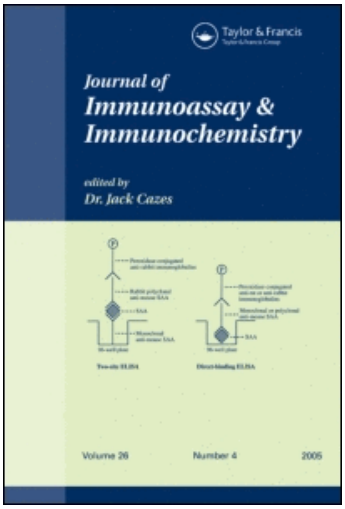

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RADIOIMMUNOASSAYS FOR THE ENANTIOMERIC COMPONENTS OF INDACRINONE AND THEIR PHENOLIC METABOLITES

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(KEY WORDS: Indacrinone, MK-286, Stereospecific Radioimmunoassays)

ABSTRACT

MK-286 is a 90:10 mixture of the (+) and (-)- enantiomers of indacrinone, a combination which induces diuresis while maintaining isouricemia. The principal (phenolic) metabolites also possess pharmacological activity and assays for the four entities were needed for clinical studies. Antisera were produced with the stereospecificity required to measure one enantiomer in the presence of the others, but significant cross-reactivity between drug and metabolite necessitated the separation of these two species by means of Sep-Pak cartridges. Recovery was assessed by concurrently fractionated reference samples. The radioligand in each case was a [I-125]-L-iodotyrosine conjugate of the analyte. Absolute assay sensitivities ranged from 2 - 20 pg/assay tube, corresponding to 0.2 - 2.0 ng/ml in serum samples. Inter-assay CV% was approximately 5% for the majority of samples. The assay was also applied to urine with analytes at concentrations > 16 ng/ml.

INTRODUCTION

Indacrinone { MK-196, [(6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-2-phenyl-1H-inden-5-yl)oxy] acetic acid} is a long acting loop diuretic and uricosuric agent. The (-)- enantiomer is the more

potent diuretic and saluretic entity, while the (+)- enantiomer is the more uricosuric. Of several combinations tested, a 90/10 mixture of the (+)- and (-)- enantiomers (MK-286) was found optimal for maintenance of isouricemia.[1,2]

Each enantiomer is metabolized to a corresponding phenol and these metabolites are pharmacologically active.[3] It is thus desirable to have the ability to measure all four species for pharmacokinetic and pharmacodynamic studies. Prior methodology has lacked either the ability to distinguish between enatiomers or applicability to low concentrations.[4] The problem of enantiomeric differentiation is made more acute by the faster metabolism of the (-)- enantiomer[5], the minor component of the 90/10 mixture.

Immunoassay development was undertaken with the expectation of sufficient stereospecificity to achieve the desired analyses. It was complicated by the unavailability of enantiomers of sufficient purity to conduct definitive cross-reaction experiments. This report describes the development and implementation of the assays.

MATERIALS AND METHODS

Equipment and Supplies

The following supplies were from the sources indicated: Bovine serum albumin, Cat. #12660, and rabbit γ-globulin, Cat. #345991 (Calbiochem); sheep or goat anti-rabbit γ-globulin antiserum (Arnel Products Co., NY); carrier-free NaI^{125} (Amersham); Sep-pak C18 cartridges (Waters Associates); disposable Sarpette tips (5 ml) and 12 x 75 mm polystyrene tubes (Sarstedt);

and glass screw-cap culture tubes with white rubber liner, 20 x 125 mm (Fisher Scientific).

Serum samples were fractionated on Sep-pak cartridges (see below) using a specially constructed polystyrene box containing a rack supporting ninety-six 20 x 125 mm culture tubes and having a lid with 96 holes each sealed with a rubber serum vial stopper pierced with a 2-inch 21-gauge syringe needle. Polyethylene tubing fitting inside the needle hub was used to connect the Sep-pak cartridge. The cartridge was topped with a Sarpette tip to contain the eluting solvent. Vacuum was applied to the box to aid flow through the Sep-pak.

Female New Zealand white rabbits and male Camm:Hartley guinea pigs were used to prepare antisera.

