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A radioimmunoassay combined with solid-phase extraction for the determination (pg ml^{-1}) of AR-C15849XX in human plasma

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Abstract

A radioimmunoassay has been developed for the determination of AR-C15849KF, a CCK-8 analogue, in human plasma. The method incorporates solid-phase sample extraction, is suitable for the determination of the analyte at pg ml^{-1} concentrations and is based on a method developed and validated for dog plasma. The solid-phase extraction, using ion-exchange aminopropyl and octadecyl sorbents sequentially, was retained for this procedure to remove matrix interferences in the plasma and to enhance method sensitivity. The calibration range is 10–500 pg ml^{-1}, using a 1 ml sample of undiluted human plasma. The method has been successfully used to generate early human pharmacokinetic data during a programme of exploratory development.

Keywords: AR-C15849KF; Anti-obesity; Human intravenous pharmacokinetic data; Human plasma; Radioimmunoassay; Solid-phase extraction

1. Introduction

AR-C15849KF (Fig. 1) is a novel and highly potent anorectic cholecystokinin octapeptide (CCK-8) receptor agonist, of possible application for the treatment of obesity. Obesity is acknowledged to be a major risk factor for morbidity and mortality [1,2]. The prevalence of obesity (body mass index > 30 kg m^{-2}) is 12% in the USA, 7% in Europe and 5% in Japan, equating to approximately 60 million people worldwide. Many of the drugs that are currently used are amphetamine-like central nervous system (CNS) stimulants with significant side-effects and/or abuse potential. There is, therefore, a clear medical and economic need for effective and safe anti-obesity drug treatment. AR-C15849KF acts peripherally following systemic absorption (limiting its potential for CNS side-effects and abuse) and, by imitating some of the effects of the naturally occurring peptide hormone CCK, triggers a feeling of satiety.

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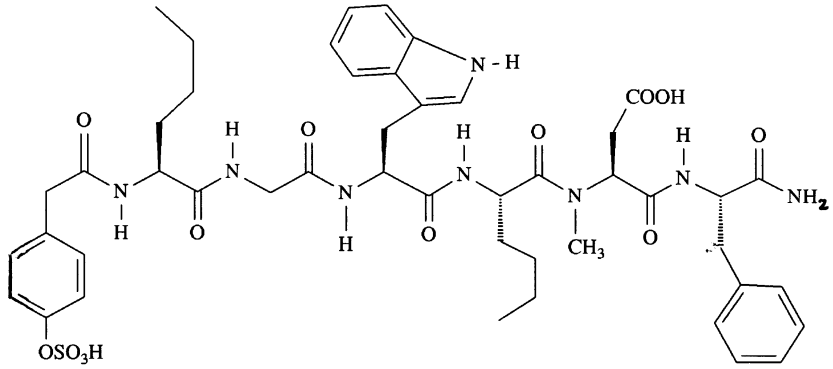

$HpaSO_3H$-Nle-Gly-Trp-Nle-MeAsp-Phe-NH_2

Fig. 1. Chemical structure of AR-C15849XX.

A radioimmunoassay (RIA) combined with solid-phase extraction (SPE) has been developed and validated for the determination of AR-C15849XX in dog plasma [3], and has been used to generate data in support of safety evaluation studies during exploratory development of the compound. A method suitable for use in human plasma has now been developed using similar methodology to enable the determination of pg ml^{-1} concentrations of AR-C15849XX present in samples of human plasma taken during exploratory clinical studies.

This paper describes the development and validation of this method, and describes the results of early pharmacokinetic studies involving intravenous administration of the compound to human volunteers.

2. Experimental

2.1. Chemicals

Chemicals were obtained from several sources. Buffer salts (Analytical grade) were purchased from Sigma UK and Fisher Scientific; methanol (HPLC grade) was supplied by Fisher Scientific. AR-C15849KF was provided by the Department of Chemistry, Astra Arcus, Rochester, NY, and by the Clinical Trials Supplies Unit, Astra Charnwood, Loughborough, UK. Radiolabelled AR-C15745XX was synthesised in the Chemical Development Department, Astra AB, Loughborough, UK. No-carrier-added sodium [^{125}I]iodide was supplied by Amersham International plc. Sheep anti-AR-C15849 antiserum was generated as described previously [3]. Donkey anti-sheep IgG antiserum was obtained from the Scottish Antibody Production Unit, Carluke, UK.

