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Measurement of the class III antidysrhythmic drug, UK-68,798, in plasma by radioimmunoassay

D.K. WALKER,*† G.W. AHERNE,‡ J.E. ARROWSMITH,§ P.E. CROSS,§ B. KAYE,† D.A. SMITH,† D.A. STOPHER† and W. WILD†

† *Department of Drug Metabolism, Pfizer Central Research, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK*
‡ *Guildhay Antisera Ltd, Unit 6, Riverside Business Centre, Walnut Tree Close, Guildford, Surrey GU1 4UG, UK*
§ *Department of Discovery Chemistry, Pfizer Central Research, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK*

Abstract: A sensitive radioimmunoassay (RIA) for the specific determination of 1-(4-methanesulphonamidophenoxy)-2-[*N*-(4-methanesulphonamido-phenethyl)-*N*-methylamino]ethane (UK-68,798), a novel class III antidysrhythmic agent, in human plasma is described. Specific antisera were raised in sheep using desmesyl-UK-68,798–succinate–ovalbumin conjugate as the antigenic hapten carrier protein. The antisera produced exhibited high specificity for UK-68,798 compared with known metabolites from animals, other antidysrhythmic agents and co-administered drugs. Good correlation was found in a comparison of the RIA method with a high-performance liquid chromatography (HPLC) method ($r = 0.997$) and a 10-fold lower limit of determination was observed for the RIA method compared with the HPLC method (0.05 and 0.5 ng ml^{-1}, respectively). The RIA method was applied to the analysis of UK-68,798 in plasma obtained from human volunteers receiving the compound.

Keywords: *Class III antidysrhythmic drug, UK-68,798; radioimmunoassay; high-performance liquid chromatography.*

Introduction

UK-68,798 (1-(4-methanesulphonamidophenoxy)-2-[*N*-(4-methanesulphonamidophenethyl)-*N*-methylamino]ethane) is a novel chemical structure which has been shown in animal studies to possess highly specific class III antidysrhythmic properties [1] according to the Vaughan-Williams classification [2]. An HPLC assay was developed for the pre-clinical discovery and development programme with the compound. Clinically, the compound may be administered at dose levels of 10 μg kg^{-1} and below. With these low dose levels, plasma concentrations of the compound are below the limit of detection of current chromatography based assays. A radioimmunoassay (RIA) was therefore developed. Initially a charcoal precipitation based assay was used for early clinical studies. Whilst these studies were ongoing the assay was refined, by use of double antibody precipitation, to allow automation. This paper details the development and performance of the radioimmunoassay for the compound. The specificity of this assay has been checked against metabolites of UK-68,798, identified following administration to rat and dog, and likely co-prescribed drugs.

Experimental

Materials

UK-68,798, substrates for the synthesis of the succinyl UK-68,798 hapten, potential metabolites of UK-68,798, D-sotalol, captopril and nifedipine were synthesized at Pfizer Central Research (Sandwich, Kent, UK). [^{3}H]-UK-68,798 was supplied by Amersham International.

Sodium chloride, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate, ammonium dihydrogen orthophosphate, orthophosphoric acid and methanol were all of "Analar" grade from BDH Chemicals Ltd. Dicyclohexylcarbodiimide, thimerosal, gelatin (type III from bovine skin), activated charcoal (100–400 mesh), warfarin, digoxin, propranolol, metoprolol, atenolol, quinidine and anti-sheep whole serum were obtained from Sigma Chemical Company. Lignocaine was obtained from Astra Pharmaceuticals Ltd. *N*-hydroxysuccinimide was from

* Author to whom correspondence should be addressed.

Fluka AG (Buchs SG). Ecoscint A scintillation fluid was from National Diagnostics (USA). Dextran T70 was supplied by Pharmacia AB. Acetonitrile (HPLC grade) was from Rathburn Chemicals Ltd. and methyl t-butyl ether (HPLC grade) from FSA Laboratory Supplies. Polyethylene glycol 6000 was from BDH Chemicals and Hionic-fluor from Packard Instrument Co.

Synthesis of [^{3}H]-UK-68,798

[^{3}H]-UK-68,798 was prepared at Amersham International by the reaction of [^{3}H]-mesyl chloride with 1-(4-methanesulphonamidophenoxy)-2-[*N*-(4-aminophenethyl)-*N*-methylamino]ethane. The product, purified by HPLC on silica gel, had a specific activity of approximately 70 Ci mmol^{-1}.

