Radioimmunoassay of the Immunomodulator *erythro*-9-(2-Hydroxy-3-nonyl)-hypoxanthine in Human Serum and Urine

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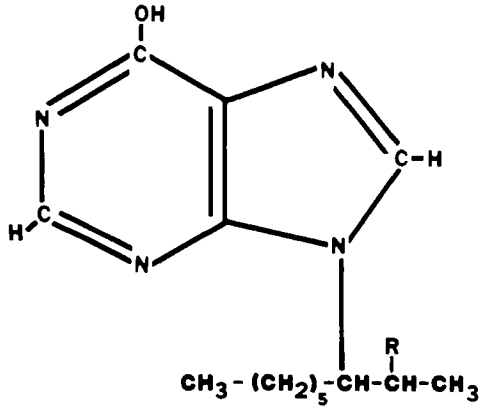
Abstract □ A radioimmunoassay was developed for the measurement in human serum and urine of *erythro*-9-(2-hydroxy-3-nonyl)-hypoxanthine. Antisera were produced in rabbits by immunization with an *erythro*-9-(2-hydroxy-3-nonyl)-hypoxanthine hemisuccinate–bovine serum albumin conjugate. The competitive antigen was *erythro*-9-(2-hydroxy-3-nonyl)-hypoxanthine labeled with carbon-14 on the purine ring. Cross-reactivities were measured against three metabolites and the naturally occurring purine bases inosine and hypoxanthine. Sensitivity of the method was 1 ng/ml in serum and 10 ng/ml in urine. Precision at clinical levels was ±15% in serum at 2 ng/ml and ±3% in urine at 200 ng/ml.

Keyphrases □ *erythro*-9-(2-Hydroxy-3-nonyl)-hypoxanthine—immunomodulator, quantitation in human serum and urine by radioimmunoassay □ Radioimmunoassay—of *erythro*-9-(2-hydroxy-3-nonyl)-hypoxanthine, quantitation in human serum and urine □ Immunomodulators—*erythro*-9-(2-hydroxy-3-nonyl)-hypoxanthine, quantitation by radioimmunoassay, human serum and urine

A considerable amount of interest in the academic and industrial community is currently being directed at the emerging discipline of immunopharmacology. The cellular immunological component of this pharmacological category includes both natural and synthetically derived substances with biological activity. Paramount among the former category are the thymic hormones (1), bacterial agents [BCG (2), *Corynebacterium parvum* (3)], transfer factor (4), and interferon (5). Levamisole (6), inosiplex (7), krestin (8), and interferon inducers (5) are examples of synthetic entities that possess a number of interesting immunomodulatory properties that have been defined in both *in vitro* and *in vivo* systems.

erythro-9-(2-Hydroxy-3-nonyl)-hypoxanthine[1] (I) is an immunopharmacologically active substance (9, 10) which is intended for use in the treatment of acquired or genetically determined deficiencies in cell-mediated immune function. Since the minimum effective dose of this compound was relatively small (0.01 mg/kg), serum and urine levels were expected to be correspondingly low. Initial analytical development, utilizing high-performance liquid chromatographic (HPLC) reverse-phase techniques and UV detection, indicated a lower limit of detectability on the order of 0.5 μg/ml in plasma. The detectability in urine, however, was even less sensitive due to interferences from normal urine constituents. Extractive procedures using various organic phases were of limited utility in improving the levels of detectability.



I: R = OH
II: R = $CO_2(CH_2)_2COOH$
III: R = $CO_2(CH_2)_2CONH$-Albumin

This paper describes a radioimmunoassay (RIA) for I. The assay is of sufficient specificity and sensitivity to determine relevant pharmacological parameters (*e.g.*, bioavailability, elimination, and absorption) at the expected therapeutic doses which would be used in clinical investigations.

