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A RIA combined with SPE for the determination of a dual D_2-receptor and β_2-adrenoceptor agonist, AR-C68397XX, in human plasma

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Abstract

A radioimmunoassay has been developed for the determination of AR-C68397XX, a dual D_2-receptor and β_2-adrenoceptor agonist, in human plasma. The method incorporates solid phase sample extraction and is suitable for the determination of the analyte at pg ml^{-1} concentrations. The antiserum was raised in Suffolk cross sheep following primary and booster immunisations with an immunogen prepared by conjugating a carboxyphenylmethyl derivative of AR-C68397XX, to bovine serum albumin. The radioligand was prepared by the ^{125}I-labelled iodination of a derivative of AR-C68397XX. The solid phase extraction procedure, using octadecyl sorbent, was introduced to remove matrix interferences in the plasma and to enhance method sensitivity. The calibration range is 20–500 pg ml^{-1}, using 0.5 ml of undiluted human plasma sample.

Keywords: AR-C68397AA; D_2-receptor and β_2-adrenoceptor agonist; Plasma; SPE; RIA

1. Introduction

AR-C68397AA (Fig. 1) is a novel and potent dual D_2-receptor and β_2-adrenoceptor agonist, currently under development for the treatment of chronic obstructive pulmonary disease (COPD) and asthma. Chronic lung diseases currently affect about 10% of the population in the western world, and their incidence is growing world-wide. Asthma is now acknowledged to be an inflammatory disease and is a risk factor in the acquisition of chronically impaired respiratory function, particularly if inadequately treated during its early stages. COPD is characterised by a persistent cough and increased mucus production, and can involve permanent changes to the bronchial mucosa leading to emphysema. COPD and asthma are still under-treated diseases in many countries,

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and there is a continuing need for new and more effective treatments.

An analytical method which employs HPLC has been developed for the determination of the free base, AR-C68397XX, in dose formulations. However, this method is unsuitable for use with plasma matrix, and has insufficient sensitivity for determination of the concentrations present in human plasma samples following inhalation dosing of the compound. A radioimmunoassay (RIA) involving a high specific activity ^{125}I-labelled radioligand, combined with solid-phase extraction (SPE) has therefore been developed to enable the determination of pg ml^{-1} concentrations of AR-C68397XX (the free base form of AR-C68397AA hydrochloride salt) present in samples of human plasma taken during exploratory clinical tolerability and pharmacokinetic studies.

This paper describes the development and validation of an RIA for the specific determination of AR-C68397XX, employing solid phase extraction for the removal of plasma interferences.

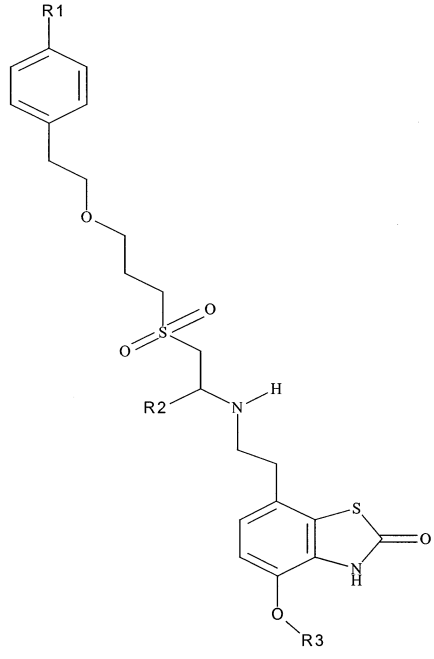

Fig. 1. Chemical structure of AR-C68397XX and related compounds.

2. Experimental

2.1. Chemicals

Chemicals were obtained from several sources. Buffer salts (analytical grade) were purchased from Sigma and Fisher; methanol (HPLC grade) was supplied by Fisher. AR-C68397AA, AR-C68397-bovine serum albumin (BSA) conjugate and radiolabelled AR-C68397XX were synthesised in the Department of Medicinal Chemistry, Astra Charnwood, Loughborough, UK. Potential cross-reacting compounds used for the specificity studies were also obtained from the same laboratories. No-carrier-added sodium ^{125}I-iodide was supplied by Amersham International. Common comedicant drugs were obtained from Sigma, Poole, Dorset. BSA (RIA grade) and Freund's incomplete adjuvant were purchased from Sigma. BGC vaccine, BP was obtained from Evans. Solid phase extraction cartridges supplied by International Separation Technology Ltd, were obtained from Jones Chromatography. Deionised water (>18 Mohm cm^{-1}) was obtained using a Milli-Q system.