Synthesis of hapten

The synthetic route for the succinyl desmesyl-UK-68,798 hapten (UK-76,906) is shown in Fig. 1. The precursor (**I** in Fig. 1) was prepared as previously described (European Patent Application EP-245997A). Compound **I** (350 mg, 0.96 mmol) was heated with succinic anhydride (100 mg, 1 mmol) at 130°C for 5 h. The residue was pre-adsorbed onto silica and purified by column chromatography on silica, eluted with methylene chloride containing methanol (gradient 0–100%). Fractions containing the product were combined and

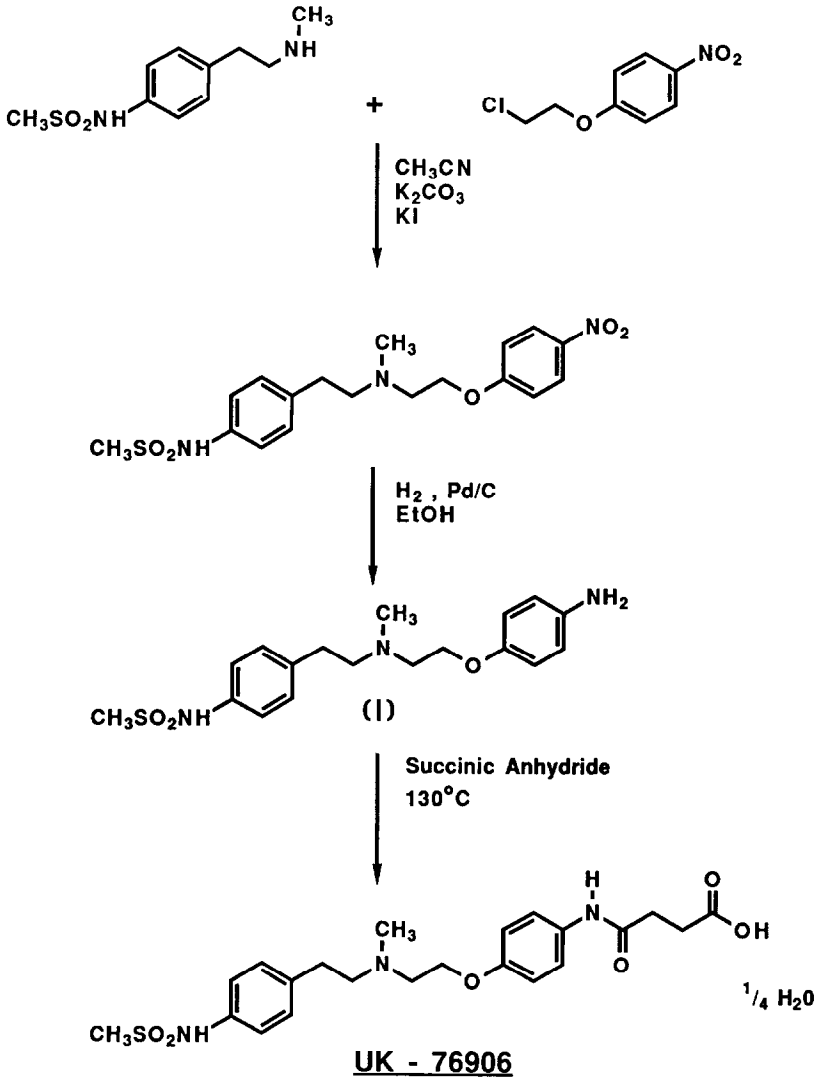


Figure 1
Synthetic route for the succinyl desmesyl-UK-68,798 hapten.

evaporated to give an oil which was triturated with ethanol to afford the hapten UK-76,906; yield 63 mg (14%); melting point 176–177°C; elemental analysis ($C_{22}H_{29}N_3O_6S \cdot \frac{1}{4}H_2O$) Found: C, 56.4; H, 6.3; N, 9.1. Calc. C, 56.4; H, 6.4; N, 9.0. R_f, 0.07 on silica eluted with methylene chloride–methanol, 80:20.