EXPERIMENTAL

Chemicals and Materials—Reagent-grade solvents and chemicals were used except for the HPLC solvents, which were HPLC grade. Freund's adjuvant[2] (complete and incomplete) was purchased in sealed glass ampules. Bovine serum albumin[3] was RIA grade. Sodium [^{14}C]-formate[4] (99+% radiopure, specific activity 51 mCi/mm) was packaged in a glass ampule in 70% ethanol under a nitrogen atmosphere. *erythro*-5-Amino-4-chloro-6-(2-hydroxy-3-nonyl)-pyrimidine[5] contained 1.5% of the *threo* isomer. The *erythro*-9-(2-hydroxy-3-nonyl)-hypoxanthine used in these assays was reference standard grade, containing 0.2% of the *threo* isomer.

Chromatographic Systems—Preparative TLC was performed on 2-mm silica plates[6] using *n*-butyl alcohol–2 *N* ammonia (5:1) as a solvent (system A). TLC systems were silica[7] with *n*-butyl alcohol–acetic acid–water (4:1:2) (system B) and cellulose F-254 with isopropyl alcohol–water (4:1) (system C).

HPLC separations were carried out using a gradient liquid chromatograph[8], a 5-μm reverse-phase 250 × 4.6-mm column[9], and a detector[10] operating at 254 nm. The eluant used was typically 0.05 *M* H_3PO_4 in water–methanol (60:40) (system D).

Scintillation Counting—Radioactivity measurements were performed on a liquid scintillation counter[11]. An open channel was used for the carbon-14 counts. Scintillation fluid[12] (2.5 ml) was added to 15 × 45-mm glass vials containing the [^{14}C]protein precipitate from the urine assay. For the serum assay precipitate, 10 ml of cocktail was added to 21 × 68-mm glass vials.

Preparation of the Immunogen—*Synthesis of Erythro-9-(2-hydroxy-3-nonyl)-hypoxanthine Hemisuccinate (II)*—Compound I (300

[1] NPT 15392.

[2] Grand Island Biological Co., Grand Island, N.Y.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Rosechem Products, Los Angeles, Calif.
[5] Dr. A. Giner-Sorolla, Sloan-Kettering Institute for Cancer Research, Rye, N.Y.
[6] Silica 60 F-254, E. Merck, Darmstadt, West Germany.
[7] Silica IB-F, J. T. Baker, Phillipsburg, N.J.
[8] Altex Model 332, Beckman Instruments, Irvine, Calif.
[9] Ultrasphere, Beckman Instruments, Irvine, Calif.
[10] Model LS-8000, Beckman Instruments, Irvine, Calif.
[11] Waters Associates, Milford, Mass.
[12] Aquasol, New England Nuclear, Boston, Mass.

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Table I—Recoveries of I from Human Serum

Amount of I Added, ng/ml	Estimated I Recovered, ng/ml						Mean	CV^b, %
	A[a]	B	C	D	E	F		
1	1.0	0.8	0.5	1.1	1.1	1.2	0.95	27.2
2	2.3	2.2	1.8	1.9	1.5	1.9	1.93	14.9
5	4.2	4.5	3.4	4.8	4.2	5.0	4.35	13.0

[a] Individuals providing serum samples. [b] Coefficient of variation for six individual observations.

mg) and succinic anhydride (2 g) were dissolved in 4 ml of pyridine. The mixture was stirred overnight at 40°. The pyridine was removed under reduced pressure, and the residue was dissolved in a minimum amount of ethanol. Concentrated ammonium hydroxide was added until precipitation ceased. The supernatant was removed, and the precipitate was washed three times with small volumes of ethanol. The supernatant and ethanol washes were pooled and applied to chromatographic system A. After a 15-hr development, the major UV-absorbing band at R_f 0.34 was isolated and eluted with 15 ml of ethanol. This elution was repeated three times. The ethanol was removed under reduced pressure, and the residue was dissolved in 50% ethanol–water prior to ultrafiltration[13]. The solvent was again removed under reduced pressure to give 124 mg (30% yield) of the hemisuccinate II. The identity of II was confirmed by base hydrolysis (1 *N* NaOH, 70° for 1 hr), which gave total conversion to the parent compound, I.