2.2. Preparation of radioimmunoassay working solutions

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

Working solutions of the AR-C68397XX 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 assay diluent; 100 μl of this solution contained approximately 12 000 dpm. Antiserum working solution was prepared by diluting the neat antiserum 250 000-fold in assay diluent. Primary stock standard solutions 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 3 months.

Phase separation of antibody-bound and free (unbound) radioligand was carried out by second antibody precipitation of the bound radioligand with diluted donkey anti-sheep IgG antiserum. This reagent (1:4 dilution of neat antiserum in assay diluent) was prepared and used on the day of assay.

2.3. Apparatus

Solid scintillation counting was performed by means of a Packard Cobra 5010 γ counter with integral data reduction software. The four-parameter logistic (4PL) or smoothed spline algorithms were employed to convert radioactivity measurements, expressed as counts min^{-1} (cpm), into concentration values (pg ml^{-1}).

Solid-phase extraction was carried out using IST cartridges (3 ml) containing octadecyl end-capped adsorbent (500 mg), 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 ethylenediamine tetraacetic acid (EDTA) as anticoagulant. The plasma was separated from red cells by centrifugation.

All plasma samples were stored at or below -20°C and assayed within three freeze–thaw cycles. Under these conditions, the results of stability studies showed that AR-C68397XX is stable for up to 6 months.

2.5. Methods

2.5.1. Synthesis of [^{125}I]AR-C68397XX monoiodophenol radioligand

A high-specific activity ^{125}I-labelled radioligand for use in radioimmunoassay development was synthesised in a two-step reaction. The structure of the product is shown in Fig. 1. The product was stored at or below -20°C prior to use in the radioimmunoassay.

2.5.2. Antiserum production

2.5.2.1. Preparation of the immunogen. In view of the relatively low molecular weight of this compound (464.6 as the free base), conjugation to a suitable protein was considered necessary to obtain a satisfactory antigenic response. Cationised BSA was found to give an improved antigenic response compared to the unmodified protein. Preparation of cationised BSA and conjugation of the ligand was carried out as detailed below.

2.5.2.2. Preparation of BSA-AR-C68397 conjugate. A solution of ethylene diamine was prepared (1 litre flask used) by diluting ethylene diamine (67 ml) with water (500 ml). The pH of this solution was adjusted to pH 4.75 by the addition of hydrochloric acid (6 M, ca. 330 ml). When the solution had cooled to room temperature, a solution of BSA (RIA grade, Sigma, 5 g) in water was added and the pH adjusted to 4.75. EDAC (1-ethyl-3[(3-dimethylaminopropyl)-carbodiimide) hydrochloride (1.8 g), was added in one portion and the pH adjusted to 4.75. The pH was maintained at 4.75 for 2 h and then the reaction quenched with sodium acetate buffer (pH 4.75, 30 ml, 4 M). Dialysis was carried out in Spectra Por dialysis tubing ($2 \times 1 \times 45$ mm, molecular weight cut off 14 000) against running water for 24 h. The dialysis was repeated for a further 24 h and the solution freeze-dried over 72 h to yield the cationised product (4.2 g). Cationised BSA (42 mg) was dissolved in water (1 ml) and triethylamine (50 μl) added. THF (0.5 ml) was added and the pH adjusted to 6–7 with 10% HCl (Solution A). 7-[*N*-(4-carboxyphenylmethyl)-2-aminoethyl]-4-hydroxybenzothiazolone (17.2 mg) was dissolved in water (1 ml), THF (1 ml) and triethylamine (50 μl) and the pH adjusted to 6–7 with 10% HCl (solution B). Solutions A and B were mixed and then EDAC (9 mg) added. After a further 5 min, 2 mg of EDAC was added. After ca. 10 min, the solution became opalescent. It was filtered rapidly through paper tissues and chromatographed on Sephadex G-25 eluting with water. The fractions were examined by TLC (silica gel, ethyl acetate/isopropanol/water 10:7:6 v/v) to confirm that excess hapten had been removed

from the protein fractions. The fractions containing the protein were identified by spraying the TLC plate with ninhydrin reagent and applying heat. The protein fractions were combined and freeze-dried, yielding 34.5 mg of the final product. The epitopic density of this conjugate was calculated by UV–vis spectrometry and was found to be 7 mol hapten per mol cBSA.