Preparation of conjugate

The hapten UK-76,906 (21 mg, 45 μmol) and *N*-hydroxysuccinimide (5.2 mg, 45 μmol) were dissolved in dry dimethylformamide (800 μl), and a solution of dicyclohexylcarbodiimide (27.8 mg, 135 μmol) in dry dimethylformamide (200 μl) was added with stirring. The reaction mixture was left for 20 h at room temperature in the dark without agitation. Crystals of dicyclohexyl urea were formed. An aliquot (0.5 ml) containing the activated ester was taken from above the crystals and added to a solution of ovalbumin (25 mg) in water (2 ml), equivalent to a molar ratio of 20:1 assuming complete conversion of hapten into active ester. After 24 h at 4°C, the protein solution was dialysed against three changes of 1 l water. The cloudy protein solution was diluted to 5 mg ml^{-1}, 0.1% sodium azide added and stored at 4°C.

Immunization of animals

Two Suffolk cross sheep were immunized with the ovalbumin conjugate at six intramuscular sites. 1 ml of conjugate solution (5 mg) was emulsified with 2 ml of complete non-ulcerative Freund's adjuvant (Guildhay Antisera Ltd). Blood was obtained from the jugular vein 9 days later and at appropriate intervals. Further intramuscular injections consisting of 2.5 mg conjugate emulsified with incomplete adjuvant were given 9, 18, 43 and 74 weeks later. Antisera were separated from blood by centrifugation and stored at 4°C with 0.1% sodium azide added.

RIA procedure

All dilutions of standard, tracer and antisera were made using 0.05 M phosphate buffer (pH 7.4) containing 0.01% (w/v) thimerosal, 0.6% (w/v) sodium chloride and 0.1% (w/v) gelatin. To samples and standard solutions containing UK-68,798 (4–120 pg) and [^{3}H]-UK-68,798 (c. 8000 dpm and 28 pg) in a total volume of 0.5 ml containing up to 0.1 ml plasma, was added diluted antiserum (0.1 ml). The mixture was incubated at 4°C for 2 h before separation of bound and unbound drug by the addition of dextran coated charcoal suspension in 0.05 M phosphate buffer (pH 7.4) (0.1 ml). Following mixing, samples were allowed to stand at 4°C for 10 min, and centrifuged at 1730***g***, 4°C for 10 min. Supernatant (0.5 ml), containing bound drug, was transferred to a plastic counting vial containing Ecoscint A scintillant and counted for radioactivity.

The RIA calibration curve for UK-68,798 was constructed using a final dilution of 1:6000 of the antiserum (i.e. 100 μl of 1:1000 dilution of the antiserum added to 500 μl buffer containing sample and tracer) and taking mean values of duplicate determinations at each standard concentration (Fig. 2). A computer program based on ALLFIT was used to fit an unweighted sigmoidal curve to the data [3]. The limit of determination was 0.05 ng ml^{-1} of plasma.

Automated method

The assay was automated by using a Tecan 5032 automatic sample processor to carry out all steps prior to the addition of scintillant. The initial steps of the automated procedure were as described above except that the concentrations of [^{3}H]-UK-68,798 and antiserum were doubled. After incubation at room temperature for 1.5 h bound and unbound drug were separated by the addition of anti-sheep serum (0.1 ml of a 1:8 dilution of a vial of freeze dried serum reconstituted in 2 ml buffer) and PEG 6000 [0.2 ml of a 10% (w/v) aqueous solution]. After mixing tubes were maintained at 4°C for 2 h and then centrifuged at 1730***g*** for 10 min at 4°C. Supernatant containing unbound drug was transferred to a plastic counting vial (20 ml) and after addition of methanol (1 ml) and Hionic-fluor (10 ml) the radioactivity was measured by scintillation counting for 10 min.

Extraction procedure for HPLC analysis

Plasma samples (2.0 ml) were buffered with 0.1 M phosphate buffer (pH 7.4, 2 ml), UK-68,798 and internal standard (compound **23** in ref [4]) were extracted with t-butyl methyl ether (5 ml) for 5 min prior to back extraction into 0.2% (v/v) phosphoric acid (1.0 ml), neutralization with 0.1 M phosphate buffer (pH 7.4, 2.0 ml) and re-extraction into t-butyl methyl ether (5 ml). The ether layer was evaporated to dryness under a stream of nitrogen and the residue redissolved in 100 μl mobile phase for HPLC analysis.