Preparation of the Bovine Serum Albumin Conjugate (III)—A solution of bovine serum albumin (23 mg) in 1.5 ml of water was stirred and room temperature and the pH was adjusted to 5.5 with a dilute sodium hydroxide solution. The hemisuccinate II was added (11 mg in 0.5 ml of water) and the pH was readjusted to 5.5. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (21 mg) was added to the aforementioned solution and the resulting turbid mixture was stirred overnight at room temperature. The suspension was removed and dialyzed against phosphate-buffered saline at 4°. The dialysate was changed frequently.

The product was characterized in the following manner:

1. An aliquot of the product was ultrafiltered, and the ultrafiltrate was analyzed by system D for I. Only a trace amount was found.
2. The ultrafiltrate was hydrolyzed with base and then analyzed for I. Again, only a trace amount of the drug was found.

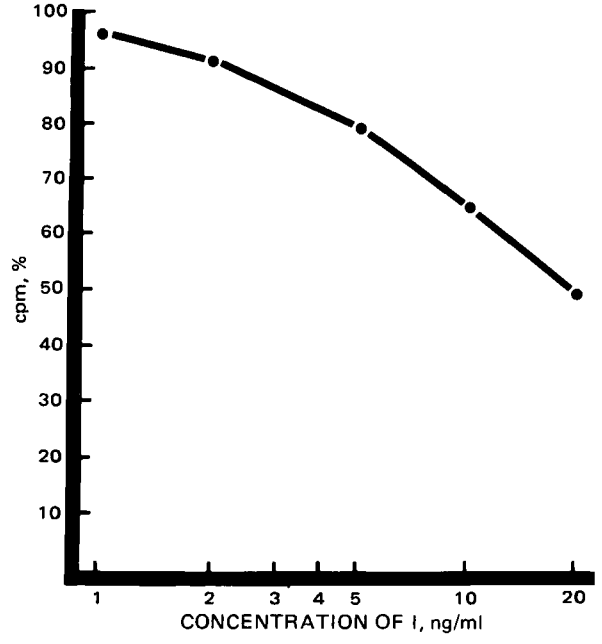

Figure 1—*Serum RIA standard curve. Known amounts of I were added to control serum at 0, 1, 2, 5, 10, and 20 ng/ml, the RIA was carried out, and the percent cpm was calculated. Percent cpm was defined as cpm sample ÷ cpm control (×100).*

[13] CF-50 Membrane Cone, Amicon Corp., Lexington, Mass.

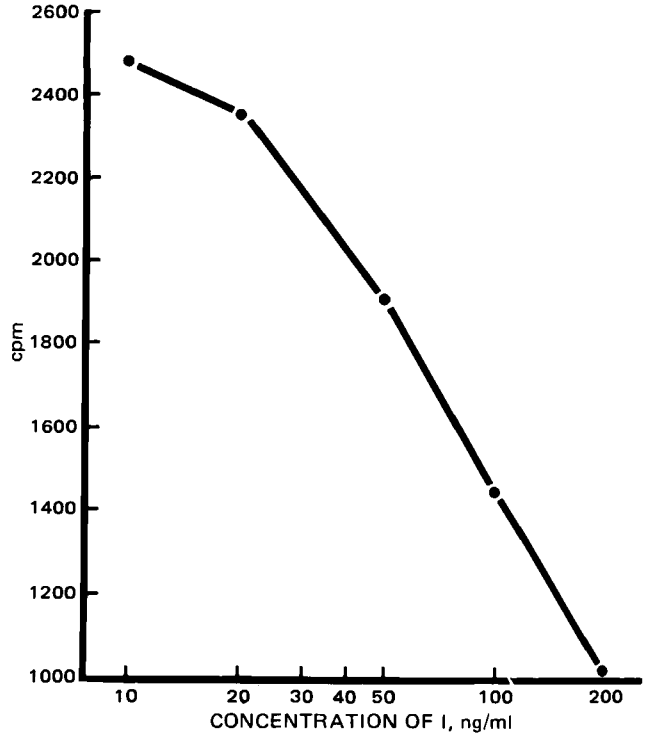

Figure 2—*Urine RIA standard curve. Known amounts of I were added to water at 10, 20, 50, 100, and 200 ng/ml, and the RIA was carried out as described in the text.*

