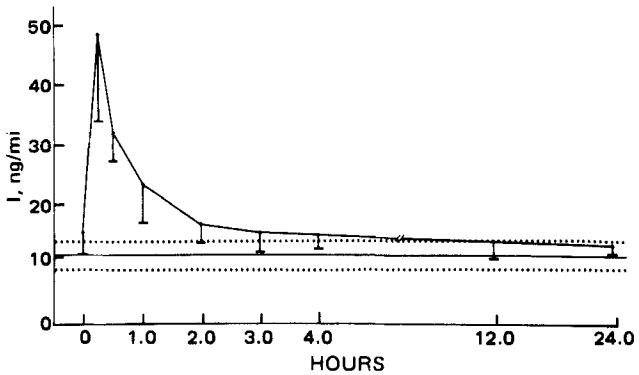

Figure 2—*Average plasma levels of I (Δ^9-THC) for 11 marijuana smokers (not corrected for contribution of I-d_3) and 10 nonsmokers. The solid horizontal line represents the average plasma level of I found in nonusers and is due to the impurity present in the internal standard, whereas the 99% confidence limits for this value are represented by the horizontal dotted lines. The vertical lines at each data point represent the lower half of the confidence limits for those 11 determinations made in the marijuana smokers at the appropriate time interval.*

99% confidence limits of I found as a contaminant in the internal standard.

Figure 2 is a plot of the average uncorrected plasma I levels of 11 male volunteers who smoked one marijuana cigarette of known I content. The maximum blood I level occurred at 0.25 hr. Also the I level in the control or 0-hr sample was positive. This fact can be explained on the basis of the type of subject used in the marijuana smoking studies. All subjects were moderate marijuana users. Each was confined 12 hr prior to smoking and was asked not to smoke marijuana for 2 days prior to the study. The analytical data obtained on the plasma of each subject indicated that only five individuals conformed to the established protocol.

The 99% confidence levels, as determined by the Student distribution (t-value) method, are shown by the vertical bars at each data point. The horizontal lines represent the average value (dark line) for 10 nonsmokers of marijuana as well as the 99% confidence limits (dotted lines). Wherever the confidence limits for data from both marijuana smokers and nonsmokers coalesce, a time period exists where marijuana use could not be assigned with 99% confidence. Thus, for up to 1 hr after marijuana smoking, the levels of I are clearly determinable and marijuana use can be assigned with 99% confidence using the present assay method.

The HPLC–mass spectrometric methodology described would be applicable to the study of other drug entities in biological fluids where very low detection levels are required along with precise specificity. However, for such determinations, the stable isotopic form of the drug is needed.

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ACKNOWLEDGMENTS AND ADDRESSES

Received June 1, 1976, from the *Bio-Analytics Laboratory, School of Pharmacy and Department of Physics, University of Missouri–Kansas City, Kansas City, MO 64108.*

Accepted for publication November 4, 1976.

Supported by Contract DOT HS 4 00968 from the Department of Transportation and the National Institute on Drug Abuse and in part by a Faculty Research Grant from the University of Missouri.

The authors are grateful for the assistance provided by Ms. Phyllis Lessin and Mr. Robert Nowlan of the Marijuana Research Institute, School of Medicine, University of California–Los Angeles. The authors also thank the School of Medicine, University of Missouri–Kansas City, for providing facilities for this work.

[x] To whom inquiries should be directed.

Radioimmunoassay of Minoxidil in Human Serum

MAX E. ROYER [x], HOWARD KO, TERRY J. GILBERTSON, JOHN M. McCALL, KAREN T. JOHNSTON, and RONALD STRYD

Abstract □ A simple, sensitive, and specific radioimmunoassay for determining minoxidil was developed. Antiserums to two minoxidil haptens were compared for cross-reactivity and assay levels on human serums. One antiserum had little cross-reactivity with minoxidil metabolites. The radioimmunoassay is specific for determining minoxidil directly in serum without extraction. Human serum minoxidil levels were determined from a single oral dose.

Keyphrases □ Minoxidil—radioimmunoassay, human serum □ Radioimmunoassay—minoxidil in human serum □ Antihypertensives—minoxidil, radioimmunoassay in human serum

Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) (I) is a potent hypotensive agent useful in the treatment of severe hypertension (1, 2). A sensitive assay was needed for determining serum I levels. Attempts with a GLC procedure indicated that sizable amounts of the several derivatives tried were lost on the column. High-pressure liquid chromatography (HPLC) would have adequate sensitivity for only the higher serum concentrations encountered at normal dosage levels of I. Chromatographic methods for I in serum also require extraction and a preliminary chromatographic cleanup step. Such procedures are tedious and time consuming. Radioimmunoassay, however, permits analysis of many samples without extraction and with adequate sensitivity.