Immunogens and Antisera

The best available preparations of individual enantiomers of both drug and metabolite (from α-methylbenzylamine salts) were treated with an equimolar quantity of N-hydroxysuccinimide (NHS) and a 10% excess of dicyclohexylcarbodiimide in dry dioxane to prepare the corresponding "active" NHS esters. After standing overnight, dicyclohexylurea was removed by filtration and the supernatant liquid was frozen in aliquots and stored at -80°C. Coupling to bovine serum albumin (BSA) was performed by dropwise addition of the active ester (25 moles/mole BSA) to BSA (22 mg/ml in 0.1 M phosphate, pH 9.0) cooled in ice-water and stirred. The mixture was refrigerated overnight prior to exhaustive dialysis against water. By UV absorption at 302 nm, the conjugates were

estimated to contain about 12 moles drug and 15 moles metabolite per mole of protein. Antisera were produced in rabbits by initial immunization with 1 mg of the conjugate as an emulsion with complete adjuvant distributed between i.m., s.c., and multiple i.d. sites. Subsequent injections, at 1 month or greater intervals, were with 0.5 mg immunogen in incomplete adjuvant. Guinea pigs were similarly immunized.

Radioligands

I-125-labeled iodotyrosine was prepared from L-tyrosine, 20 µg/ml in 0.2 M borate pH 8.5, 10 µl; 20 µl 0.5 M phosphate pH 7.5; 2 mCi I-125, 20 µl; 10 µl chloramine T, 2.5 mg/ml. After 60 seconds, 20 µl sodium metabisulfite, 1.5 mg/ml in 0.5 M K_2HPO_4 was added. Aliquots were used to couple to active esters of drug and metabolite. Tyramine was iodinated as follows: Chloramine-T, 10 µl, 5 mg/ml in 0.2M borate pH 8.5, was added to tyramine, 10 µl, 16.4 µg/ml in 0.1N HCl; NaOH, 10 µl, 0.1N; 0.2M borate, pH 8.5, 20 µl; 1mCi, NaI-125, 10 µl. After sixty seconds, sodium metabisulfite, 10 µl, 12 mg/ml, was added. Aliquots were used to couple the active esters of drug and metabolite.

I-125-iodotyrosine conjugates, prepared (overnight, room temperature) from 20 µl aliquots of radiolabeled iodotyrosine and active esters of drug and metabolite enantiomers (5×10^{-8} moles, 10 µl in dioxane), were purified by HPLC (Altex Model 110 solvent metering pump, Tracor Model 960 UV detector, Rheodyne Model 7125 injection valve and a Thomson 5 µ ODS column, 0.46 x 25 cm). Drug radioligands were eluted isocratically with 57% methanol in 0.1% acetic acid, pH adjusted to 5.6 with triethylamine, flow rate 1

ml/min. Retention times were 15.3 minutes and 16.5 minutes for (+)-drug and (-)-drug conjugates, respectively. For the metabolite conjugates, the solvent contained 50% methanol and retention times were 18.5 and 12.0 minutes for the (+)- and (-)-metabolite conjugates, respectively. Forty drop fractions were collected and pooled as appropriate.

Non-radioactive conjugates were prepared on an analytical scale only to establish HPLC purification conditions. With L-iodotyrosine, racemic drug and metabolite yielded the expected diastereomeric products. Retention times for L-iodotyrosine, racemic drug and diastereomeric drug conjugates, were 3.8, 5.5, 15.3, and 16.5 minutes, respectively. For the metabolite, the corresponding retention times were 4.2, 5.7, 12.0, and 18.5 minutes. Iodotyramine conjugates were prepared similarly.

Enantiomer Purification

Repurification of the original enantiomer preparations was effected by derivatization with L-leucine to form diastereomeric amides followed by separation of diastereomers by HPLC and hydrolysis to the desired materials (S. J. deSolms and C. Homnick, manuscript in preparation).

(+)- Enantiomers of parent drug and its p-hydroxy-metabolite were further purified on a Pirkle covalent phenylglycine column (4.6 x 250 mm) eluted with an isocratic mixture of acetonitrile:water:acetic acid (70:30:0.1), pH 7.8 (adjusted with sec. butylamine), at a flow rate of 1 ml/min. Under this condition, almost baseline separation (K_1' = 5.13 and K_2' = 5.67

for the drug; K_1' = 6.13 and K_2' = 6.80 for the metabolite) of (+)- and (-)- enantiomers for each compound was obtained. For both compounds, the early eluting peak was due to the (+)-enantiomer. Compounds purified by chiral columns contained chiral phase as contaminant. In order to remove the chiral phase, both (+)- enantiomers were purfied on a μBondapak ODS column (4.6 x 300 mm) eluted with 40% methanol in water containing 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min.