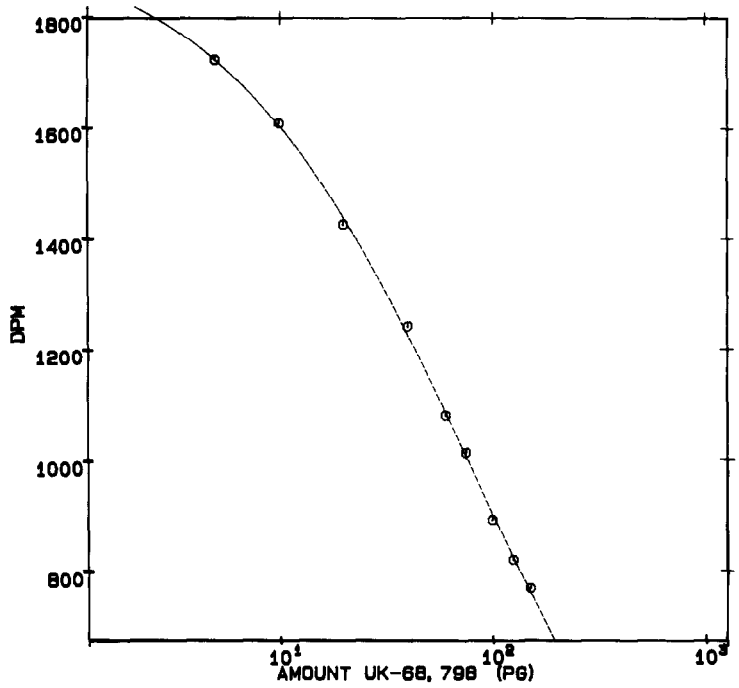


Figure 2
Calibration curve of 4-120 pg UK-68,798 produced by a modified version of the computer program ALLFIT. Each calibration point represents the mean value of duplicate determinations. Concentrations in unknown samples, in up to 100 μl of human plasma, suitably diluted with assay buffer, were calculated from this curve from the mean of duplicate determinations.

HPLC analysis

The HPLC system comprised of a 25 × 0.5 cm analytical column filled with Spherisorb, 5 μm silica packing material (Hichrom, Reading), the mobile phase was acetonitrile–20 mM ammonium phosphate, pH 7.0 (40:60, v/v) pumped at 1.0 ml min^{-1} with UV detection at 230 nm. The limit of determination was 0.5 ng ml^{-1}.

Results and Discussion

The production of antisera in the two sheep was assessed by the dilution of antisera (in 100 μl buffer) giving 50% binding to 28 pg of [^{3}H]-UK-68,798 (in 500 μl buffer). The antiserum used in the assay was from sheep 1641 following the final boost (Fig. 3).

The specificity of the antiserum was assessed by the competition in binding to the antibody between UK-68,798 and its known metabolites from animal experiments (Fig. 4) and other likely co-prescribed drugs. The results are listed in Table 1, in which the cross-reactivity is expressed as a concentration ratio of the test compound to UK-68,798 to give a 50% displacement of [^{3}H]-UK-68,798. The only metabolite giving a significant degree of cross reactivity was metabolite **I**, the *N*-desmethyl-UK-68,798. HPLC analysis has not detected plasma concentrations of this compound in man likely to influence the assay (Fig. 5). The only drug to demonstrate any cross-reactivity was sotalol, which has the same methyl-sulphonamide function as UK-68,798. Whilst the cross reactivity of sotalol is lower than that

Table 1
Specificity of the antiserum

Compound	Cross-reactivity (%)
UK-68,798	100
Metabolite **I**	11
Metabolite **II**	1.5
Metabolite **III**	0.2
Metabolite **IV**	<0.002
Warfarin	<0.002
Digoxin	<0.002
Propranolol	<0.002
Metoprolol	<0.002
Amiodarone	0.06
D-Sotalol	<0.002
Lignocaine	<0.002
Atenolol	<0.002
Nifedipine	<0.002
Quinidine	<0.002
Captopril	<0.002

