Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 4, pp. 483–492, 1994

0731–7085/94 $7.00 + 0.00

Determination of L-691,121, a new Class III antiarrhythmic, and its principal metabolite in plasma by differential radioimmunoassay

T.F. GREBER,* T.V. OLAH, J.D. GILBERT, A.G. PORRAS and M. HICHENS

Merck Research Laboratories, West Point, PA 19486, USA

Abstract: A sensitive and specific method based on radioimmunoassay (RIA) has been developed for the analysis of L-691,121, a new antiarrhythmic agent, and its major metabolite, L-692,199, in plasma. Two RIAs using immunogens and radioligands prepared from different derivatives of L-691,121 were used in conjunction to determine both parent compound and metabolite concentrations by solving simultaneous equations, since neither assay alone was adequately specific. Variable cross-reactivity factors were incorporated into the calculations to correct for non-parallel drug and metabolite displacement curves. The direct assay using 30 μl of plasma is sensitive to 0.1 ng ml^{-1} and has sufficient precision, accuracy and specificity for the analysis of clinical samples.

Keywords: *L-691,121; Class III antiarrhythmic; radioimmunoassay (RIA); differential radioimmunoassay.*

Introduction

The compound, L-691,121 (methanesulphonamide, *N*-[1′-[2-(5-benzofurazanyl)ethyl]-3,4-dihydro-4-oxospiro[2*H*-1-benzopyran-2,4′-piperidin]-6-yl]-,monohydrochloride) is a selective Class III antiarrhythmic agent [1, 2] designed for the treatment and prevention of ventricular tachyarrhythmias and potentially for the prevention of sudden cardiac death (SCD) [3]. SCD is generally defined as death occurring within 24 h after the onset of an acute cardiac illness and common in individuals predisposed for ventricular arrhythmia due to a previous cardiac ischemic insult. A category of substances known as Class I antiarrhythmics which act by modulating the transfer of sodium ions have been shown to be unconvincingly effective in decreasing long-term mortality [4, 5]. There is reason to suspect that selective Class III antiarrhythmic agents (potassium channel blockers) would be more effective in preventing episodes of malignant arrhythmias leading to SCD.

Pharmacological studies on L-691,121 have been described recently [6]. An analytical method based on radioimmunoassay (RIA) was needed to support clinical trials, since the potency of the drug precluded the development of less sensitive methods such as LC or even LC/MS. Preclinical studies demonstrated the extensive conversion of the drug to its alcohol metabolite, L-692,199 by the reduction of the ketone [7]. This conversion was so efficient that metabolite–drug ratios reached 50:1 following an oral dose in dogs, imposing a challenge to immunoassay specificity.

Rabbit antisera were raised against immunogens prepared by conjugating drug derivatives to bovine serum albumin (BSA) via two different sites on the molecule. The corresponding ^{125}I-radioligands were also prepared from these derivatives. Two RIAs were developed. One had similar reactivity for both drug and metabolite and while the other had good specificity for the drug, the overabundance of metabolite made it also unsuitable. This paper describes the development and performance of a differential RIA technique [8], in which the data from two assays of differing specificities were used together to calculate the concentrations of both the parent drug and its metabolite in plasma.

Materials and Methods

Reagents

The following reagents were purchased from the indicated vendors: BSA and rabbit gammaglobulin from Calbiochem (San Diego, CA, USA); Crotein SPC from Croda, Inc. (Parsippany, NJ, USA); chloramine T, N-FMOC-L-

* Author to whom correspondence should be addressed.

alanine, oxalyl chloride and L-tyrosine from Sigma (St Louis, MO, USA); sheep anti-rabbit gamma-globulin from Arnel Products Co. (New York, NY, USA); Na-^{125}I from Amersham (Arlington Heights, IL, USA) and ^{125}I-Bolton-Hunter reagent from Dupont (Wilmington, DE, USA); *N*-hydroxysuccinimide, carboxymethoxylamine hemihydrochloride, triethylamine, 3,5-difluoro-dinitrobenzene and 1,3-dicyclohexylcarbodiimide from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). The following compounds were obtained within Merck Research Laboratories: L-691,121, L-692,199 and L-692,194, (Fig. 1, structures **I**, **II** and **VII**, respectively). Remaining agents were widely available and not dependent on the source.