Fractionation of Serum and Urine

Serum, 0.5 ml, was acidified with 25 μl 3.3 N HCl. Sep-pak cartridges were pre-conditioned with 2 ml methanol followed by 4 ml water, and the acidified sample was applied. After a 2 ml hexane wash, the drug was eluted with 7 ml of a mixture of hexane-toluene-acetic acid, 50:50:1. A further 2 ml hexane wash was added to the drug fraction. Metabolite was eluted with 3.5 ml methylene chloride-acetic acid, 50:1, and then with 1 ml methylene chloride.

For urine, 200 μl aliquots were diluted with 1 ml water acidified with 25 μl 3.3 N HCl and fractionated in the same way. Each of the fractions was back-extracted with 2 ml 0.65 N NaOH and the aqueous phase was transferred to capped 12 x 75 mm polystyrene tubes for storage at -20°C until assayed.Each batch of unknowns was accompanied by a comprehensive set of recovery controls consisting of racemic drug and metabolite in serum or urine, which was used to apply a recovery factor and to detect any dependence of recovery upon concentration.

Radioimmunoassay

See Table 1 for the reagents and samples used. Using a Micromedic automatic pipetting station, 0.1 ml antiserum, 0.1 ml labeled ligand premixed with anti-rabbit γ-globulin, sample and buffer to a total volume of 0.8 ml were added to 12 x 75 mm glass tubes. Total radioligand was 20,000 cpm per tube. After room temperature incubation for 18-24 hours, the tubes were centrifuged at 800 g for 45 minutes and supernatants were discarded. The pellets were counted for 3 minutes each using a Micromedic 4/600 counter or a Packard Model 5160 or 5360.

All samples were assayed in triplicate. One complete set of replicates was pipetted then the second and the third, and assay tubes remained in that order for work-up and counting so that any assay drift would be detected as variability between replicates.

Reference materials were the racemates, stored in aliquots in aqueous solution. Dilutions were made in assay buffer for each run. Quality control was effected by routine assay of reference material in the assay buffer, prepared as a pool at four different concentrations (zero, low, medium, high) and stored in aliquots for routine analysis as unknowns.

Data Processing

A first degree polynomial of the conventional logit/log type was available as well as optional second or third degree polynomials of this type:

$$y = a_0 + a_1 x + a_2 x^2 + a_3 x^3$$

where $y = \log_e$ analyte concentration, $x = \log B/B_0$ (B_0 = bound counts with zero analyte, B = bound counts for an analyte

TABLE 1

Assay Conditions

	(+)-Drug	(-)-Drug	(+)-Metabolite	(-)-Metabolite
Buffer[1]	0.2 M borate	0.2 M borate	0.1 M phosphate	0.1 M phosphate
Antiserum #	1582	1572	1584	1591
Dilution[2]	50,000	75,000	25,000	75,000
Serum extract dilution[3]	50	undiluted	10	undiluted
Volume assayed, µl	40	20	40	40
Reference range, ng/ml[4]	0.1-40	0.2-160	0.1-40	0.2-80
I_{50}, ng/ml[5]	1.4	3.0	1.5	4.5
Effective range, ng/ml[6]	20-8000	0.8-640	4-1600	0.8-320
Urine extract dilution[3]	8	8	8	8
Volume assayed, µl	4	40	8	40
Reference range, ng/ml[4]	0.4-160	0.2-80	0.4-160	0.2-80
Effective range, ng/ml[6]	32-12,800	16-6400	32-12,800	16-6400

1 Buffer also contains 0.05 M EDTA, 0.22 M sodium acetate, 0.125 mg/ml BSA; final pH for borate is 8.5, for phosphate, 7.5.

2 Antisera are diluted in assay buffer containing 50 µg/ml rabbit γ-globulin.

3 Extracts are diluted in assay buffer.

4 Reference samples prepared from the racemate of the analyte. Stated concentrations are 50% of the nominal concentrations, being those of the enantiomers.

5 Concentration of reference for 50% inhibition of binding.

6 Indicates effective range for original sample, uncorrected for recovery.

Assay tubes contained 0.1 ml antiserum, 0.1 ml radioligand combined with second antibody (anti-rabbit γ-globulin), sample and buffer to a total volume of 0.8 ml.

concentration of e^y). The equation was derived by least-squares regression and evaluated by reading-back standards as though unknowns. Generally, the second or third degree polynomial was most satisfactory, although the linear logit-log equation could be used for a restricted concentration range. Quality control and recovery data were stored cumulatively in computer files to provide assay statistics.