Solid-phase extraction cartridges, supplied by International Separation Technology Ltd., were obtained from Jones Chromatography. Borosilicate glass tubes (12 mm × 75 mm) were obtained from Corning. Deionised water (> 18 MΩ cm^{-1}) was obtained using a Milli Q system.

The numbering system for the compound formerly known as ARL 15849XX changed to AR-C15849XX after the publication of the original method [3]. These two designations refer to the same compound.

2.2. Preparation of RIA working solutions

The RIA diluent consisted of 0.1 M sodium phosphate buffer, pH 6.0, containing 0.05% (w/v) bovine serum albumin. This solution was stored for up to 1 month at a nominal 4°C.

Working solutions of the AR-C15745XX radioligand and antiserum (described below) were prepared on each day of analysis. Radioligand working solution was prepared by adding approximately 100 μl of the stock solution to 10 ml of

Table 1
Concentrations of AR-C15849XX measured in individual human plasma samples

Sample details	Determined concentration (pg ml^{-1})							
	1	2	3	4	5	6	Mean	CV%
Spiked plasma (100 pg ml^{-1})	98.3	87.4	103	94.2	86.8	88.9	93.1	7.1
Blank plasma	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	–
Blank B/B$_0$ value (%)	102	99.3	98.5	102	97.0	100	99.8	2.0

RIA diluent; 100 μl of this solution contained approximately 15000 dpm. Antiserum working solution was prepared by diluting the neat anti-AR-C15849 antiserum 400000-fold in assay diluent. Primary aqueous stock standard solutions (60 μg ml^{-1}) were prepared at intervals of approximately 3 months and stored at or below −20°C. Plasma calibration standards and quality control samples were stored at or below −20°C for up to 12 months.

Phase separation of antibody-bound and free (unbound) radioligand was performed by second antibody precipitation of the bound radioligand with diluted donkey anti-sheep IgG antiserum. This reagent (1:4 dilution of neat antiserum in RIA diluent) and the non-immune sheep serum (1:50 in RIA diluent), added to improve pellet formation, were prepared and used on the day of analysis.

2.3. Apparatus

Solid scintillation counting was performed by means of a Packard Cobra 5005 gamma counter with integral data reduction software. The four parameter logistic or smoothed spline algorithms were employed to convert radioactivity measurements, expressed as counts per minute (cpm), into concentration values (pg ml^{-1}).

Solid-phase extraction was carried out using 3 ml IST cartridges containing 500 mg of aminopropyl adsorbent, followed by 3 ml IST cartridges containing 500 mg of octadecyl end-capped adsorbent, and performed using a 24 position vacuum box. The final sample extract was reduced to dryness under nitrogen in a Turbovap evaporator.

2.4. Blood sample collection

Blood samples, for the preparation of a normal human plasma pool (control plasma) and for generation of individual plasma samples, were taken from healthy volunteers into tubes containing lithium heparin as anticoagulant. The plasma was separated from the red cells by centrifugation.

All plasma samples were stored at or below −20°C and assayed within three freeze–thaw cycles.

Table 2
Interassay precision and accuracy of validation samples

	Determined concentration of AR-C15849XX (pg ml^{-1})							
	Validation sample (pg ml^{-1})							
	5	10	20	50	100	200	500	1000
Accuracy (%)	68.0	95.0	106	103	106	95.5	99.0	137
Precision (%CV)	82.4	18.3	17.7	12.0	16.3	12.8	9.3	47.2

Table 3
Intra-assay precision and accuracy of validation samples

	Determined concentration of AR-C15849XX (pg ml^{-1})							
	Validation sample (pg ml^{-1})							
	5	10	20	50	100	200	500	1000
Accuracy (%)	176	116	107	99.2	118	107	92.8	73.6
Precision (%CV)	80.5	9.9	12.6	16.6	3.9	2.5	7.1	23.4

2.5. Methods

2.5.1. Synthesis of [^{125}I]AR-C15745XX radioligand

AR-C15745XX was radioiodinated in the hydroxyphenylacetic acid moiety (Fig. 1) via direct electrophilic radioiodination utilising sodium [^{125}I]iodide as the ^{125}I source and chloramine-T as oxidant, as described previously [1]. The required product was purified by preparative high-performance liquid chromatography.