Preparation of Immunogens

Immunogen IX. Twenty milligrams (47 μmol) of **VII** was dissolved in 0.4 ml dimethylsulphoxide. Ten milligrams of 3,5-difluoro-dinitrobenzene (DFDNB) was added directly and the solution was stirred for 4 h at room

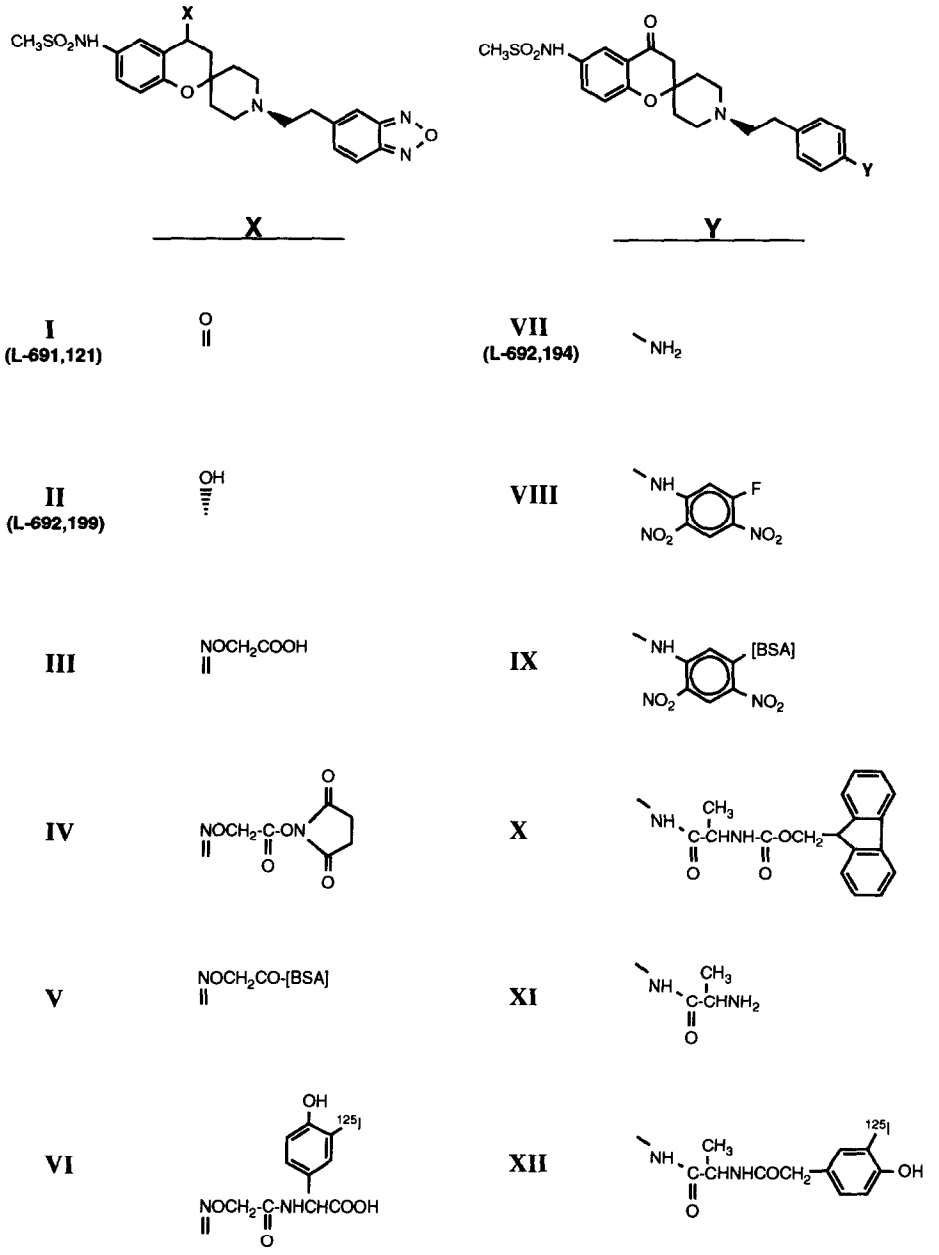

Figure 1
Chemical structures of the compounds described in the text.

temperature and then overnight at 4°C. The product, **VIII** was isolated on a silica gel 60 column prepared in methylene chloride and eluted with toluene–methanol (10:3, v/v). Fractions were collected and analysed by TLC (toluene–methanol, 10:3) and those containing **VIII** (R_f = 0.5) were combined, evaporated to a yellow semisolid and redissolved in 2.0 ml dimethylformamide (DMF). Fast atom bombardment mass spectroscopy (FABMS) data were consistent with a pseudomolecular molecular ion $(M + H)^+ = 614$. The concentration of **VIII** (determined spectrophotometrically) was 17 μmol ml^{-1}.

To 26 mg (0.4 μmol) BSA in 2.0 ml potassium phosphate (pH 8.6; 0.125 M) precooled to 0–4°C, were added either 1.0 ml (17 μmol) **VIII** in DMF or 1.0 ml DMF (control) in small aliquots over 3 h. The solutions were kept overnight at 0–4°C and then dialysed exhaustively for 96 h against distilled water. Difference spectra (versus the control) showed the incorporation of 34 mol of hapten per mol of BSA.

Immunogen V. L-691,121·HCl (**I**; 35.4 mg, 71.8 μmol) was dissolved in 1.0 ml dry pyridine. Following the addition of 15.6 mg of carboxymethoxylamine hemi-hydrochloride (71.5 μmol) in 0.78 ml pyridine, the solution was refluxed for 0.5 h. Two additional equivalents of reagent were added over a period of 3.5 h and the reaction mixture was refluxed for a further 6.0 h.