2.5.2.3. Antiserum production in sheep. Antisera were raised in sheep (Suffolk-cross) using Freund's complete (FC) and incomplete (FIC) adjuvants. The immunogen (200 μg per animal) was emulsified with Freund's complete adjuvant and used to immunise three Suffolk cross sheep by the intramuscular route. The animals were subsequently boosted at 1, 2 and 3 months after the initial immunisation, with further 200-μg amounts of the conjugate emulsified in Freund's incomplete adjuvant. Test bleeds were taken approximately 10 days after each booster immunisation, and acceptable titres, corresponding to 50% maximum binding of the radioligand, were found in all sheep after the second boost. The serum from the second bleed from Sheep VS 76 (titre approximately 1:750 000) was judged to be the most suitable for development of an RIA method, and was stored at or below −20°C.

2.5.3. Solid-phase extraction radioimmunoassay (SPE-RIA)

2.5.3.1. Extraction. A single solid-phase extraction steps was employed prior to RIA; octadecyl end-capped sorbent was used as a combined purification and concentration step to remove interferents and enhance the sensitivity of the method. The order of additions to the octadecyl cartridges was as follows:

1. Methanol (100% v/v, 1 × 2 ml);
2. Water (1 × 2 ml);
3. Diluted plasma sample (1.5 ml);
4. Sodium phosphate buffer, pH 6.0 (100 mM, 3 ml);
5. 100% methanol (3 ml).

After each addition, the fluid was allowed to run into the column until the top of the bed was just dry. At the end of the extraction, eluates from the methanol elution step were evaporated to dryness under nitrogen at 45°C. Extracts were reconstituted in 1 ml assay diluent by vortex mixing.

2.5.3.2. RIA. Calibration standards were prepared over the range 10–1000 pg ml^{-1} AR-C68397XX 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 prior to RIA. Analysis of the extracts was carried out in duplicate using borosilicate glass tubes (Corning, 12 × 75 mm), 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 assay diluent, 0.1 ml radioligand working solution and 0.1 ml antiserum working solution. Non-specific binding (NSB) 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 donkey anti-IgG antiserum working solution (0.1 ml, 1:4 dilution) and non-immune sheep serum (0.1 ml, 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 rev. min^{-1}, 20 min, 15°C). After transfer to a suitable holding rack, the supernatants were decanted to waste by inversion of the rack. Tubes were then transferred to γ counter racks and counted for at least 2 min. The SPE-RIA procedure was used for method validation studies, and to analyse human plasma samples from phase I and phase II clinical studies.

2.5.4. Validation of the SPE-RIA method

The SPE-RIA was validated in accordance with recommendations [1–4] 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.

2.5.4.1. Specificity studies

Cross-reactivity. The specificity of the antiserum, with regard to potential cross-reacting compounds was determined by analysing aliquots of control plasma which had been spiked with the compound at concentrations up to 2000 pg ml^{-1}. Cross-reactivity, where quantifiable, was calculated from the measured apparent concentration in the RIA as a percentage of the nominal concentration. The only metabolite so far identified as being present in significant quantities in human plasma or urine is the glucuronide conjugate, attached via the phenolic –OH moiety of the benzothiazolone ring. The following compounds were tested (Fig. 1): (**1**), (**2**), (**3**). In addition, the compound 4-hydroxy-2-oxo-(3H)-benzthiazole-7-acetic acid (**4**), was tested.

In addition to investigating cross-reactivity with respect to closely related structures, a range of common comedicants used in asthma therapy was investigated as follows: salbutamol, salmeterol, beclamethasone dipropionate, ipatropium bromide, sodium cromoglycate, nedocromil sodium and paracetamol.

Interference by endogenous plasma components. Control-plasma samples taken from six different subjects, were analysed by SPE-RIA to assess any interference by endogenous material.

2.5.4.2. 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-C68397XX at each of the concentrations of the calibration standards. The inter-assay investigations were performed over five separate 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 validation samples.