Antiserums were developed in rabbits against two bovine serum albumin conjugates of the N-4-glutaryl (II) and

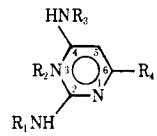
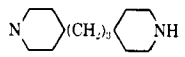
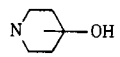


Table I—Specificity of Antiseruns to Bovine Serum Albumin Conjugates of Compounds II and III

Compound	R_1	R_2	R_3	R_4	Cross-Reactivity[a], % Anti-II	Anti-III
I	H	O	H		100	100
II	H	O	$C(CH_2)_3COH$ (O, O)		144	2.3
III	H	O	H		<0.1	760
IV	H	O	H		0.7	28
V	$CH_3C(=O)$	O	$CH_3C(=O)$—		5.1	<0.1
VI	H	—	H		<0.1	<0.1
VII	—[b]	—[b]	—[b]		1.4	1.3
VIII	H	O	H	$N(CH_2)_4COH$ (O)	<0.1	0.4

[a] Defined as: (100 × concentration of I at 50% binding)/concentration of compound tested at 50% binding. [b] Position of the glucuronic acid attachment not established.

dipiperidine (III) derivatives of minoxidil (Table I). These antiserums were compared for specificity, and one was chosen for further development of the radioimmunoassay.

EXPERIMENTAL

Synthesis of II and Conjugation to Bovine Serum Albumin—Two grams of ^{14}C-minoxidil and 1.09 g of glutaric anhydride were refluxed for 2 hr in 150 ml of dry acetone. The residue was filtered and washed to give 2.8 g of II. TLC in methanol–benzene–ammonium hydroxide (100:100:1) indicated one component. The NMR shift of the proton at the 5-position and X-ray crystallography of the methyl ester of II indicated a single substitution on the amino group at the 4-position.

Anal.—Calc. for $C_{14}H_{21}N_5O_4$: C, 53.40; H, 6.87; N, 20.76. Found: C, 53.51; H, 6.96; N, 21.07.

Compound II, 165 mg, and 300 mg of bovine serum albumin were dissolved in 10 ml of 50% pyridine (3). Over 15 min, 193 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added slowly; the mixture was then stirred for 16 hr. After dialysis and freeze drying, 383 mg of material was recovered.

A portion of the conjugate was washed exhaustively with acetone followed by chloroform to remove any undialyzed II. The amount of ^{14}C-II remaining on bovine serum albumin indicated that approximately 29 moles of II/mole of bovine serum albumin was covalently linked.

Preparation of III and Conjugation of Bovine Serum Albumin and III to Agarose Gel Support—A solution of 3.0 g of 2,4-diamino-6-chloropyrimidine 3-oxide in 11.15 g of 4,4′-trimethylenedipiperidine was heated at 80° and then stirred with aqueous sodium bicarbonate. The product (III) was dissolved in methanol and precipitated by addition of acetonitrile.

Compound III was attached to a modified agarose gel[1] which, in turn, was conjugated to bovine serum albumin analogous to the preparation of solid phase immunogens described by Trump (4). A slurry of 430 mg of modified agarose gel and 360 mg of bovine serum albumin in 20 ml of water was stirred for 3 hr. A solution of 430 mg of III in 20 ml of dimethylformamide was added. After 3 hr, 50 mg of sodium bicarbonate was added. After 12 hr, the conjugate was filtered and washed. The material was stored as a wet slurry.

Immunization and Antiserum Production—The conjugate of II (8 mg), 4 ml of 0.9% saline, and 4 ml of Freund's complete adjuvant[2] were emulsified in a blender[3]. A total of 1 ml was injected intradermally at multiple sites in the back and flanks of each of six New Zealand White rabbits. The rabbits were challenged again at 4 months with a similar injection except that Freund's incomplete adjuvant was used[2]. Only one rabbit gave significant antibody titer. Of the eight bleedings during 289 days after the first injection, the bleeding at Day 129 had the highest titer and was used for the radioimmunoassay.

Blood was collected by cutting the ear vein and applying gentle suction. The blood was allowed to clot 1–2 hr at room temperature and then was centrifuged. The serum was divided into aliquots and stored at −70°.

The immunization and bleeding schedules were carried out in a similar manner for the antiserum to the conjugate of III.

Assay Reagents—*Buffer A*—One hundred and ninety-two grams of tris(hydroxymethyl)aminomethane, 1 g of thimerosal[4], and 1 liter of 1.0 *N* HCl were diluted to make 10 liters of pH 7.8 (0.1 μ) buffer.