The cold reaction mixture was concentrated to an oil under a stream of nitrogen, diluted with 3.0 ml of distilled water, adjusted to pH 11 with 2 M potassium hydroxide and extracted with 3 × 3.0 ml aliquots of ethyl acetate. The aqueous phase was acidified with 0.1 M hydrochloric acid, and the resulting precipitate of the desired carboxymethyloxime (**III**) was consolidated by centrifugation. The supernatant was discarded and the pellet was washed with 2 ml of 0.05 M hydrochloric acid and 2 × 2 ml portions of water. The pellet was resuspended in 2 ml absolute ethanol and 2 ml dry toluene. The solvents were removed under a stream of nitrogen and the remaining solid was dried over phosphorus pentoxide in a vacuum desiccator. The yield was 22.4 mg (42 μmol), 58%.

The *N*-hydroxysuccinimide ester (**IV**) was prepared by incubating 21.9 mg (41 μmol) of **III** with 4.7 mg (41 μmol) of *N*-hydroxysuccinimide and 27.9 mg (135 μmol) of dicyclohexylcarbodiimide in 4.2 ml dry DMF at room temperature for 48 h. Without isolation, one-half of the product (**IV**) was concentrated to 1.3 ml under a stream of nitrogen and added to BSA (26 mg, 0.40 μmol) in 1.0 ml potassium phosphate (pH 8.50; 125 M); 1.3 ml DMF was added to a control solution of BSA. Additions were made in small aliquots with stirring at 0–4°C over a 6 h period. The solutions were diluted with 1.0 ml distilled water and allowed to stand overnight at room temperature. Exhaustive dialysis against water was carried out for 96 h at 0–4°C. Difference spectra (versus control BSA) indicated the incorporation of approximately 31 mol of hapten per mol of BSA.

Immunization

Immunogen solutions were emulsified with an equal volume of Freund's complete adjuvant. Female New Zealand White rabbits were immunized with approximately 1 mg protein by multiple intradermal injections plus single intramuscular (i.m.) and subcutaneous (s.c.) injections. Boosting with one-half quantity of protein was performed with emulsions in incomplete adjuvant (i.m. and s.c. only) at 1, 3 and 7 months post-initiation, and antisera obtained prior to the latter two injections were stored at −70°C.

Radioligands

Radioligand XII. *N*-(9-fluorenylmethyloxycarbonyl)-L-alanine (fmoc-ala) 4.5 mg (14 μmol) was dissolved in 1.2 ml dry toluene, 60 μl (699 μmol) oxalyl chloride was added and the reaction mixture was heated at 60°C for 1.0 h. The toluene and excess reagent were removed under a stream of nitrogen. After drying, the residual oil was redissolved in 180 μl dry chloroform and heated at 60°C for 1 h with 5 μmol of **VII** free-base. The solvent was removed under nitrogen and the residue was redissolved in 1.0 ml hydrochloric acid (0.1 M) and extracted with ethyl acetate. The pH of the aqueous layer was adjusted to 11 with potassium hydroxide (1 M) and the product **X**, was extracted into ethyl acetate. After removal of solvent under a stream of nitrogen, the product was examined by TLC (R_f = 0.24; chloroform–methanol–ammonium hydroxide 90:10:1, v/v/v). The protecting group was removed by heating for 1 h at 50°C with two drops of morpholine in a

total volume of 200 μl DMF and the product was eluted from the TLC plate with methanol. FABMS showed the expected pseudomolecular molecular ion $(M + H)^+$ at 501 dalton corresponding to **XI**.

This product (120 μg) was dissolved in 60 μl sodium borate (pH 8.5; 0.1 M) to yield a final concentration of 4 μmol ml^{-1}. Benzene was removed from the vial of [^{125}I]-Bolton-Hunter reagent [9] by evaporation under a stream of dry nitrogen immediately prior to use. To the residue (1 mCi, 0.45 nmol) was added 20 μl (40 μg) of the pre-cooled (0–4°C) **XI** solution. The reaction mixture was incubated with stirring in an ice bath overnight. Purification of the radioligand was carried out by HPLC on a Waters μBondapak ODC column (3.9 × 300 mm, 10 μm) with a flow rate of 1 ml min^{-1}, eluting with a 60-min linear gradient of 20–50% methanol in 0.1% acetic acid–water adjusted to pH 4.22 with triethylamine. Radioactivity was detected with a flow-through detector (Beckman, Model 170, Irvine, CA, USA). The eluent was collected as 60 drop fractions using a fraction collector (Gilson Medical Electronics Inc., Model 203, Middleton, WI, USA). The retention times for **XI** and **XII** were 17 and 75 min, respectively. The fractions containing the **XII** peak were tested for binding to the specific antibody raised against **IX**, pooled, and stored at −20°C.