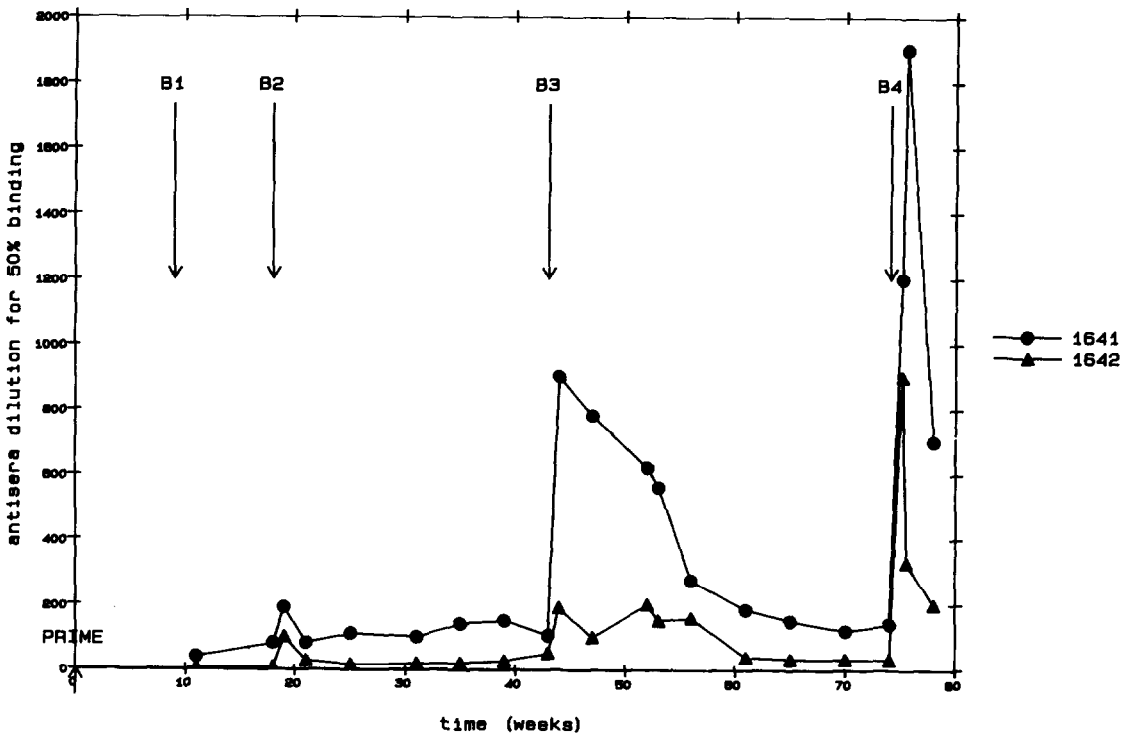
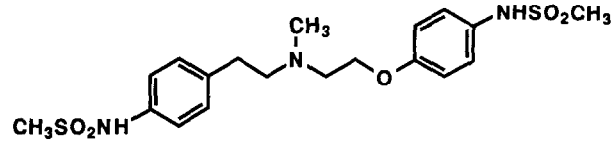
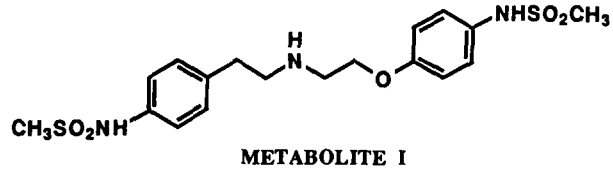


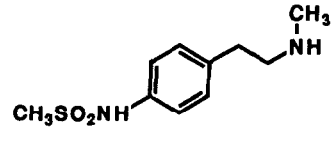
Figure 3
Production of antisera to UK-68,798 in two sheep following priming and booster doses of the succinyl desmesyl-UK-68,798 ovalbumin conjugate. Antisera titre were assessed as the dilution of antisera in 100 µl which bound 50% of approximately 28 pg [^{3}H]-UK-68,798 in a total volume of 600 µl.



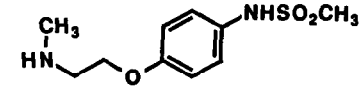
UK-68798



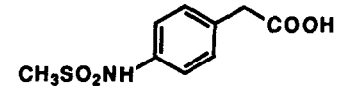
METABOLITE I



METABOLITE II



METABOLITE III



METABOLITE IV

Figure 4
Structure of UK-68,798 and likely metabolites which have been assessed for cross-reactivity by the RIA procedure.