2.5.2. Solid-phase extraction radioimmunoassay (SPE–RIA)

2.5.2.1. Extraction. Two solid-phase extraction steps were employed prior to RIA: an initial ion-exchange extraction on aminopropyl sorbent was followed by a desalting and concentration step on octadecyl endcapped sorbent. The order of additions to the aminopropyl cartridges was as follows: (1) 1×2 ml 100% (v/v) methanol; (2) 5×3 ml 1 M sodium acetate buffer, pH 5.5; (3) 4×3 ml 50 mM sodium acetate buffer, pH 5.5; (4) 1 ml plasma sample (diluted with 2 ml water); (5) 3 ml 50 mM sodium acetate buffer, pH 5.5; (6) 3 ml 50 mM sodium citrate buffer, pH 6.0.

Eluates, containing AR-C15849KF, were collected during step 6 and applied directly to octadecyl endcapped cartridges conditioned with 2 ml 100% (v/v) methanol, followed by 2 ml water. After a 2 ml wash with 10% (v/v) methanol, the compound was eluted with 3 ml 100% methanol and evaporated to dryness under nitrogen at 45°C. Extracts were reconstituted in 0.5 ml assay diluent by vortex mixing. Extraction of the compound from plasma using the above procedure is identical to that reported for the dog plasma analysis method [3]. The extract is reconstituted in a reduced volume of diluent in order to increase the sensitivity of the method.

2.5.2.2. RIA. Calibration standards were prepared over the range 10–2000 pg ml^{-1} AR-C15849XX in pooled human plasma. A zero plasma calibration standard and plasma quality control samples at three levels were also analysed. All plasma samples were extracted, as described above, before RIA. Analysis of the extracts was carried out in duplicate using borosilicate glass tubes for all calibration standards, quality controls and test samples. In each instance, the final RIA incubation contained 0.1 ml sample extract, 0.1 ml radioligand working solution and 0.1 ml antiserum working solution. Additional non-specific binding tubes, designed to assess the extent of precipitation of the radiolabel in the absence of primary antibody, and total radioactivity tubes were included with each batch of analyses.

The assay tubes were incubated overnight for at least 15 h at a nominal 4°C. After allowing the tubes to reach ambient temperature, phase separation was then carried out by addition of 0.1 ml donkey anti-IgG antiserum working solution (1:4 dilution) and 0.1 ml non-immune sheep serum (1:50 dilution), each prepared in assay diluent. Each of the tubes was vortex mixed and incubated at ambient temperature for 30 min, then centrifuged (3500 rpm, 20 min, 15°C). After transfer to a suitable holding rack, the supernatants were decanted to waste by inversion of the rack over a suitable sink. Tubes were then transferred to gamma counter racks and counted for at least 2 min.

Table 4
Stability of AR-C15849XX in human plasma stored under different conditions

Parameter	Storage condition (nominal added concentration 100 pg ml^{-1})							
	Baseline	Freeze–thaw × 3	24 h Ambient temp.	24 h 4°C	24 h −20°C	24 h −20°C Extr.	24 h 4°C Extr.	12 months −20°C
% Nominal concentration	86.6	95.5	110	126	117	109	121	110