RESULTS

Specificity

Initial cross-reactivity data were obtained using the enantiomers supplied for immunogen preparation. For the drug, apparent cross-reactivity between enantiomers was compatible with the probable enantiomeric impurity and revealed nothing certain about true cross-reactivity. In the case of the metabolite, however, it appeared that cross-reactivity was inordinately high. Purification of reference materials via diastereomeric derivatives resulted in a much improved spectrum of cross-reactivities, while subsequent repurification by chiral HPLC reduced the observed cross-reactivity between enantiomers still further (Table 2).

It was concluded that drug and metabolite separation was necessary but that (-)-enantiomers could be determined in the presence of considerable excess of the (+)-enantiomers. This was confirmed experimentally (Table 3). Metabolite could be assayed without substantial interference by a 40-fold excess of the (+)-enantiomer while the drug assay tolerated up to a 100-fold

TABLE 2

Effect of Enantiomer Repurification on the Apparent Cross Reactivity[1]

Assay	Enantiomer Preparation[2]	Cross-Reacting Species (+)-Drug	(-)-Drug	(+)-Metab.	(-)-Metab.
(+)-Drug	1	100[3]	1	0.9	0.06
	2	100	0.75	0.42	0.054
	3	100	ND[4]	ND	ND
(-)-Drug	1	1	100	1	1
	2	0.34	100	0.086	0.43
	3	0.06	100	ND	ND
(+)-Metab.	1	2	<0.3	100	6.8
	2	1.9	<0.1	100	0.2
	3	ND	ND	100	ND
(-)-Metab.	1	<0.2	1	4.6	100
	2	<0.17	0.65	0.26	100
	3	ND	ND	0.08	100

1 Cross reactivities are expressed in terms of % relative reactivity at 50% depression of binding. Critical data are those underlined (for the (+)-enantiomer in the (-)-enantiomer assay).

2 Preparation 1 = purification via crystallization of diastereomeric α-methylbenzylamine salts
Preparation 2 = further purification by HPLC separation of amides with L-leucine
Preparation 3 = further purification by chiral HPLC

3 By definition

4 Not determined

TABLE 3

Determination of (-)-Enantiomer in the Presence of Excess (+)-Enantiomer

Assay	Ratio (+)/(-)	% of Nominal Conc Found 0.2 ng/ml	1 ng/ml	12.5 ng/ml
(-)-Drug	0	100%	100%	99%
	1	95%	99%	100%
	3	100%	99%	105%
	10	100%	103%	100%
	30	100%	103%	106%
	100	100%	111%	107%
	300	130%	130%	114%

	Ratio (+)/(-)	% of Nominal Conc Found 0.46 ng/ml	4 ng/ml	40 ng/ml
(-)-Metab.	0	102%	103%	102%
	1.3	113%	105%	104%
	4	104%	104%	96%
	13	102%	102%	103%
	40	104%	107%	115%
	132	113%	120%	115%

excess. The permissible ratios of enantiomer concentrations were not exceeded in clinical samples assayed.

Assay Optimization

Acetic acid was an essential component of eluting solvents but the acetate in the final extracts increased both non-specific binding and net binding. The results of a study of the effects of increasing acetate concentrations are shown in Figure 1. Since