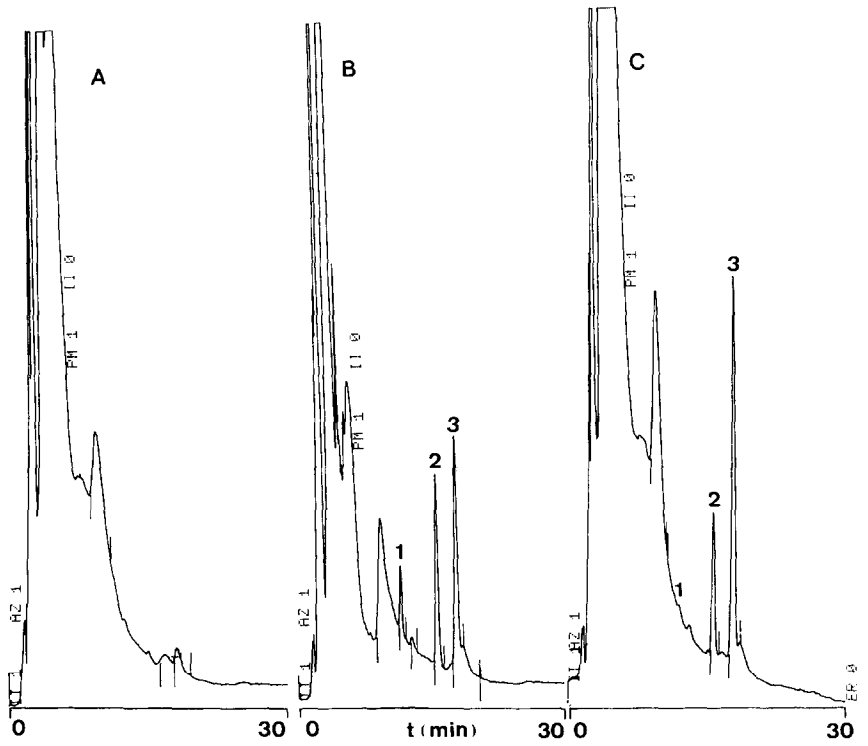


Figure 5
HPLC traces of human plasma extracts to confirm the absence of the *N*-desmethyl metabolite of UK-68,798 (metabolite I) in human plasma following doses of UK-68,798 to human volunteers. Trace A is an extract of 4 ml control human plasma. Trace B is an extract of 4 ml control human plasma to which was added 5 ng ml^{-1} metabolite I (peak 1), 5 ng ml^{-1} UK-68,798 (peak 2) and 10 ng ml^{-1} internal standard (peak 3). Trace C is an extract of 4 ml pooled plasma (1–6 h) from a human volunteer following a single oral dose (12.5 μg kg^{-1}) of UK-68,798 to which 10 ng ml^{-1} internal standard has been added.

seen for metabolites of UK-68,798 a concentration of 1–2 μg ml^{-1} sotalol would give an equivalent response to 0.1 ng ml^{-1} of UK-68,798. Concentrations in excess of 2 μg ml^{-1} sotalol are observed following oral doses of 200–800 mg sotalol [5]. This assay would therefore be unsuitable for UK-68,798 in the presence of sotalol unless a pre-separation stage was performed. However, the two compounds are most unlikely to be co-prescribed.

Accuracy and precision of the assay on a day-to-day basis were determined by analysis of quality assurance samples of human plasma to which known amounts of UK-68,798 had been added (Table 2). These samples were analysed daily together with unknown samples. Intra-daily accuracy and precision are shown in Table 3. Accuracy and precision of the assay were of the same order as that observed in many RIA procedures where only low levels of radioactivity are present (e.g. [6]) and are therefore suitable for the analysis of samples. The assay was not affected by changes in plasma volumes up to 100 μl.