Radioligand VI. Twenty microlitres (2 mCi) of carrier-free Na-^{125}I and 5 μl (12.5 μg) chloramine-T solution were added to 0.2 μg of L-tyrosine [1.1 nmol in 1 μl hydrochloric acid (0.01 M), 4 μl borate buffer (pH 8.5; 0.2 M)] and 10 μl potassium phosphate (pH 7.5; 0.5 M). The reaction was stopped after 60 s by the addition of 15 μg (79 nmol) of sodium metabisulphite in 10 μl of water. Five microlitres (50 nmol) **IV** in DMF, 10 μl of potassium phosphate, dibasic (0.5 M) and 10 μl of DMF were added and the reaction proceeded overnight. Purification of **VI** was carried out by LC as described for **XII** but using a two-step linear gradient of 20–50% methanol from 0 to 30 min followed by 50–70% methanol from 30 to 60 min all in 0.1% acetic acid–water. The retention times for the monoiodinated (**VI**) and the diiodinated products were 42 and 48 min, respectively. Coelution with the matching ^{127}I derivatives confirmed their identity. The fractions containing the **VI** peak were tested for binding to the specific antibody raised against **V**, pooled, and stored at −20°C.

Radioimmunoassay

Two assays were developed. RIA_A used the specific antiserum raised against the **IX** immunogen and the **XII** radioligand. Working standards of L-691,121 and L-692,199 were stored as 50 ng ml^{-1} and 5 μg ml^{-1} concentrations, respectively in assay buffer at −70°C. L-691,121 was diluted in buffer to yield concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng ml^{-1} for the standard curve with quality control samples at concentrations of 0, 0.5, 1 and 5 ng ml^{-1}. A standard curve of L-692,199 was prepared by assaying standards at concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng ml^{-1}. Quality control samples contained 0, 20, 50 and 200 ng ml^{-1}.

RIA_B used the specific antiserum raised against immunogen **V** and radioligand **VI**. L-691,121 was diluted in buffer to yield concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng ml^{-1} for the standard curve with quality control samples at concentrations of 0, 0.5, 1 and 5 ng ml^{-1}. The standard curve for L-692,199 was prepared by analysis of standards at concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng ml^{-1}. Quality control samples contained 0, 0.5, 2 and 10 ng ml^{-1}.

The assay buffer was potassium phosphate (0.05 M), containing ethylenediamine–tetraacetic acid (0.05 M), Crotein (2%), sodium azide (0.01%), adjusted to pH 7.0. Primary antisera were diluted appropriately in assay buffer containing 50 μg ml^{-1} rabbit gamma-globulin. Second antibody (sheep anti-rabbit gamma-globulin) was diluted according to titre (generally 1:40) in assay buffer containing the radioligand at 200,000 cpm ml^{-1}. All reagents were pipetted simultaneously: binding at equilibrium is similar whether the primary or secondary antibodies are added simultaneously or sequentially. Reagents were added to 12 × 75 mm glass tubes using an automatic pipetting station (Micromedic APS, Horsham, PA, USA) as follows: 0.05 ml standard solution, test plasma sample or quality control; 0.05 ml normal plasma was added to standards and quality controls. All tubes received 0.1 ml of the specific antibody/gamma-globulin reagent, 0.1 ml of the second antibody/radioligand, and 0.7 ml of assay buffer. Non-specific binding (NSB) was determined in tubes lacking only the primary antibody. After overnight in-

cubation at room temperature (approx. 18 h), the tubes were centrifuged for 45 min at 800 *g*, the supernatant was discarded and the radioactivity in the consolidated pellet determined by counting for 3 min in a gamma counter (Micromedic Model Apex 10/600, Horsham, PA, USA). Assays were performed in triplicate. Calibration curves were constructed by plotting the fraction of control binding (B − NSB)/(Bo − NSB) against concentration using a third degree polynomial as a variant of the conventional logit–log transformation. Appropriateness of fit was evaluated by reading standards as though unknowns. The concentration of analyte in test samples was determined by interpolation from the standard curve.

Differential RIA calculations

Assuming that the assay results, obtained in both the specific assay (RIA_A) and the non-specific assay (RIA_B), represent the sum of the concentrations of drug and cross-reacting metabolite (times its appropriate cross-reactivity factor, CR), then the following equations hold:

$$RIA_A = [Drug] + ([Metabolite] \times CR_A) \quad (1)$$

$$RIA_B = [Drug] + ([Metabolite] \times CR_B). \quad (2)$$

Solving these two equations yields the relationships:

$$[Metabolite] = \frac{RIA_B - RIA_A}{CR_B - CR_A} \quad (3)$$

$$[Drug] = RIA_A - ([Metabolite] \times CR_A) \quad (4)$$

Results

Antisera

Specific antisera were raised in five rabbits for each immunogen. All antisera were tested 3 months after immunization for titre against various radioligands as well as reactivity in the resulting assay of both the parent drug, L-691,121 and its alcohol metabolite, L-692,199. These results are summarized in Table 1. The antisera were stored at −70°C either undiluted or as small aliquots diluted 1:300 in assay buffer. These small aliquots were routinely used to prepare assay reagents when needed.

