, which was reported to be a minor metabolite of pseudoephedrine (11) in humans, unexpectedly cross-reacted only to the extent of 0.8–5.0% (Table II). In the nor compound, a methyl group was lacking on the nitrogen atom on which the bridge to protein in the immunogen was attached. The low cross-reactivity in the derivatized assay with the γ label is easier to understand in that norpseudoephedrine would presumably react with methyl acrylate in the derivatization step to give a bis adduct, which would then be recognized less effectively by the antiserum.

Several recent reports described the ability of antiserum to discern stereochemical differences in drug molecules. Cook *et al.* (27) described the development of stereoselective radioimmunoassay procedures for (*R*)- and (*S*)-warfarin, Gross and Soares (28) prepared antiserum to *d*-amphetamine, and Kawashima *et al.* (29) reported radioimmunoassay procedures for *l*- and racemic propranolol. The *d*-pseudoephedrine antiserum developed in the present study also possesses a high degree of stereospecificity, as shown by the very low cross-reactivities of *l*-pseudoephedrine, *d*- and *l*-ephedrines, and *l*-norephedrine. A change in the aromatic ring of the ephedrine series, such as in *dl*-*p*-hydroxyephedrine, essentially eliminates recognition by the antiserum. Changes in side-chain structure, as represented by ephedrine, *N*-methylphenylethanolamine, methamphetamine, metaraminol, methoxamine, phenylethylamine, and *l*-phenylephrine, are well recognized by the antiserum, resulting in very low cross-reactivities. A representative catecholamine, epinephrine, caused no inhibition of radioligand binding, nor did triprolidine and codeine, which may, on occasion, be coadministered with pseudoephedrine.

The pharmacokinetics of *d*-pseudoephedrine in humans have not yet been investigated in detail, although some data relating plasma levels to pharmacological effects were reported (12). A major limitation to the pursuit of such studies has been the lack of suitable analytical methodology. The applicability of the present radioimmunoassay technique to pharmacokinetic studies is shown by the data in Figs. 4 and 5. The day-to-day plasma levels in this bioequivalence study were measured easily with the direct radioimmunoassay procedure with polyethylene glycol 6000 as the separation system. As seen in Fig. 4, the assay demonstrated that steady-state plasma concentrations of pseudoephedrine were approached by Day 3 of dosing with either formulation.

The bioequivalence of the immediate- and sustained-release preparations in these subjects was demonstrated by the lack of significant difference between the mean area under their plasma concentration–time curve (*AUC*) values over the entire 126-hr period of the study [43,796 ± 2738 (*SEM*) ng/ml hr for the sustained release and 47,910 ± 3085 (*SEM*) ng/ml hr for the immediate release]. Since this *AUC* comparison was approximate due to the infrequent blood collections during Days 1–4, a similar *AUC* comparison was made for 8 am–8 pm on Day 5, when steady-state concentrations had been achieved and additional blood samples were collected. From these data (Fig. 5), *AUC* calculations again indicated bioequivalence of immediate- and sustained-release preparations [5744 ± 395 (*SEM*) ng/ml hr for the sustained release and 5674 ± 354 (*SEM*) ng/ml hr for the immediate release].

Figure 5 clearly shows that, although the observed maximum plasma concentrations following each formulation were not significantly different, the mean concentrations were maintained in the 450–550-ng/ml range for ~8 hr with the sustained-release preparation, thus avoiding the trough at 6 hr and the peak at 7–8 hr that occurred with the two-dose regimen of the immediate-release preparation. Thus, the radioimmunoassay has been used to demonstrate the rationale for this type of sustained-release preparation. Recently, studies of similar pseudoephedrine formulations have produced similar results (30, 31).

These radioimmunoassay procedures are being applied currently to detailed studies of the disposition of *d*-pseudoephedrine in animals and humans.

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Comparative Teratogenicity of Cortisone and Phenytoin in Mice

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Abstract □ Single administrations of cortisone or phenytoin to pregnant mice on Days 11–14 of gestation caused similar skeletal and dissimilar soft tissue fetal anomalies. Cortisone reduced both maternal and fetal weight, whereas phenytoin only reduced fetal weight without adversely affecting maternal weight. A correlation between fetal weight reduction and cleft palate incidence was evident for each drug. Because probit analysis of dose–response regression lines did not deviate from parallelism after drug challenge, it was concluded that cortisone and phenytoin may produce palatal anomalies in the mouse fetus by a similar mechanism.

Keyphrases □ Teratogenicity—effects of cortisone and phenytoin on mice □ Cortisone—teratogenic effects on mice □ Phenytoin—teratogenic effects on mice □ Antiepileptics—teratogenic effects of phenytoin on mice

The experimental use of various agents to elicit and inhibit cleft palate has been pursued vigorously in recent years to determine its cause and to prevent its occurrence in human newborns. Although cortisone is the prototypal agent employed for the induction of this anomaly in lower species (1), phenytoin is of particular interest because of the purported link between its use as an antiepileptic drug during pregnancy and the appearance of cleft palate in offspring (2, 3).

The purpose of this investigation was to compare the teratogenic effects of phenytoin with those of cortisone by means of dose–response relationships in mice and to ascertain whether the mechanism involved in cleft palate inception by cortisone and phenytoin is similar.

EXPERIMENTAL

Animals—CF-1 albino mice[1] (25–35 g) were used. Females were confined in groups of 10 to aggregate cages for at least 2 weeks prior to mating. Males were placed individually in metal cages (12.5 × 15 × 10 cm) with a wire mesh front and floor[2]. Adult mice were maintained on a commercial diet[3] and tap water *ad libitum*. The breeding room was equipped with an electrical system[4], which provided 12 hr of light (7:00 am–7:00 pm) and 12 hr of darkness. Room temperature was maintained between 22 and 26°.

Preparation of Solutions—Saline and cortisone acetate solutions were prepared commercially[5,6]. Fresh aliquots (10 ml) of phenytoin solution were prepared as needed on the day of injection by dissolving the drug[7] in a stock solution of 70% propylene glycol[8] (in saline). Phenytoin solutions were prepared containing 10, 14, 16, or 18 mg of phenytoin/ml of vehicle in injection volumes of 0.1–0.3 ml. All injections were made with a glass syringe[9]. The bone-staining solution was prepared by dissolving 18 mg of alizarin red-S in each liter of 1% KOH in distilled water. A 50:50 mixture of 70% ethyl alcohol and 100% glycerin was used to fix and clear the specimens. Fetuses chosen for soft tissue analysis were fixed in Bouin's solution.

Breeding Procedure and Treatment Regimen—The breeding procedure was previously described (4). Gravid females were randomly

[1] Charles River Breeding Laboratories, Wilmington, Mass.
[2] RD-T unit, Norwich Wire Works, Norwich, N.Y.
[3] Rodent Laboratory Chow 5001, Ralston-Purina Co., St. Louis, Mo.
[4] Astronomic dial time switch with "skipper," model V-45073, International Register Co., Spring Grove, Ill.
[5] Sodium chloride injection USP (0.9%), lot B7D091A, McGraw Laboratories, Division of American Hospital Supply Corp., Irvine, Calif.
[6] Cortone, lot 0613A, Merck Sharp and Dohme, West Point, Pa.
[7] Dilantin Sodium, lot PE338, Parke-Davis Co., Detroit, Mich.
[8] Lot P-810, Amend Drug and Chemical Co., Irvington, N.J.
[9] B-D 1-ml tuberculin syringe with 1.27-cm, 26-gauge needle.

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