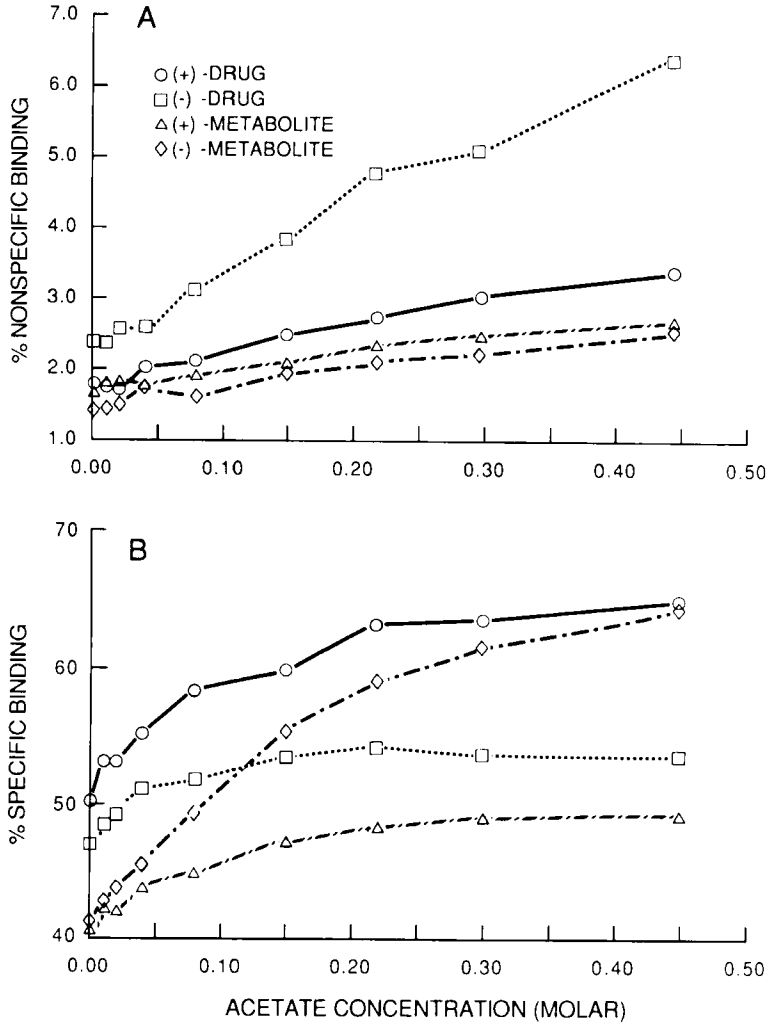

Figure 1. Effect of acetate ion on (A) % non-specific binding and (B) % specific binding

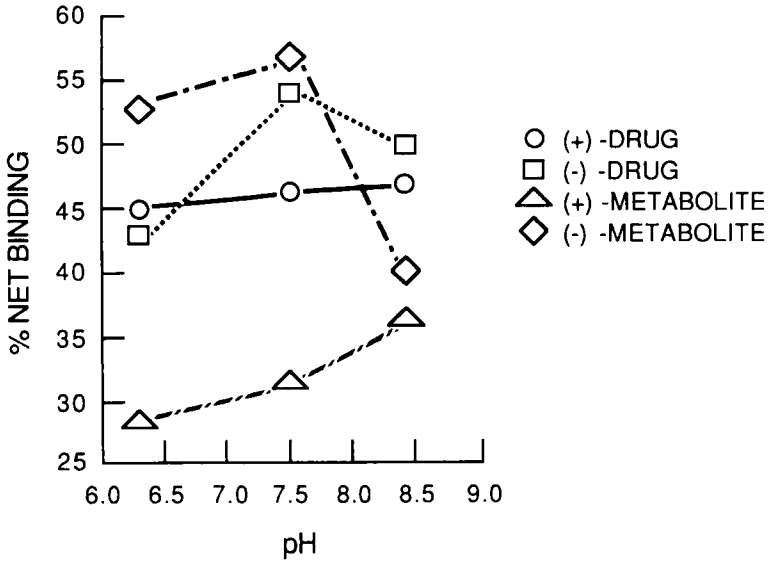

Figure 2. Effect of pH on % net specific binding

net binding was almost constant when acetate concentrations exceeded 0.2 molar, the assay buffer was supplemented with 0.22 molar acetate. The effect of additional acetate in the extracts was then negligible except in the case of the (-)-metabolite. An undiluted plasma extract would increase the acetate concentration to 0.25 molar and cause a measurable change in ligand binding. In this case, RIA standards were prepared in an extract of drug-free plasma to provide an equivalent matrix. Figure 2 illustrates the effect of pH on ligand binding. Effects were enantiomer-dependent. For the sake of uniformity, pH 8.5 was selected for drug assays and pH 7.5 for the metabolite, although these did not furnish maximum binding for all enantiomers.