Radioligands

The incorporation of total radioactivity into **VI** ranged from 7 to 56% for four separate iodinations. For **XII**, the range was 26–49%. When stored at −20°C both radioligands were generally usable for 2 months before new batches were prepared.

Assay buffer

Buffers of pH 6–9 were tested. The highest binding for all assays was obtained at pH 7. Initially, BSA at 1 mg ml^{-1} was included in the assay buffer but was replaced by 2% Crotein when it was shown that metabolite interference in RIA_A was reduced at the more sensitive regions of the displacement curves (Fig. 2). The same effect was achieved with the BSA buffer if 100 pg of the metabolite was added to each tube. This measure had no significant effect on the assay containing Crotein.

Specificity

The approximate cross-reactivity of the metabolite in the RIA_A and RIA_B assays measured at 50% B/Bo was 0.8 and 80%,

Table 1
Titration of antisera from individual rabbits used in the development of RIA_A and RIA_B. These antisera were collected from the animals 7 months after immunization

RIA	Immunogen	Radioligand		Rabbit 1	2	3	4	5
A	**IX**	**XII**	Titre:	13,000	110,000	35,000	17,000	13,000
			CR:	1.3%	0.8%	1.5%	1.3%	0.8%
B	**V**	**VI**	Titre:	1,800	2,100	3,000	2,100	4,000
			CR:	60%	45%	80%	71%	80%

The titre is defined as the dilution of antisera which yields 50% binding (Bo/T) of radioligand. The cross-reactivity (CR) of the major metabolite, L-692,199, measured at 50% of control binding (B/Bo).

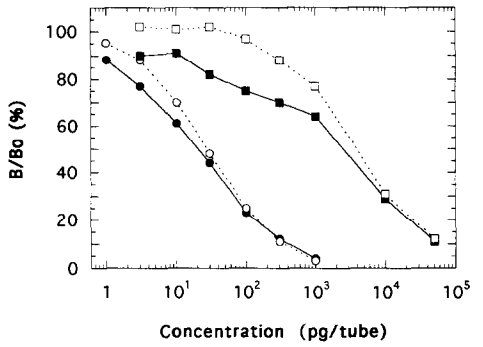

Figure 2
Effect of BSA (0.1%, closed symbols) and Crotein (2%, open symbols) on the displacement curves of L-691,121 (circles) and L-692,199 (squares) in RIA_A.

respectively. However, this relationship was not constant throughout the assay range and is illustrated by the displacement curves for drug and metabolite shown in Fig. 3. Figure 4 shows the relationship between cross-reactivity and %B/Bo with a line representing a third degree polynomial fit. These polyomials were used to generate a cross-reactivity factor for each individual sample based on its position on the standard curve. To determine whether this cross-reactivity relationship would hold for various combinations of drug and metabolite concentrations, six solutions containing drug and metabolite at ratios in the range of (200:1) to (5:1) were diluted so that four to five determinations covering the entire assay range for both assays at each given ratio could be made. All combinations of results from the two assays for each ratio were calculated in two ways, using single cross-reactivity (CR) factors based on the cross reactivity at 50% of control binding and using the polynomial to generate variable CR factors. For example, five determinations in each of the assays would result in 25 possible combinations at a given metabolite to drug ratio. Table 2 contains the mean results and variability of these calculations for drug and metabolite.

Assay sensitivity and precision

Representative standard curves for both assays are illustrated in Fig. 3. A typical assay included standard curves for both L-691,121 and L-692,199 along with quality controls at three concentrations near the upper, middle and lower regions of the calibration range. The intra- and inter-assay accuracy and precision data are shown in Table 3. The sensitivity limit for the parent drug was 0.1 ng ml^{-1}. This

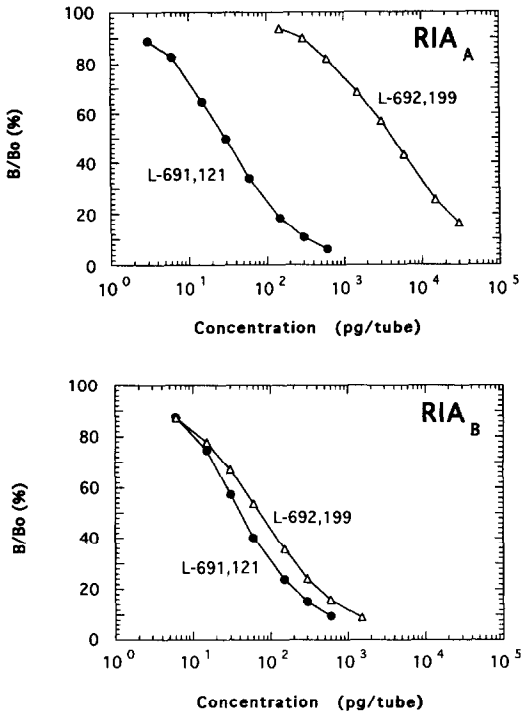

Figure 3
Typical standard curves for both L-691,121 and L-692,199 for RIA_A and RIA_B.