3. An aliquot of the product was hydrolyzed with base and ultrafiltered. A large amount of I was found in the ultrafiltrate, corresponding to 4.21 μmoles or ~13 molecules bound to a molecule of bovine serum albumin in the product.
4. Base hydrolysis of bovine serum albumin alone did not interfere with the HPLC assay. The results of the first two experiments indicated only small amounts (<1 μg) of I and II were present in the product. Two other coupling agents used under various conditions did not yield a conjugate. Agents found not to be effective were 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-ethyl-5-phenylisoxazolium-3′-sulfonate.

The conjugate (III) was diluted with sterile phosphate-buffered saline to a final concentration of 1 mg/ml and frozen in aliquots. It was determined by the aforementioned HPLC procedure that a freeze–thaw cycle did not alter the integrity of the conjugate.

Immunization—The solution of III was thawed and emulsified thoroughly with an equal volume of Freund's complete adjuvant. Subcutaneous injections of the emulsion (1.6 ml) were made to the back area of each of four New Zealand White rabbits. After 3 weeks, a second injection was administered with incomplete Freund's adjuvant. Four weeks after the second injection, all rabbits showed a workable titer of antibodies to I. Blood was removed from the ear vein, allowed to clot at room temperature for 1 hr, and stored overnight at 4°. The serum was removed and used in the RIA. Periodic booster shots of the original preparation of III were administered to the rabbits for over 1 year, and each time the antiserum titer showed a anamnestic (2-week) rise to original levels.

Preparation of [8-^{14}C]*erythro*-9-(2-Hydroxy-3-nonyl)-hypoxanthine (IV)—The 70% ethanol solvent was removed under reduced pressure from sodium [^{14}C]formate (5 mCi, 51 mCi/mm). The sodium [^{14}C]formate was then dissolved in 100 μl of phosphoric acid, and an excess of *erythro*-5-amino-4-chloro-6-(2-hydroxy-3-nonylamino)-pyrimidine (55 mg) was added with vortexing. The reaction was heated at 95–100° in an oil bath for 4 hr, and then cooled. Methanol (300 μl) and then concentrated ammonium hydroxide (180 μl) were added with vortexing. This solution was applied to system A and developed overnight. The UV-absorbing band corresponding to I (R_f 0.51) was isolated and eluted into 175 ml of methanol. Analysis by HPLC (system D) determined a chemical purity of 97.9% IV, which contained 1.4% of the *threo* isomer; yield was 13.7 mg of IV (50%). Radiochemical purity analyses using the same system showed 96.5% of IV and 1.6% of the *threo* isomer. Analyses in systems B and C revealed 99.6 and 98.6% overall radiopurity, respectively. The TLC systems were unable to resolve the *threo* isomer.