Buffer B—Human serum albumin[4] (0.1%) was added to Buffer A.

Counting Solution—Five hundred milliliters of nonionic detergent[5], 170 ml of scintillator fluid[6], and 3.79 liters of toluene (analytical reagent) were mixed.

Primary Antibody—Rabbit antiserum was diluted 1:100.

Secondary Antibody—Goat antirabbit antibody was diluted to give maximal precipitation of the primary antibody (1:8).

Label—3′,4′,5′(*n*)-^3H-Minoxidil, 25.6 Ci/mmole (5), approximately 13,000 dpm/ml in Buffer B, was the label.

Standards—Minoxidil was diluted in Buffer B.

Assay Procedure—Buffer B (for unknown samples) and pretreatment serum (for standards) were added to separate 12 × 75-mm glass culture tubes[7] with a 50-μl pipet[8]. The unknown sample or standard, 50 μl, and label, 500 μl, were added with an automatic pipet[9], and the tubes were incubated 1–2 hr at 25°. A normal standard curve consisted of duplicate standards with concentrations of 1, 2, 4, 8, 16, 32, 64, and 128 ng/ml. Four blanks (B_0) with no unlabeled drug and two infinity samples (>30,000 ng/ml) were also run. Approximately 75% of the label was bound at B_0. When the expected concentration of I was high, only 10 μl of the unknown was sampled and 40 μl of pretreatment serum was added to give a fivefold

[1] Affi-Gel 10, Bio-Rad Laboratories, Richmond, Calif.

[2] Difco Laboratories, Detroit, Mich.
[3] Sorvall, Ivan Sorvall, Inc., Newton, Conn.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] BioSolv, Beckman Instruments, Fullerton, Calif.
[6] Liquifluor, New England Nuclear, Boston, Mass.
[7] Dispo, Scientific Products, McGaw Park, Ill.
[8] Eppendorf, Brinkmann Instrument Co., Westbury, N.Y.
[9] Model 25004, Micromedic Systems Inc., Philadelphia, Pa.

Table II—Recovery and Within-Day and Between-Day Coefficients of Variation (*CV*) (*n* = 66)

Actual Amount, ng/ml	Amount Found, ng/ml	Recovery (*SEM*), %	*CV*, % Within Day	*CV*, % Between Day
8	7.12	89.0 ± 0.8	5.4	5.7
64	64.76	101.2 ± 1.1	6.5	6.5
256	253.68	99.1 ± 0.9	7.0	3.4

dilution. Primary antibody, 200 μl, was added to each tube, and the tubes were incubated 16–24 hr at 25°; then 200 μl of secondary antibody was added, and the tubes were incubated 40–72 hr at 5°.

The tubes were centrifuged at 1000×*g* for 30 min at 5°, and the supernate was immediately poured into counting vials[10]. The tubes were left to drain at an angle in the counting vials for about 30 min and then were discarded (6). Counting solution (15 ml) was added, and the samples were counted on a liquid scintillation counter[11] for 10,000 counts or 10 min. The counts of samples that may have contained quenching materials (*e.g.*, hemoglobin) and that had changes in counting efficiencies greater than ±0.5% from the median efficiency were corrected for counting efficiency by the external standard method.

The results were calculated by computer[12] using the Rodbard and Lewald (7) program. Six control samples also were run as unknowns at 8, 64, and 256 ng/ml. Since 256 ng/ml is outside the normal standard curve range, 40 μl of Buffer B was added to the tubes and only 10 μl was sampled (5× dilution).

The cross-reactivity of the antiserums was determined from plots of the standard curves measured for each compound tested. Serum, 50 μl, was added to each standard tube to approximate the conditions normally found in the assay.

RESULTS AND DISCUSSION

The following general considerations were followed in the radioimmunoassay development: (*a*) the antibodies should not cross-react significantly with known metabolites; (*b*) the assay should require a minimum of sample preparation; (*c*) where possible, precision and accuracy should be emphasized rather than sensitivity and speed; and (*d*) the assay should be generally applicable to measuring I in serum at many dosage levels.

Specificity—The major urinary metabolites of I are the glucuronide (VII) in humans and the 4′-hydroxy metabolite (IV) in dogs (8–10). The reduced analog (VI) and carboxy derivative (VIII) are also known metabolites of I. The cross-reactivities of these metabolites and several other I derivatives with antiserums to both II and III are shown in Table I. Both antiserums had the same titer and had adequate specificity against all known metabolites, with the exception of the 28% cross-reactivity of IV using the antiserum against III.