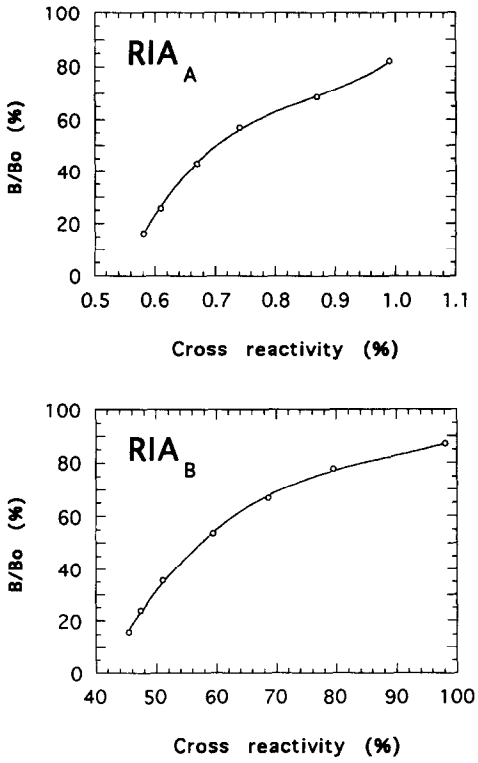

Figure 4
The relationship of B/Bo (%) and the cross-reactivity of L-692,199 in RIA_A and RIA_B expressed as a per cent of L-691,121.

Table 2
Mean accuracy (% found/expected) of drug and metabolite measurement using the differential RIA technique comparing the use of single and variable cross-reactivity factors

Ratio metabolite/drug	Single factor		Variable factor	
	Mean	RSD (%)	Mean	RSD (%)
Drug (L-691,121)				
200	113.9	45	106.5	14
100	115.1	19	106.1	4
50	107.5	11	105.4	4
20	100.8	5	99.2	8
10	99.9	5	98.8	5
5	97.8	2	97.7	2
Metabolite (L-692,199)				
200	95.5	27	102.7	4
100	86.2	18	105.2	3
50	92.2	21	100.7	9
20	95.3	20	105.6	9
10	91.8	14	116.1	5
5	106.3	19	119.3	3

Table 3
Calculation of intra- and inter-assay accuracy and precision of the measurement of L-691,121 and L-692,199 by RIA_A and RIA_B. The former statistic was calculated using 10 replicates within a single assay while the latter was the result of four assays on different days

RIA	Compound	Concentration (ng ml^{-1})	Intra-assay		Inter-assay	
			Recovery* (%)	RSD (%)	Recovery* (%)	RSD (%)
A	L-691,121	0.2	94.6	6.8	92.1	10.0
		1.0	102.8	2.3	96.3	5.3
		5.0	99.5	2.0	98.8	1.0
A	L-692,199	20.0	100.3	10.5	105.6	9.8
		50.0	97.4	4.7	93.9	5.2
		200.0	101.7	1.7	98.2	6.6
B	L-691,121	0.2	117.5	4.3	104.3	4.9
		1.0	105.6	2.2	95.0	3.9
		5.0	105.0	3.3	97.7	5.3
B	L-692,199	0.5	110.4	5.8	100.9	3.4
		2.0	100.0	4.1	104.0	2.5
		10.0	96.2	2.9	104.0	1.4

*Defined as $\frac{[\text{Found}]}{[\text{Added}]} \times 100\%$.

concentration typically resulted in <90% of control binding (B/Bo) using 30 μl of plasma. The inter-assay relative standard deviations (RSD) at 0.2 ng ml^{-1} were 10.0 and 4.9% for assays A and B, respectively.

Parallelism and recovery

Two samples of plasma taken from a human volunteer receiving L-691,121 orally were diluted in control human plasma together with a control containing both L-691,121 and L-692,199 in control plasma. These samples were diluted serially with control plasma and assayed for both drug and metabolite. Satisfactory proportionality was observed and is shown in Fig. 5.

Recovery was assessed by adding reference standard of both compounds to two clinical plasma samples in amounts close to the endogenous quantities previously measured. The results in Table 4 indicate satisfactory recovery.