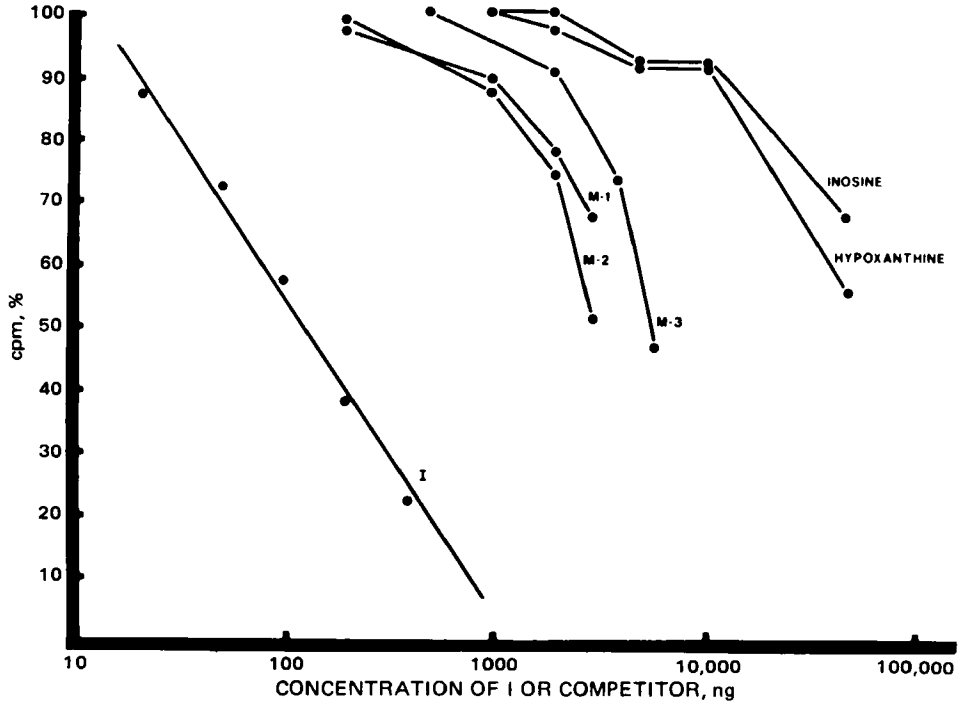

Figure 3—*RIA competition curves for the drug (I), three of its metabolites (M-1, M-2, M-3), inosine, and hypoxanthine. The serum RIA was carried out as described, and either I, a metabolite, inosine, or hypoxanthine was added as a competitor.*

RIA from Human Serum—A stock solution of IV was prepared in water at an activity of ~2×10^7 dpm/ml. A stock solution of 0.5 *M* sodium phosphate buffer, pH 7.5, was also made.The assay mixture was prepared by mixing 1.6 μl of stock solution IV, 2 ml of buffer, 50 μl of antiserum, and diluting to a final 10 ml volume. This mixture was freshly prepared and used immediately. In practice, the antiserum was diluted to give a total binding of IV in the presence of control serum of 70%.

The assay mixture (1 ml) was added to 2 ml of serum, and each sample was run in duplicate. Standards of I were prepared in serum at 0, 1, 2, 5, 10, and 20 ng/ml (also in duplicate) and analyzed concurrently. The solutions were thoroughly mixed and allowed to incubate overnight at 4°. After warming to room temperature, saturated ammonium sulfate was added slowly with vortexing to a final dilution of 40% saturation. The precipitate was allowed to stand 5 min, then centrifuged. The supernatant was aspirated, and the precipiate was washed with 1 ml of 50% saturated ammonium sulfate. Water (0.5 ml) was used to dissolve the precipitate, and the radioactivity (in cpm) was measured.

Quantitation of the amount of drug present in serum was determined from a standard curve of percent cpm *versus* ng/ml of I added. Percent cpm for a given sample was calculated by dividing the sample cpm by the control cpm and multiplying by 100. A typical standard curve is illustrated in Fig. 1.

RIA from Human Urine—The assay mixture was identical to that for the serum RIA except human control serum was also added (ml serum per 10 ml assay mixture) to ensure adequate levels of precipitable protein. To duplicate 1-ml urine samples, 2 ml of ethyl acetate was added; this was vortexed over a 5-min period. Duplicate standards of 0, 10, 20, 50, 100, and 200 ng/ml of I in water were prepared, extracted, and run concurrently. One milliliter of the top layer was removed, and the solvent was evaporated under a stream of nitrogen. The assay mixture (1.5 ml) was added and incubated at 4° overnight. After warming to room temperature, 1 ml of saturated ammonium sulfate was added slowly while vortexing. The precipitate was allowed to stand for 5 min, then centrifuged to a pellet. The supernatant was aspirated and discarded. Water (0.2 ml) was used to dissolve the precipitate, and the radioactivity (in cpm) present in the pellet was measured. Quantitation of the amount of drug present in the urine was derived from a graph of the standard curve of cpm *versus* ng/ml of I added. A typical standard curve is illustrated in Fig. 2.