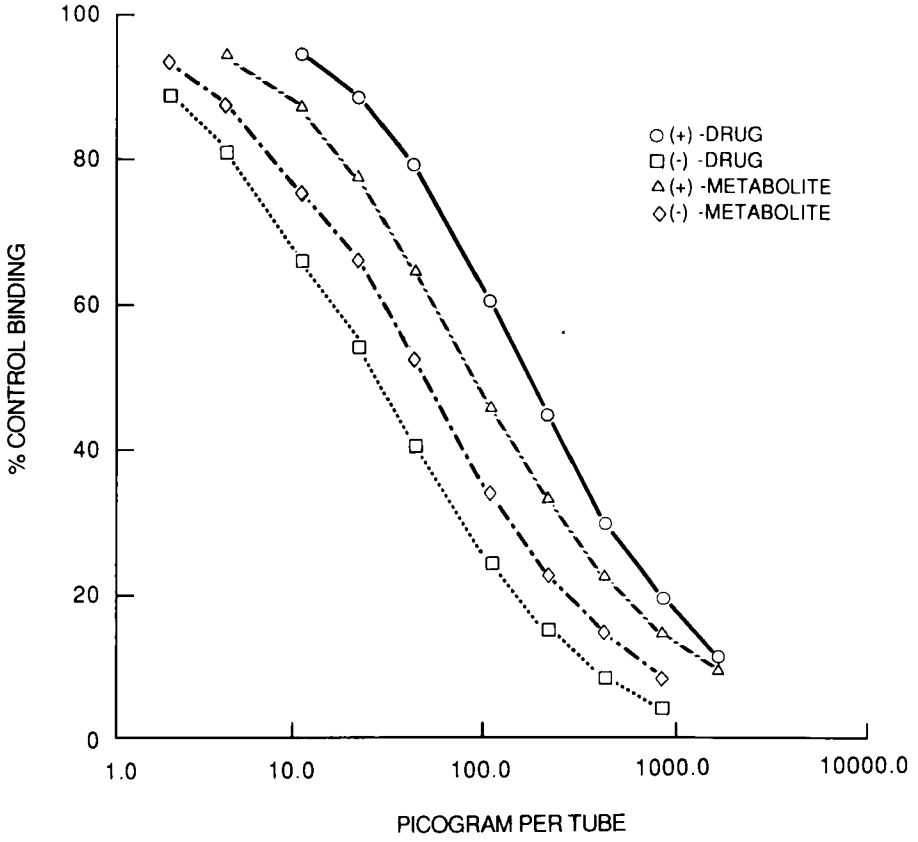

Figure 3. Representative standard curves for (+)- and (-)- enantiomers of drug and metabolite

Standard Curves

Figure 3 shows typical standard curves for all four analytes. Limits of measurement were 2 and 4 pg/assay tube for (-)-drug and (-)-metabolite, and 8 and 20 pg for (+)-metabolite and (+)-drug. Extracts of biological samples were routinely diluted or not as in Table 1, to ensure that the majority of

samples would be in the valid range of the assay [(+)-enantiomers in plasma and all urine samples] or to obtain maximum sensitivity [(-)-enantiomers in plasma].

Assay Performance

Estimates of assay variability were obtained from accumulative data for the recovery controls included in each assay. Since these were taken from a pool, their concentrations were not subject to between-assay variability except insofar as recovery might be variable. A mean recovery for all concentrations was used to determine recovery and this factor was applied to all unknowns as well as to the individual recovery controls. Thus statistics derived from recovery controls are those applicable to unknowns. Data in Tables 4 and 5 indicate that recovery was not concentration-dependent and that the assay was reproducible. Intra-assay variability represents the imprecision of the assay itself while inter-assay variability represents the additional effect of recovery variation. These data have been accumulated in the course of the routine analysis of clinical samples. Recoveries of the drug ranged from 90-100% and were 75-85% for the metabolite. Within a single assay, the CV for mean recovery was generally 3-6%. In one assay, 23 samples each of four concentrations of racemic metabolite in serum were processed together. Variance was not concentration-dependent, and the overall CV% was 5.13% for (+)-metabolite and 3.07% for (-)-metabolite.

Insofar as mean recovery is used to correct calculated concentration of unknowns, recovery controls constitute the

TABLE 4

Assay Statistics for Serum Recovery Controls

Assay	Nominal Conc	Mean	Intra-[1] Assay CV%	Inter-[2] Assay CV%	N
(+)-Drug	40 ng/ml	39.4	5.0	5.0	20
	100	97.7	5.4	5.5	21
	200	201	2.5	6.6	20
	400	407	1.8	3.9	21
	1 µg/ml	1.01	2.3	2.9	20
	2	1.99	3.0	4.6	20
	4	3.98	3.5	4.6	18
(-)-Drug	2 ng/ml	2.01	8.0	15.3	19
	4	3.78	4.3	7.8	22
	10	9.86	3.3	6.4	22
	20	20.0	2.3	4.2	21
	40	40.8	2.9	6.3	22
	100	102	4.0	5.5	21
	200	199	4.0	7.2	21
	400	404	4.8	5.5	17
(+)-Metab.	10 ng/ml	9.5	5.6	6.2	19
	20	19.1	4.2	4.9	19
	40	40.9	2.7	3.0	19
	100	102	2.4	2.8	19
	200	204	2.5	3.5	19
	400	396	2.3	3.2	18
(-)-Metab.	2 ng/ml	1.95	8.3	15.0	21
	4	3.97	5.7	6.8	21
	10	10.3	3.8	4.3	21
	20	20.2	2.7	3.8	21
	40	39.9	3.2	4.2	21
	100	101	2.7	4.1	21
	200	201	3.0	4.4	21