The manual method was used for early clinical trials with UK-68,798. However, with the increasing number of samples for assay during compound progression, the need for a less labour intensive method resulted in the development of the automated method using the Tecan 5032 automatic sample processor. Separation of bound and unbound radioactivity was performed by the double antibody procedure as this was more amenable to automation than charcoal precipitation. An improvement in inter- and intra-daily accuracy and precision of the assay was found using the automated method (Tables 4 and 5).

To confirm the specificity and validity of the RIA method, a number of samples from human volunteers were analysed by both RIA and HPLC methods and the results plotted as shown in Fig. 6. To extend the validation to higher concentrations more applicable to the HPLC method a number of samples from dogs were also included. A good correlation between the two methods is indicated (r = 0.997).

Table 2
Inter-daily accuracy of the analysis of UK-68,798 by RIA (manual method using charcoal precipitation)

Concentration added (ng ml^{-1})	Mean determined concentration (ng ml^{-1})	Relative standard deviation (%)	Relative mean error (%)
0.80	0.78 (n = 8)	14	3
1.60	1.51 (n = 7)	18	6
2.80	2.60 (n = 7)	5	7
3.60	3.20 (n = 7)	9	11
4.40	4.04 (n = 7)	9	8

Table 3
Intra-daily accuracy of the analysis of UK-68,798 by RIA (manual method using charcoal precipitation)

Concentration added (ng ml^{-1})	Mean determined concentration (ng ml^{-1})	Relative standard deviation (%)	Relative mean error (%)
0.10	0.09 (n = 5)	25	10
0.50	0.55 (n = 5)	8	10
1.00	1.01 (n = 5)	4	1

Table 4
Inter-daily accuracy of the analysis of UK-68,798 by RIA (automated method using double antibody separation)

Concentration added (ng ml^{-1})	Mean determined concentration (ng ml^{-1})	Relative standard deviation (%)	Relative mean error (%)
0.10	0.093 (n = 7)	12	7
0.30	0.311 (n = 7)	12	4
0.55	0.504 (n = 7)	18	8

Table 5
Intra-daily accuracy of the analysis of UK-68,798 by RIA (automated method using double antibody separation)

Concentration added (ng ml^{-1})	Mean determined concentration (ng ml^{-1})	Relative standard deviation (%)	Relative mean error (%)
0.10	0.098 (n = 7)	3.4	2.0
0.30	0.286 (n = 7)	2.2	4.7
0.50	0.521 (n = 7)	6.4	4.2

Figure 7 shows mean plasma concentration profiles of UK-68,798, as determined by RIA analysis, in human volunteers following single oral and intravenous (10 μg kg^{-1}) administration of UK-68,798. Following intravenous administration, plasma levels exhibited a bi-exponential decline with a terminal elimination rate of 0.075 h^{-1}. After oral dosing the mean peak plasma concentration was 2.5 ng ml^{-1} at 3.0 h and declined with a terminal elimination rate of 0.081 h^{-1}. The assay allowed monitoring of plasma concentrations up to 48 h after single oral and intravenous doses of 10 μg kg^{-1} UK-68,798, at which time concentrations were in the range 0.05–0.12 ng ml^{-1}. Thus following a dose of 10 μg kg^{-1} the assay is sensitive enough to measure concentrations of the drug over four times the elimination half-life of the compound. The assay therefore is suitable for future monitoring of UK-68,798 concentrations in clinical studies and will provide reliable results for planned pharmacokinetic studies.

In conclusion, the RIA of UK-68,798 provided greater sensitivity than that available by HPLC using a smaller volume of plasma. The procedure was sufficiently specific that pretreatment of samples to isolate UK-68,798 from known animal metabolites and endogenous components of plasma was not required.