Table II—Recoveries of I from Human Urine

Amount of I added, ng/ml	Estimated I Recovered, ng/ml					Mean	CV^b, %
	A[a]	B	C	D	E		
0	<10	<10	<10	<10	13		
10	10.5	23	12.5	8	24	15.6	47.4
20	14	32	21.5	11	39	23.5	50.5
50	41	63	51	56	66	55.4	18.0
100	112	112	108	103	120	111	5.6
200	205	210	202	215	220	210	3.5

[a] Individuals providing urine samples. [b] Coefficient of variation for five individual observations.

RESULTS AND DISCUSSION

The accuracy and precision of the immunoassays were evaluated and the results are given in Tables I and II. For the serum RIA, samples were collected from six untreated individuals, and I was added at levels of 1, 2, and 5 ng/ml. Control serum was also run for each individual. Percent cpm (cpm sample/cpm control × 100) was calculated, and the concentration of drug present (in ng/ml) was read from a graph of a standard curve. The standard curve was generated from similarly treated pooled human sera. Average recoveries in serum at levels of 1, 2, and 5 ng/ml were 95, 97, and 87%, respectively. Precision was directly related to the concentration. The coefficient of variation (*CV*) decreased from 27% at 1 ng/ml (the sensitivity of the assay) to 15% at 2 ng/ml and 13% at 5 ng/ml. The method clearly discriminates between control (blank) serum and drug-treated serum at low levels (1 ng/ml) and yields quantitatively acceptable results at higher levels.

Various statistical methods were attempted to improve the accuracy of quantitation in serum. These methods include linear regression of log ng/ml *versus* percent cpm and polynomial least-squares fit to ng/ml *versus* percent cpm. Simple graphical interpolation gave results comparable to the computer-generated data.

The serum RIA requires a control sample from each individual for the calculation of percent cpm. However, it was possible to obtain quantitative results without the control sample by a plot of cpm *versus* ng/ml of the standard and deriving the concentration from the uncorrected cpm of the sample. These results were considerably less accurate than those obtained from the percent cpm method, probably due to variable amounts of competing materials in the individual sera. The interference was not removed by solvent extraction or chemical or membrane deproteinization. Since control sera are generally available from pharmacological studies, their requirement is not a great limitation. A calculation of percent cpm was not necessary for the standard curve of the urine RIA. Acceptable results were obtained from a plot of uncorrected cpm *versus* ng/ml of I in water for the urine quantitation.

The urine RIA recovery data are given in Table II. For this study, urine from five individuals was sampled, and I was added at levels of 10, 20, 50, 100, and 200 ng/ml. Control urine was also analyzed for each individual. The assays were carried out over a period of several weeks, with each

individual being analyzed on a separate day using a concurrent standard curve. The urine assay standards of I were made in water, extracted, and treated identically to the urine samples.

Average recoveries of drug in urine at levels of 10, 20, 50, 100, and 200 ng/ml were 156, 118, 111, 111, and 105%, respectively. The high recovery values were due to small constant amounts of background in the normal urine (5–10 ng/ml) and were mostly due to two individuals (B and E). The interference, of course, becomes less important at higher drug levels (100–200 ng/ml). The precision also improves considerably at higher levels, ranging from a *CV* of 50% at 10–20 ng/ml to 3% at 200 ng/ml.

For the urine RIA, an extraction was necessary to increase the sensitivity level. Without an ethyl acetate extraction, a background of 50–100 ng/ml was present in control urine. The cause of this interference has not been determined. The highest background level noted in this study was in subject E at 13 ng/ml, which has subsequently been found to be the highest level observed in any normal urine examined. In every other normal urine assayed, the amount of interference was well below the lowest concentration on the standard curve (usually 10 ng/ml).