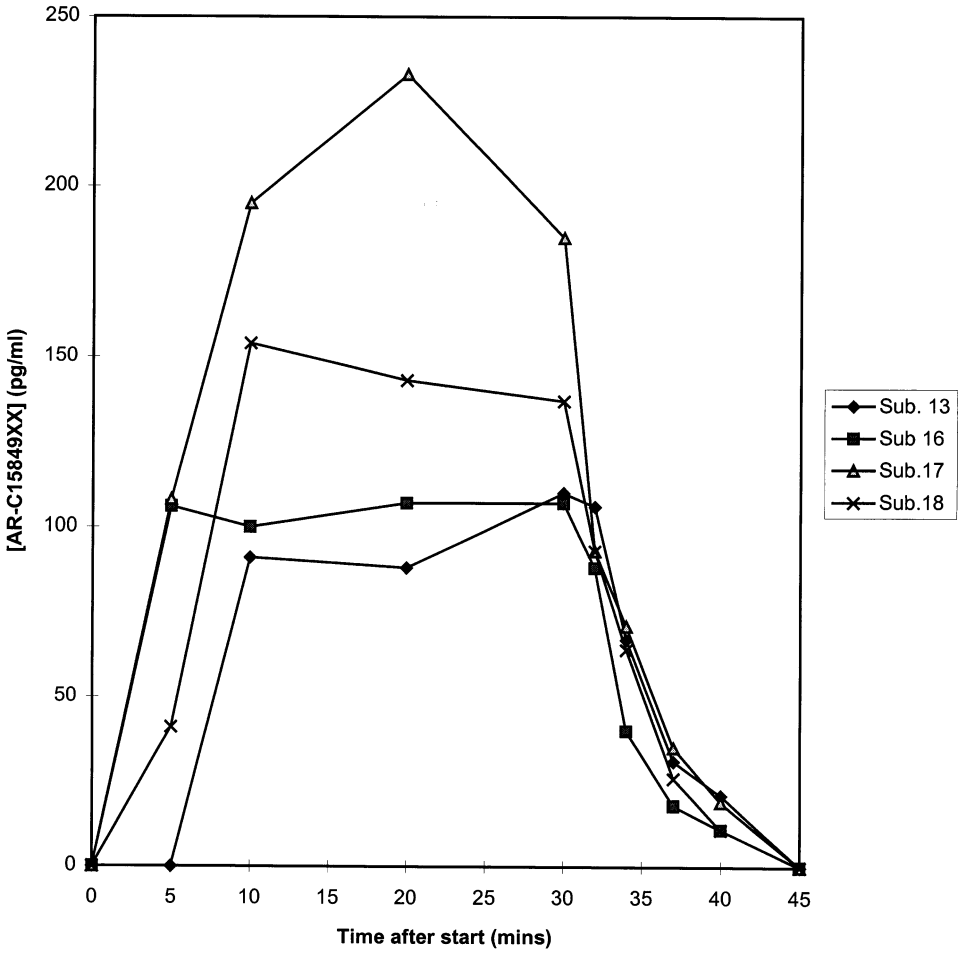

Fig. 2. Plasma AR-C15849XX concentration profiles in four human volunteers following intravenous administration at 4.0 ng kg^{-1} min^{-1}.

The SPE–RIA procedure was used for method validation studies and to analyse human plasma samples from clinical studies.

2.5.3. Validation of the SPE–RIA method

The SPE–RIA was validated in accordance with recommendations [4–7] which assess the performance of a method with regard to its specificity, sensitivity, accuracy and precision. Quality control samples were included throughout these validation studies and in each test sample analysis batch. In addition, the stability of the compound in plasma under a variety of conditions was established. The specificity assessment in this case was limited to an examination of individual matrix samples for effects on recovery of the compound. This is justifiable in view of the extensive cross-reactivity screen carried out for the method in dog plasma [3], and the similarity of the methodology used for the two matrices.

2.5.3.1. Assay accuracy and precision. The inter- and intra-assay accuracy and precision of the method were determined by analysis of aliquots of control plasma spiked with AR-C15849KF at each of the concentrations of the calibration standards. The interassay investigations were performed over five independent analysis batches using identical spiked samples that had been aliquoted and stored at −20°C prior to analysis.

Intra-assay performance was assessed by six replicate determinations of the spiked control plasma.

2.5.3.2. Stability. Aliquots of control plasma spiked with AR-C15849KF were analysed immediately following preparation, then after storage under the following conditions: 24 h at a nominal 4°C, at or below −20°C, and at ambient temperature; up to 12 months at or below −20°C. The stability to up to three freeze–thaw cycles was investigated, as was the stability of the sample extracts at or below −20°C.

2.5.3.3. Analysis of diluted extracts. The concordance between results obtained by analysing extracts at different dilutions in assay diluent was investigated at dilutions up to 20-fold in order to extend the range of the method.