1 Based on mean of triplicates

2 Means of triplicates compared between assays

TABLE 5

Assay Statistics for Urine Recovery Controls

Assay	Nominal Conc	Mean	Intra-[1] Assay CV%	Inter-[2] Assay CV%	N
(+)-Drug	200 ng/ml	201	7.8	8.0	13
	400	405	3.5	6.7	13
	1 µg/ml	1.02	3.2	3.1	13
	2	2.03	2.3	2.3	12
	4	3.96	1.9	4.5	12
	10	9.92	2.7	4.9	13
(-)-Drug	50 ng/ml	49.0	5.4	6.5	12
	100	101	5.1	4.5	12
	200	196	4.3	5.3	14
	400	403	2.1	5.1	14
	1 µg/ml	1.00	2.4	4.5	14
	2	2.05	2.8	3.7	13
	4	4.00	3.5	5.8	14
(+)-Metab.	200 ng/ml	205	3.6	6.4	14
	400	410	4.2	5.1	14
	1 µg/ml	1.02	2.4	4.1	14
	2	1.95	1.7	4.4	13
	4	3.82	2.0	6.5	13
	10	9.67	3.7	8.4	13
(-)-Metab.	50 ng/ml	50.2	5.8	11.9	13
	100	100.6	3.5	8.7	13
	200	210	3.1	5.8	13
	400	393	2.4	6.7	13
	1 µg/ml	1.00	2.7	6.3	12
	2	1.98	2.7	5.3	13
	4	3.88	5.3	4.7	13

[1] Based on mean of triplicates

[2] Means of triplicates compared between assays

primary reference. Nevertheless, variations in recovery could detract from the quality of a standard curve in a potentially non-detectable fashion. The standard curve for each assay was therefore calculated on the basis of a set of reference solutions diluted from a stock solution in each assay. The goodness-of-fit

TABLE 6

Assay Statistics for Buffer Quality Controls[1]

Assay	Nominal Conc.(ng/ml)	Mean	Intra Assay CV%	Inter Assay CV%	N
(+)-Drug	0.50	0.49	2.87	6.39	5
	2.5	2.50	4.87	6.19	33
	25	25.6	3.30	5.21	33
(-)-Drug	0.50	0.48	3.71	7.27	7
	2.5	2.45	3.08	4.99	31
	25	25.5	3.07	5.03	31
(+)-Metab.	0.50	0.50	4.77	4.10	6
	2.5	2.61	2.73	4.02	31
	25	25.1	3.50	4.31	31
(-)-Metab.	0.50	0.50	6.55	5.57	7
	2.5	2.61	3.45	5.50	31
	25	25.1	2.54	5.75	31

[1] All statistics are based upon individual values for triplicate determinations in each assay and expressed relative to the mean values for triplicate determination.

of the calculated equation was evaluated by a read-back of standards as though unknowns. Correlation coefficients for the relationship between nominal and read-back values (mean of triplicates) were customarily 0.9997 to 0.99991 and slopes were 0.99 to 1.01. Quality was also monitored by QC samples prepared in bulk in assay buffer and stored in aliquots for routine use. Statistics for these appear in Table 6.

Validation

The radioimmunoassays for all four analytes in serum and urine were validated by an independent gas-liquid chromatographic (GLC)

TABLE 7

Validation of Radioimmunoassays for Enantiomeric Drug and Metabolite in Human Serum and Urine

Assay	Serum	Urine
(+)-Drug	1.04 ± 0.06 (18)[1] 0.987[2]	1.00 ± 0.05 (9) 0.998
(-)-Drug	1.00 ± 0.06 (18) 0.997	0.98 ± 0.06 (9) 0.999
(+)-Metabolite	1.03 ± 0.11 (14) 0.993	0.91 ± 0.07 (8) 0.993
(-)-Metabolite	1.05 ± 0.32 (15) 0.954	1.04 ± 0.09 (9) 0.988

[1] Each value represents mean ± S. D. (N) of concentration by RIA over concentration by GLC

[2] Correlation-coefficient

assay[4]. Selected samples from a single enantiomer clinical study were used for comparative purpose since GLC method is non-stereoselective. The results are shown in Table 7.