Analysis of clinical plasma samples

This differential RIA method was used to analyse plasma samples from a clinical study in which volunteers received 15 mg of L-691,121, orally. Figure 6 shows the mean concen-

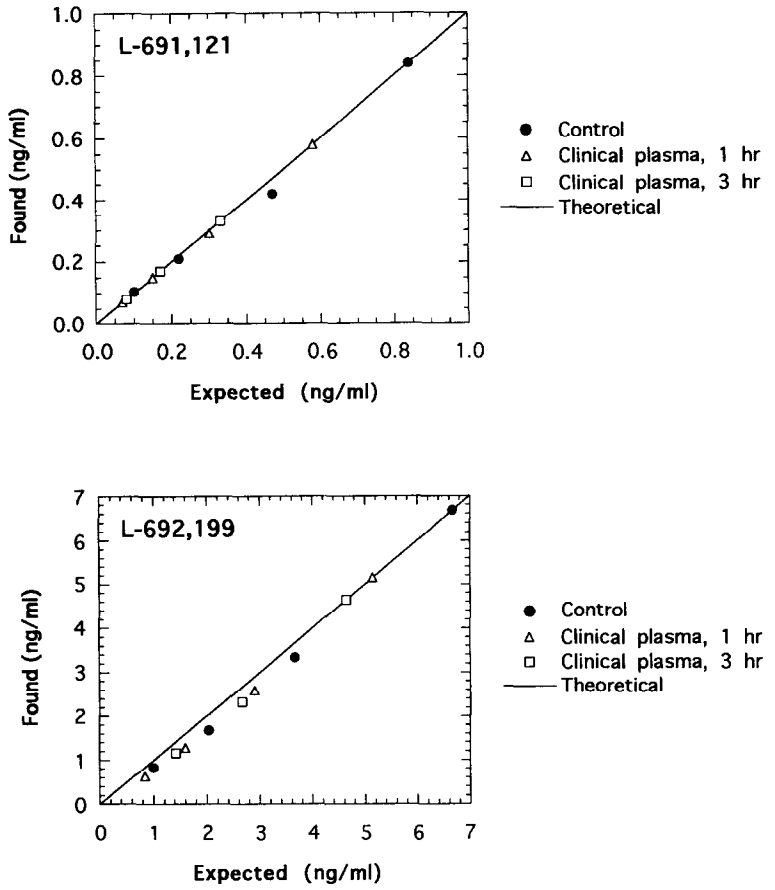

Figure 5
Proportionality of measured concentrations of L-691,121 and L-692,199 to dilution. The control (circles) was normal human plasma spiked with both compounds. The clinical plasma was obtained from a human clinical study in which the volunteer received 15 mg of L-691,121, 1 h (triangles) and 3 h (squares) before these samples were collected. The line represents the theoretical.

Table 4
Recovery of L-691,121 and L-692,199 in clinical samples to which measured quantities of both compounds were added. The plasma was obtained from a human clinical study in which the volunteer received 15 mg of L-691,121 orally 1 and 3 h before these samples were collected

Time post dose (h)	Original (ng ml^{-1})	Added (ng ml^{-1})	Measured (ng ml^{-1})	Accuracy* (%)
Drug (L-691,121)				
1	0.58	0.39	0.92	94.8
3	0.33	0.39	0.69	95.8
Metabolite (L-692,199)				
1	5.15	3.42	8.02	93.6
3	4.64	3.42	7.90	98.0

*Defined as $\frac{[\text{Measured}]}{[\text{Original}] + [\text{Added}]} \times 100$.

tration–time profiles of both drug and metabolite.

Discussion

When developing the RIA for the drug, L-691,121, the alcohol metabolite, L-692,199, had already been identified in dog urine and an authentic standard was available. Therefore early RIA development focused on specificity for the drug in the presence of this metabolite. The approximate cross-reactivities of the meta-

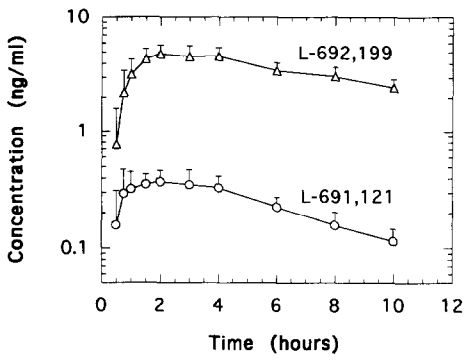

Figure 6
Mean concentration–time profiles of L-691,121 and L-692,199 in the plasma of six volunteers following oral administration of 15 mg of L-691,121. The error bars represent one standard deviation from the mean.

bolite in the two RIAs (measured at 50% B/Bo) were 0.8 and 80%. Metabolism studies in dogs showed that at 6 h post-dose the ratio of metabolite to drug was *ca* 50:1. Ordinarily, a cross-reactivity of <1% would be acceptable, but with this excess of metabolite a 40% overestimation of drug could result even using the more specific assay. One solution considered was to separate the drug from its metabolite by a liquid- or solid-phase extraction prior to RIA, but it was not possible to resolve the two compounds adequately. However, using two RIAs with widely different cross-reactivities the results of both assays were used to calculate drug and metabolite concentrations.