3. Results and discussion

3.1. Solid-phase extraction RIA (SPE–RIA)

Early investigations indicated that significant interferents to ligand antibody binding were present in unextracted human plasma; this, and the extreme sensitivity required of the method for analysis of the compound in clinical samples following intravenous dosing at rates at or below 4 ng kg^{-1} min^{-1}, made use of a direct RIA method impracticable. It was therefore necessary to include a suitable solid-phase extraction procedure for purification and concentration of the sample prior to RIA. The procedure described here effectively removes plasma interferents, and has the additional advantage of concentrating the compound of interest, thereby increasing the sensitivity of the method.

Table 5
Effect of sample extract dilution on measured AR-C15849XX concentration

	Determined AR-C15849XX concentration (pg ml^{-1})			
Dilution factor	1.0	5.0	10	20
Concentration	506	572	486	452
% nominal concentration	101	114	97.2	90.4

3.2. Validation of the SPE–RIA method

3.2.1. Specificity studies

3.2.1.1. Interference by endogenous plasma components. Plasma samples from six different volunteers were analysed. The results are presented in Table 1, and show all plasma samples to be free of any interfering components detectable above the limit of quantification (LOQ) of the method.

3.2.2. Method accuracy, precision, calibration range and sensitivity

Summary inter- and intra-assay accuracy and precision data for the determination of AR-C15849XX in human plasma are presented in Tables 2 and 3. The accuracy and precision data were used to define the LOQ of the method. Both inter- and intra-assay precision and accuracy data for the 5 pg ml^{-1} validation sample were clearly outside conventional limits of acceptability for a bioanalytical method at its proposed LOQ (20% for precision and 80–120% for accuracy). The values at 10 pg ml^{-1}, however, did both fall within these limits. Accordingly, the LOQ of the method was set at 10 pg ml^{-1}. In a similar way, the upper reporting limit for undiluted sample extracts was set at 500 pg ml^{-1}, based on unacceptable performance at 1000 pg ml^{-1}.

3.2.3. Stability

Data obtained for the stability of AR-C15849XX in human plasma in samples containing 100 pg ml^{-1} AR-C15849XX are presented in Table 4. The results indicate minimal effect of storage on the measured concentration of the compound in human plasma, either at ambient temperature or stored frozen at or below −20°C, over a 24 h period. Plasma samples and sample extracts stored at 4°C did show a slight rise in measured concentration over this period, but sample extracts stored frozen at or below −20°C had acceptable stability. Recent investigations have

confirmed the stability of the compound in plasma for periods up to 1 year at this temperature.

3.2.4. Analysis of diluted sample extracts

The effect of sample dilution on measured AR-C15849XX concentrations in extracts of a 2000 pg ml^{-1} validation sample analysed undiluted, and diluted 5-, 10- and 20-fold with assay diluent, are shown in Table 5. Agreement between dilutions up to 20-fold is good. This, together with the extract stability data at or below −20°C reported in Table 4, justifies the practice of re-analysing samples with measured concentrations above the working range of the calibration curve, using diluted sample extracts.

3.2.5. Application of the method to analysis of samples from clinical exploratory development studies involving intravenous administration

Samples were taken from healthy volunteers during an ascending dose intravenous tolerability study and analysed using the method reported here. Plasma concentrations were of considerable interest, since this represented the first administration of the compound to man. The data are summarised in Fig. 2, which represents the data for individual volunteers dosed by intravenous infusion at the highest rate administered (4.0 ng kg^{-1} min^{-1}). Preliminary pharmacokinetic analysis of the data suggests the half-life of the compound in plasma is around 3 min.

4. Conclusions

A radioimmunoassay, incorporating a solid-phase sample extraction procedure, has been developed and validated for the determination of AR-C15849XX in human plasma. The method is highly sensitive (LOQ 10 pg ml^{-1}), highly specific, and has levels of precision and accuracy acceptable for early development. It has been used to demonstrate acceptable stability of the compound in plasma, and is sufficiently sensitive for specific determination of the AR-C15849XX in plasma samples from low dose clinical studies carried out in support of the exploratory development programme.

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