Since this might be a source of error in assaying for I in dog serum, the antiserum against II was selected for further assay development. At time points (2–4 hr after oral administration) at which metabolites in human serum would likely be observed, the apparent concentrations of I measured by the two antiserums (II and III) were not significantly different ($p > 0.05$). Either antiserum should have adequate specificity to determine I in human serum.

Factors Affecting Accuracy—If 50 μl of buffer were substituted for serum in the standards, a positive bias of about 2–11% would result. Because little was known about the subject-to-subject variation in protein binding and its effect on the radioimmunoassay, 50 μl of a subject's own pretreatment serum was added to each standard tube. A separate standard curve was run before and after each set of unknowns. This procedure ensured that the standards and samples contained as nearly as possible the same constituents and that systematic errors in pipetting or equilibrating during an assay averaged out.

The long equilibration times at each step ensured equilibrium conditions at each stage of the assay (11). Although shorter equilibration times may be permissible, the long equilibration times do not seriously affect the number of samples that can be run over a long period.

No binding loss of I to glass was observed after 10 sequential transfers from tube to tube. To ensure that I remained in solution, 0.1% human serum albumin was used as diluent for the label and standard solutions (11). Serum that contained significant amounts of hemoglobin gave low counts due to quenching. Corrections were made only if the counting efficiency was changed by more than ±0.5% from the median value of the subject's samples.

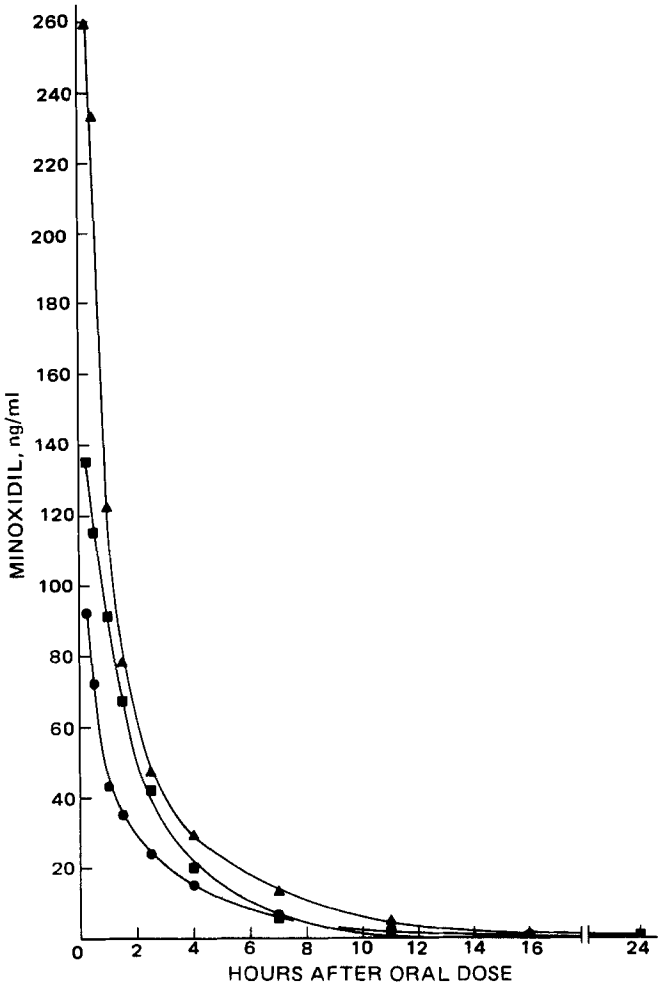


Figure 1—*Serum minoxidil levels after a single oral dose. Key:* ●, *Patient A, 10 mg;* ■, *Patient P, 20 mg; and* ▲, *Patient J, 30 mg.*

Assay Statistics—The mean limit of detection, as determined by the Rodbard and Lewald (7) program, for 18 standard curves was 3.02 ± 1.52 ng/ml (*SD*). With a primary antibody dilution of only 1:100, the assay as run was not set up for maximal sensitivity. The detection limit would be much lower if the primary antibody was further diluted and/or larger sample volumes were taken.

The recovery and the within-day and between-day coefficients of variation are shown in Table II. At 8 and 64 ng/ml, the percentage of label bound relative to that for B_0 was approximately 80 and 20%, respectively.

Human Serum Minoxidil Levels—Levels of ^{14}C-minoxidil and its metabolites previously were determined in human, dog, and monkey serum (8, 9). The radioimmunoassay offers a convenient means of determining I in human serum without administering radioactive I to the patient and without requiring subsequent chromatography of the metabolites.