The specificity of the RIA was also investigated. Three metabolites of I, designated as M-1, M-2, and M-3, were isolated from rabbits. UV spectra of the metabolites indicated the purine base was unchanged, so the amount of metabolites could be estimated using the molar absorptivity of the parent compound. These metabolites and other possible competitors containing a similar purine ring (hypoxathine and inosine) were added to an assay in varying amounts and compared with the competitive effects of I (Fig. 3). Metabolite M-2 was the most effective competitor in the assay, having a cross-reactivity of 2–4%. Metabolite M-1 had a cross-reactivity of 1.5–2%, and M-3 was the least effective competitive metabolite at 0.8–2%. Hypoxanthine and inosine were cross-reactive at ~0.1%. Percent cross-reactivity was defined as the ng of I ÷ ng of competitor (×100) when each was at a level producing the same reduction in percent cpm.

The various rabbit antisera were screened for usability. Of the four immunized rabbits, the serum from one particular animal was extremely sensitive to changes in I. When used in the serum RIA, this rabbit's antiserum gave the curve illustrated in Fig. 1, whereas the other rabbits' antiserum produced no measurable reduction in percent cpm up to a drug concentration of 10 ng/ml. At some concentrations (2–10 ng/ml), the percent cpm actually significantly increased compared with the control. This effect has been noted in other systems and has been ascribed to increasing amounts of hapten producing a conformational change in the antibody, thus increasing its binding affinity (11).

Various approaches to assay optimization were used. Incubation times from 2 to 24 hr were explored. The shorter times were usable, but the 24-hr incubation time yielded the maximum slope (sensitivity) at lower concentrations. Reversing the order of the addition of IV and sample to the antiserum had no effect. The pH of the assay buffer was incrementally varied from 5 to 7.5, but binding remained constant throughout this range. Since hypoxanthine and inosine were slightly competitive in the assay and are present in normal plasma at a level of ~600 ng/ml (12) and ~200 ng/ml (13), respectively, the effect of their enzymatic removal from the serum was studied. Purine nucleoside phosphorylase, xanthine oxidase, and uricase were added to normal serum and compared by an RIA standard curve to the same untreated serum. There were no apparent differences between the standard curves generated.

Attempts to synthesize an ^{125}I-labeled derivative of I yielded apparently unstable products. Attachment of tyrosine or tyrosine methyl ester to II gave multiple UV-absorbing products by TLC analysis. Attachment of iodine-125 to tyrosine methyl ester, then conjugating the labeled ester to II also yielded multiple-labeled products. These products were isolated and tested in the RIA, but would not bind to the antiserum. Since the ^{14}C-labeled drug gave acceptable sensitivity and excellent chemical and radiochemical stability, neither iodine-125 or tritium labeling efforts were pursued.

The RIA described in this article has been used to assess serum and urine drug levels in human volunteers participating in a tolerance trial. Patients were administered a single oral dose of either 0.7, 3.0, or 9.0 mg of I. Assays were performed on 24-hr pooled urine collections and on serum at 2 hr postdose.

Urinary levels obtained after administration of 0.7 mg of I ranged from 64 to 290 ng/ml (n = 5); after 3.0 mg the range was 190 to 640 ng/ml (n = 7); and after 9.0 mg the range was 437 to 1425 ng/ml (n = 6). Urine samples were diluted with water when necessary to bring the drug concentration into the standard curve range. Corrections were made for urine volume, and the amount and percent of dose excreted were calculated (± standard deviation). From the 0.7-, 3.0-, and 9.0-mg dose groups, 196 ± 62 μg (28.0% of dose), 355 ± 102 μg (9.6% of dose), and 850 ± 241 μg (9.4% of dose) were excreted, respectively. The percentage of dose excreted unchanged from the 0.7-mg group is significantly higher than the other two groups (ANOVA, $p < 0.001$).

The 2-hr postdose serum levels were also analyzed from the three dosage groups. The 0.7-mg group attained a mean serum level of 1.24 ng/ml (n = 4), the 3.0-mg group a level of 5.43 ng/ml (n = 6), and the 9.0-mg group had an average level of 10.7 ng/ml (n = 4). The RIA techniques, therefore, appear to possess adequate sensitivity for their intended purpose.

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