One additional complication became evident. The displacement curves for drug and metabolite were not parallel (Fig. 3) and therefore a constant CR factor was not appropriate. The results in Table 2 showed that the use of variable factors in the calculation of drug and metabolite provided a substantial benefit over the single factor method. This result was not necessarily anticipated since the factors were generated by standard curves of the single compounds alone. In unknown samples and those spiked with the two compounds simultaneously, the binding is a combination of the reactivity and mass of both compounds.

The data suggested assay limits defined by the ratio of metabolite–drug. When measuring the drug, the variability became excessive at a ratio of 200 and the accuracy of metabolite measurement was in jeopardy when the metabolite exceeded drug by <10. The latter observation is the consequence of the result being a small difference between two large numbers.

The observation concerning the effect of buffer protein on the specificity of RIA_A was unexpected. Situations are frequently encountered in which a cross-reacting compound exhibits greater interference at higher binding (B/Bo). A useful solution often involves adding a small amount of the interfering compound to the assay. However, the improvement due to a change from BSA to Crotein, a commercially available collagen hydrolysate, was not anticipated.

Ordinarily, LC-fractionated plasma samples are analysed by RIA to search for additional immunoreactive metabolites or endogenous compounds. However, in this case the concentration of analytes in human plasma was too low to permit this analysis. Nevertheless, the other tests of assay validity did not indicate any obvious defects in the method. The good recoveries of the drug and metabolite added to clinical samples suggested that there was no interference by the matrix or by unknown metabolites. The analysis of the diluted clinical samples also supported assay validity by demonstrating proportionality to dilution. One possible complication would be the presence of a glucuronide conjugate of the alcohol metabolite in plasma. This derivative would most likely be measured as the unconjugated metabolite by this method. We had no definitive means to detect it at the concentrations present in the clinical plasma samples. In preclinical studies a glucuronide was identified in the bile of dogs but not of rats [7].

The analysis of the clinical plasma samples by this method demonstrated the rapid formation of metabolite, L-692,199 following an oral dose. Since the inter-assay precision was quite satisfactory (Table 3), we believe that the high variability observed in drug concentrations during the earliest time points was related to inter-subject variation rather than assay imprecision. The concentrations of drug in the last two time points were approximately the same or lower and did not show excessive variability.

The usefulness of calculating the concentrations of two compounds in plasma from the results of two RIAs with differing reactivities with the analytes was demonstrated. A similar method has been described for morphine and its metabolites [8], although in that study the

necessity for variable CR factors was not evident.

This analytical method is labour-intensive but it is useful in certain situations such as this in which a single assay with sufficient specificity cannot be found and when prior resolution of analyte is impractical. It requires two assays: one discriminating strongly between the two analytes and the other hardly at all. It also requires the absence of significant quantities of additional immunoreactive species in the samples.

Acknowledgements — The authors thank Mr J. Butcher, Dr J. Elliott and Mr J. White for synthesizing the intermediates used to prepare the immunogens, Dr M. Goldberg and Mr E. McWilliams for providing the clinical samples, and Dr H. Ramjit for running FAB mass spectra.

References

[1] E.M. Vaughan Williams, *J. Clin. Pharmacol.* **24**, 129–147 (1984).

[2] E.M. Vaughan Williams, *Eur. Heart J.* **6**, 145–149 (1985).

[3] W.P. Newman, III, R.E. Tracy, J.P. Strong, W.D. Johnson and M.C. Oalmann, *Ann. N.Y. Acad. Sci.* **382**, 39–49 (1982).

[4] D.S. Echt, P.R. Liebson, L.B. Mitchell, R.W. Peters, D. Obias-Manno, A.H. Barker, D. Arensberg, A. Baker, L. Friedman, H.L. Greene, M.L. Huther, D.W. Richardson and the Cast Investigators, *N. Engl. J. Med.* **324**, 781–788 (1991).

[5] R.L. Woosley, *Clin. Pharmacol. Ther.* **47**, 553–556 (1990).

[6] J.M. Elliott, H.G. Selnick, D.A. Claremon, J.J. Baldwin, S.A. Buhrow, J.W. Butcher, C.N. Habecker, S.W. King, J.J. Lynch Jr, B.T. Phillips, G.S. Ponticello, E.M. Radzilowski, D.C. Remy, R.B. Stein, J.I. White and M.B. Young, *J. Med. Chem.* **35**, 3973–3976 (1992).

[7] S. Vickers, C.A. Duncan, P.H. Kari, C.F. Homnick, J.M. Elliott, S.M. Pitzenberger, M. Hichens and K.P. Vyas, *Drug Metab. Dispos.* **21**, 467–473 (1993).

[8] C.W. Hand, R.A. Moore, H.J. McQuay, M.C. Allen and J.W. Sear, *Ann. Clin. Biochem.* **24**, 153–160 (1987).

[9] A.E. Bolton and W.M. Hunter, *Biochem. J.* **133**, 529–539 (1973).

[Received for review 21 July 1993; revised manuscript received 5 October 1993]