Three volunteer patients with hypertension that was not controlled by other drugs were given single oral doses of I. Their serum levels of I are shown in Fig. 1. For 20 patients receiving a single dose of 5–100 mg, the peak serum levels were reached at 0.6 ± 0.5 hr (mean ± *SD*) and the mean (±*SD*) half-life was 1.4 ± 0.9 hr as determined by the radioimmunoassay.

Therefore, the radioimmunoassay for I has (*a*) adequate specificity for measuring serum I levels in the presence of other metabolites, (*b*) no requirement for extraction or extensive sample preparation (some dilution may be necessary at high levels), (*c*) adequate precision and accuracy for comparing serum levels and determining serum half-life, and (*d*) sufficient sensitivity and applicability to large numbers of samples.

[10] Packard Instrument Co., Downers Grove, Ill.
[11] Packard Tri-Carb model 3375.
[12] IBM 370/155 computer.

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ACKNOWLEDGMENTS AND ADDRESSES

Received September 3, 1976, from the *Drug Metabolism Research Section, The Upjohn Company, Kalamazoo, MI 49001.*

Accepted for publication November 10, 1976.

The authors thank Dr. Richard C. Thomas for the minoxidil glucuronide, Murray M. Cooper for adaption of the Rodbard and Lewald computer program, C. G. Wickrema Sinha for the X-ray crystallographic structure proof, and Dr. G. R. Lang, Dr. D. T. Lowenthal, and Dr. G. Bailey for the serum samples from volunteer patients.

[x] To whom inquiries should be directed.

Evaluation and Optimal Combination of TLC Systems for Qualitative Identification I: Sulfonamides

H. De CLERCQ, D. L. MASSART [x], and L. DRYON

Abstract □ A mathematical criterion for the evaluation of chromatographic analysis procedures is given by the information content as derived from Shannon's equation. This information content yields a numerical value representative of the merits of each chromatographic separation and thus allows selection of the optimal systems. In most cases, however, one analysis is not sufficient to allow the qualitative identification of the sample. Therefore, several chromatographic systems are combined. Two approaches allow the desired combination; one either calculates the information content of several procedures as one mathematical value or classifies the systems according to mutual resemblance by numerical taxonomy techniques. From the resulting groups of dissimilar systems, one optimal system can be chosen per group according to the information content. The results obtained by these mathematical procedures are illustrated with a practical example: the selection and evaluation of systems for the TLC analysis of sulfonamides.

Keyphrases □ TLC—systems, evaluation and optimal combination for qualitative determination of sulfonamides □ Sulfonamides, various—TLC systems for qualitative determination, evaluation and optimal combination

TLC is one major method in pharmaceutical analysis for the identification of organic compounds, and there is an enormous literature on the subject. It is not always easy to select the best TLC systems from the many that have been published, and it is more difficult to select the optimal combination of two or more systems. The reasons for this difficulty are:

1. The systems (any combination of stationary phase and solvent) are developed by many different workers, who use slightly different development procedures, saturation conditions, *etc.*

2. Most investigators do not use objective value judgments but rather state that their separation procedures yield either "good" or "excellent" or "poor" results for a group of substances. Furthermore, while it is rather easy to characterize a separation of two substances, it is often more difficult to characterize a separation of 10 substances.

3. Even if the selection of the individually best systems is possible, it is often nearly impossible to obtain, on sight, the optimal combination, since the best combination of n systems does not necessarily contain the n individually best systems.

It is necessary to create some order out of this chaotic literature. One way to do this is to compile the literature available for restricted application domains (1). Another approach consists of a comparison under standardized conditions of reported systems (and, in the present case, of some new systems). Formal methods are then used for the evaluation and optimal combination of the TLC systems.

EXPERIMENTAL

Reagents and Chemicals—All solvents were reagent grade, and reference sulfonamides were used as 0.2% (w/v) solutions in acetone. Sulfanilamide was always used as an internal standard.

Adsorbent—Precoated TLC silica gel 60 F-245 plates[1] and precoated TLC aluminum oxide 60 F-254 plates[1] (type E) were used.

Detection was by UV light (254 nm).

Apparatus—The plates were developed in carefully controlled saturation conditions[2] and standardized at 40% relative humidity.

RESULTS

The separation systems proposed in the literature and a few others were investigated (Tables I and II). Table I contains those systems for which a preliminary screening with seven sulfonamides yielded unpromising results (bad streaking of the spots, all hR_f values near 0 or 100, *etc.*).

Table II lists the systems that passed the screening stage. The sul-

[1] Merck, Darmstadt, Germany.

[2] Vario-KS-Chamber, Camag, Muttenz, Switzerland.