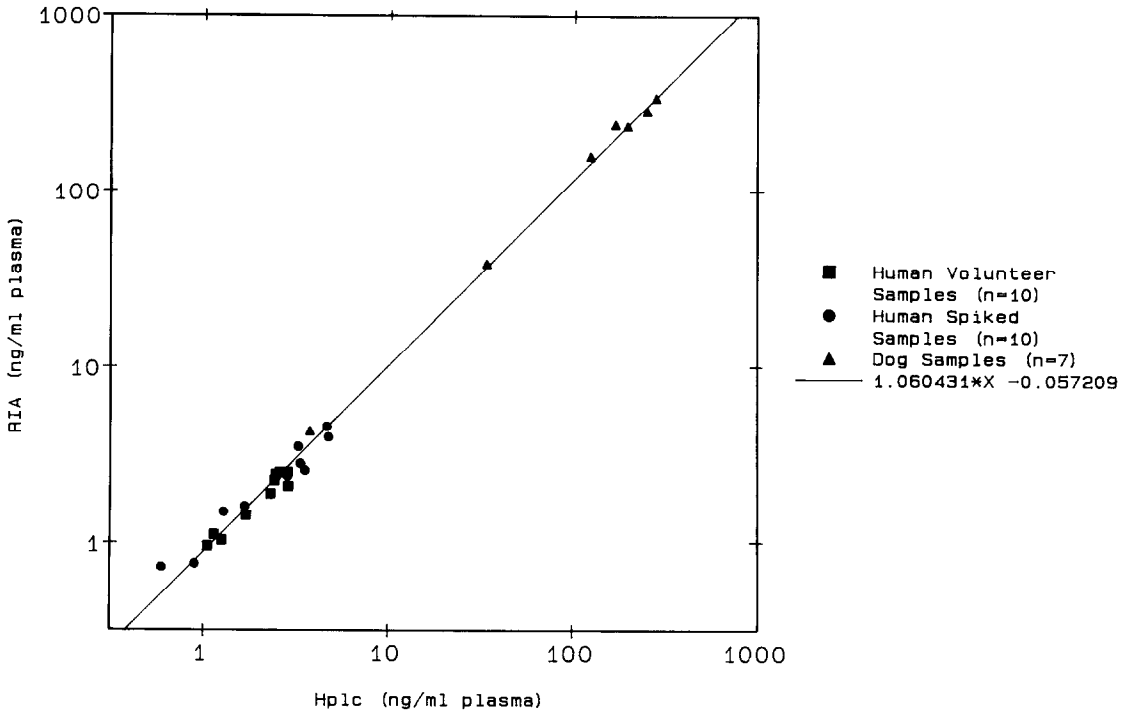


Figure 6
Correlation graph of plasma UK-68,798 concentrations measured by HPLC (x) and RIA (y) in dog and human plasma, regression coefficient $r = 0.997$.

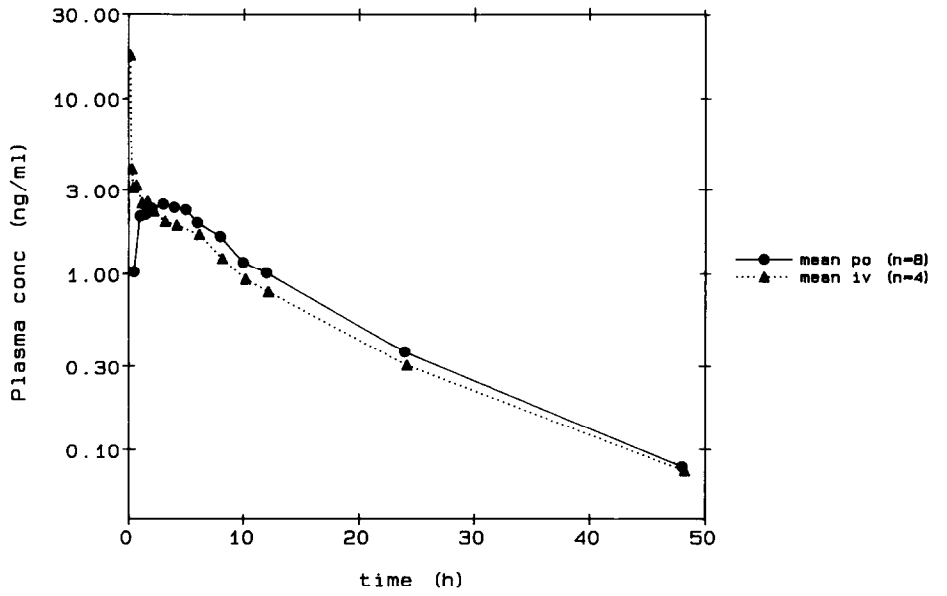


Figure 7
Mean plasma concentrations of UK-68,798 in human volunteers following single oral ($n = 8$) and intravenous ($n = 4$) doses of UK-68,798 at a dose level of 10 μg kg^{-1} as measured by RIA.

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