DISCUSSION

Key factors in the development of this assay were the specificities of the antisera, a reproducible method of fractionating samples and measuring recovery, and the removal of interference by sample extracts. The initial analytes had been prepared by fractional crystallization of α-methylbenzylamine salts. Though pure enough for many purposes, it became evident

that enantiomeric impurity precluded an exact determination of assay specificity. The principal evidence leading to repurification was the almost uniform apparent cross-reactivity between metabolite enantiomers with all rabbit and guinea pig antisera (five animals each, several bleeds). Initial attempts at definitive purification through diastereomers prepared with α-methylbenzylamine and HPLC separation were frustrated by the observation that the available enantiomers of the reagent was insufficiently optically pure to achieve the desired result. Derivatization with L-leucine followed by HPLC separation and regeneration of the drug and metabolite produced enantiomer preparations showing substantially reduced cross-reactivity. Two key enantiomers, (+)-drug and (+)-metabolite were further purified by HPLC using a chiral column and evaluated for cross reactivity in assays for the (-)-enantiomers. For the (-)-drug assay it was projected that a 100:1 excess of (+) over (-) might introduce an assay error of about 6%, and for the metabolite, 8%. Most clinical samples were expected to contain ratios less than 100:1. Although an approximate correction could be applied mathematically, it was not found necessary in practice.

The fractionation method is unconventional because conventional means (i.e., using relatively polar solvents to elute the adsorbed analytes) failed to achieve the desired separation. The adsorption from serum or urine is attributed to a hydrophobic interaction with the C18 side chains of the Sep-pak cartridge and the subsequent separation to be chromatography on the silica gel

support; indeed this step was based upon thin-layer chromatographic separations. Acetic acid was an essential component of eluting solvents, but the resulting high concentration of acetate in the back-extracted fractions interfered grossly in the assay when iodotyramine conjugates were employed as labeled ligands (data not shown). It is likely that protein-ligand interactions were responsible and the most deleterious effect was the increase in non-specific binding. Iodotyrosine was then selected as the labeled moiety of the ligands on the basis that its greater likelihood of solvation might reduce non-specific binding, and this end effect was realized. There was an added advantage in that L-iodotyrosine formed diastereomeric conjugates which were separable during the HPLC isolation of the ligands, affording a purer product. Furthermore, labeled ligands could be made with the active ester of racemates and ligands for the two enantiomers could be prepared simultaneously. Matrix effects were not entirely lost, but could be reduced to insignificance by incorporating 0.22 M acetate in the assay buffers.

Optimal pH appeared to be stereospecific, both (-)-enantiomers exhibiting greater specific binding at pH 7.5, the (+)-enantiomers having greater binding at pH 8.5. For convenience, pH 8.5 was chosen for the analysis of both enantiomers of the drug and pH 7.5 for both enantiomers of the metabolite. The (-)-drug exhibited a relatively small drop in binding at pH 8.5 while the effect on (-)-metabolite was much greater. The choice of a single buffer

for both enantiomers permitted racemic standards to be prepared and used for both assays.

Recovery controls constitute the primary standard since all values for unknowns are normalized according to the calculated recovery. It was possible to use these to construct the standard curve but variability in recovery between samples would have the potential of distorting the standard curve. If a logit-log transformation were applicable, the criteria of linearity could have been used to detect distortion, but the second or third degree polynomial lack this characteristic. Small changes in the standard curve as the radioligand aged precluded reproducibility of the curves as a criterion. For this reason, a more reliable set of standards was diluted from a stock solution for each assay. The exact concentration of those standards was not critical provided the proportionality between concentrations was precise. Buffer QC data were used to confirm that proportionality, and they also confirmed the nominal concentrations.

There was no independent method for measuring both enantiomers of drug and metabolite in clinical samples. In one clinical study individual enantiomers were administered and the selected samples were assayed by a published GLC method[4] and by RIA. Agreement between two methods was excellent, confirming validity of RIA analysis. These radioimmunoassays have been used successfully in analysis of enantiomers of indacrinone and its principal metabolite from several clinical studies.

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