2.5.4.3. Stability. Aliquots of control plasma spiked with AR-C68397XX 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 4 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 stored for longer periods at or below −20°C.

2.5.4.4. 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 made use of a direct RIA method impracticable. It was therefore necessary to develop a suitable solid-phase extraction procedure for purification and concentration of the sample prior to RIA. The procedure described here effectively removes plasma interferents.

3.2. Validation of the SPE-RIA method

3.2.1. Specificity studies

3.2.1.1. Cross-reactivity. Aliquots of blank pooled plasma samples were spiked with the following compounds to assess their potential for cross-reaction if present in test plasma samples (Fig. 1): (**1**), (**2**), (**3**), 4-hydroxy-2-oxo-(3H)-benzthiazole-7-acetic acid (**4**). The cross-reactivity data are presented in Table 1.

As might be predicted from the linkage position of the conjugate used to generate this antiserum, significant cross-reactivity was detectable for compound (**1**). There was, however, no detectable cross-reactivity with the fragment (**4**), and much reduced cross-reactivity with a carbonyl substituted compound (**2**). This indicates significant recognition beyond the benzothiazolone ring structure. The only metabolite so far identified as

Table 1
Cross-reactivity with putative metabolites and common comedicants

Compound	Apparent AR-C68397XX concentration in cross-reactivity samples (pg ml^{-1})		
	Observed concentration (pg ml^{-1})	Nominal concentration (pg ml^{-1})	% Cross-reactivity
(**1**)[a]	52.9	2000	2.6
(**2**)[a]	1040	2000	52
(**4**)[a]	<20	2000	<0.5
Salbutamol	<20	20 000	<0.1
Salmeterol	<20	20 000	<0.1
Beclamethasone dipropionate	<20	20 000	<0.1
Ipatroprium bromide	<20	20 000	<0.1
Sodium cromoglycate	<20	50 000	<0.04
Nedocromil sodium	<20	50 000	<0.04
Paracetamol	<20	1000 ng ml^{-1}	<0.005

[a] See Fig. 1 for compound structures.

Table 2
Concentrations of AR-C68397XX determined in individual human plasma samples

Sample details	Determined concentration (pg ml^{-1})							
	1	2	3	4	5	6	Mean	CV (%)
Blank plasma	<20	<20	<20	<20	<20	<20	<20	–
200 pg ml^{-1} spiked	194	224	150	203	177	198	191	13.2
Blank B/B_0 value (%)	98	103	103	103	103	105	103	2.3

Table 3
Inter-assay precision and accuracy of validation samples

Sample	Validation sample (pg ml^{-1})							
	10	20	50	100	200	350	500	1000
Accuracy (%)	102	109	104	103	107	92	103	86
Precision as % CV	22.9	7.8	14.6	10.5	11.0	15.8	17.1	35.4

present in significant quantity in plasma or urine is the glucuronide conjugate (**3**), linked at the phenolic hydroxyl moiety of the benzothiazolone ring, and this metabolite showed no detectable cross-reactivity in the assay.

There was no detectable cross-reaction with any of a range of common comedicants used in asthma or COPD therapy. The combination of antiserum specificity and SPE extraction before RIA thus results in a highly specific assay for the parent compound.

3.2.1.2. Interference by endogenous plasma components. Control plasma samples from six different subjects were analysed. The results are presented in Table 2, and show all plasmas to be free of any interfering components detectable above the limit of quantification of the method. Results from

analysis of the same plasma samples containing 200 pg ml^{-1} AR-C68397XX confirmed the absence of significant interferents.

3.2.2. Method accuracy, precision, calibration range and sensitivity

Inter- and intra-assay accuracy and precision data for the determination of AR-C68397XX in human plasma are summarised in Tables 3 and 4. The accuracy and precision data were used to define the lower limit of quantification (LOQ) of the method. The LOQ was defined as 20 pg ml^{-1} on the basis of acceptable inter- and intra-assay performance of the validation sample spiked at 20 pg ml^{-1}. The upper reporting limit for undiluted sample extracts was set at 500 pg ml^{-1}.

3.2.3. Stability

Data obtained for the stability of AR-C68397XX in human plasma in samples containing 100 pg ml^{-1} AR-C68397XX are presented in Table 5. The results indicate minimal effect of storage on the measured concentration of the compound in plasma at or below 4°C. Plasma extracts likewise show good stability over a 1-month period at −20°C.

3.2.4. Analysis of diluted sample extracts

The effect of sample dilution on measured AR-C68397XX concentrations in extracts of a 500 pg ml^{-1} validation sample analysed undiluted, and diluted 2-, 5-, 10- and 20-fold with assay diluent,

Table 4
Intra-assay precision and accuracy of validation samples

Sample	Validation sample (pg ml^{-1})					
	10	20	100	200	500	1000
Accuracy (%)	118	113	104	107	110	85
Precision as % CV	15.4	16.7	8.0	13.7	13.6	44.7

Table 5
Stability of AR-C68397XX in human plasma stored under different conditions

Parameter	Storage conditions (nominal added concentration 200 pg ml^{-1})						
	Base-line	Freeze–thaw ×3	24 h ambient	24 h 4°C	24 h −20°C	1 month extracts	6 month −20°C
Concentration	235	248	216	175	220	216	228
% Nominal concentration	118	124	108	87.5	110	108	114

Table 6
Effect of sample extract dilution on measured AR-C68397XX concentration: samples diluted before extraction versus diluted extracts

	Determined AR-C68397XX concentration (pg ml^{-1}) at dilution factor of				
	1	2	5	10	20
Sample diluted before extraction	565	456	497	487	–
% Nominal concentration	113	91	99	98	–
Extract diluted before RIA	537	560	511	496	547
% Nominal concentration	107	112	102	99	109

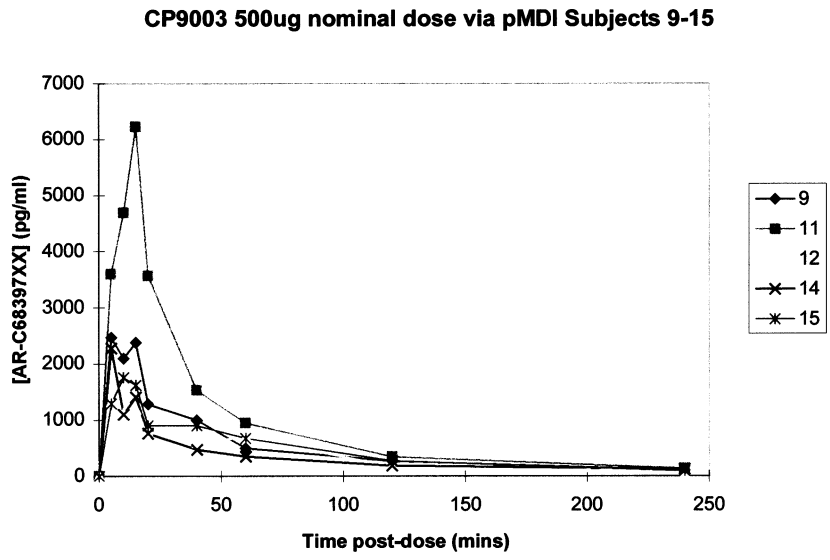

Fig. 2. Concentrations of AR-C68397XX in human plasma samples from an ascending dose clinical safety and tolerability study using the metered dose inhaler formulation.

are shown in Table 6. Agreement between dilutions up to 20-fold is good. This, together with the extract stability data reported in Table 5, justifies the practice of re-analysing samples with measured concentrations above the working range of the calibration curve, using diluted sample extracts. The data for plasma samples diluted up to 10-fold with control human plasma before extraction is also acceptable.

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

Samples were taken from healthy volunteers [5] during an ascending dose i.v. 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 via the metered dose inhaler. The data are summarised in Fig. 2, which represents the data for individual volunteers administered a single dose of 500 μg AR-C68397AA by inhalation. Preliminary PK analysis of the data suggests the half-life of the compound in plasma is around 45 min, with peak plasma concentrations reached approximately 10 min after dosing.

4. Conclusions

A radioimmunoassay for the determination of AR-C68397XX in human plasma, incorporating a solid-phase sample extraction procedure, has been developed and validated. The method is highly sensitive (LOQ, 20 pg ml^{-1}), specific, and has levels of precision and accuracy acceptable for clinical support during compound development. The method has been used to demonstrate stability of the compound in plasma under a variety of conditions, and is sufficiently sensitive for the determination of the AR-C68397XX in plasma samples from clinical studies carried out in support of the current development programme.

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