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A time-resolved fluorescence immunoassay for the determination of a novel respiratory therapeutic agent, AR-C68397XX (Viozan™) in human plasma

Vivian J.C. Willson [a,*], William J.S. Lockley [b], Andrew Mather [b], Jaspal Singh [a], Charles M. Gilbert [a], Mark A. Bayliss [c], David Wilkinson [a]

[a] *Department of Drug Development Metabolism and Bioanalysis, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leics., LE11 5RH, UK*
[b] *Department of Medicinal Chemistry, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leics., LE11 5RH, UK*
[c] *Department of Drug Metabolism and Pharmacokinetics, Pfizer Central Research, Sandwich, Kent, UK*

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Abstract

A dissociation-enhanced lathanide fluorescence immunoassay (DELFIA™) method has been developed for the determination of AR-C68397XX, a novel respiratory therapeutic agent, in human plasma. The method is a 'direct' immunoassay and provides an alternative to the solid phase extraction RIA described in a previous publication, which employs the same specific antiserum. The DELFIA method is suitable for the determination of the analyte at pg ml^{-1} concentrations. The non-isotopic label was prepared by complexation of a DTPA derivative of AR-C68397XX with free europium cation (Eu^{3+}). Plasma samples were diluted at least 5-fold prior to analysis to eliminate matrix interference. The calibration range is 10–2000 pg ml^{-1}, and the LOQ of the method is 50 pg ml^{-1} using 50 μl of diluted human plasma sample.

Keywords: AR-C68397AA; Respiratory therapy; COPD; Plasma analysis; DELFIA

1. Introduction

AR-C68397AA (Viozan™) (Fig. 1) is a novel and potent respiratory therapeutic agent with combined D_2 and β_2-receptor agonist properties, currently under development for the treatment of chronic obstructive pulmonary disease (COPD). Chronic lung diseases currently affect about 10% of the population in the western world, and their incidence is growing worldwide. 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

* Corresponding author. Fax: 44-1509-645535.
E-mail address: viv.willson@astrazeneca.com (V.J.C. Willson).

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changes to the bronchial mucosa leading to emphysema. Asthma and COPD are still undertreated diseases in many countries, and there is a continuing need for new and more effective treatments.

A specific and highly sensitive radioimmunoassay involving solid phase extraction (SPE) has been developed and validated [5]. This analytical method has been used for the determination of AR-C68397XX in plasma samples from phase I and early phase II studies in support of compound development. However, although relatively robust, this method does involve a time-consuming SPE stage, which limits batch sizes and sample throughput during routine analysis. The development of a non-extraction method involving DELFIA technology [7] was therefore undertaken in an attempt to increase the potential for rapid throughput, without compromising the sensitivity requirements of clinical support. An additional advantage was the enhanced stability of the non-isotopic tracer, which obviates the regular radioiodination required to maintain the RIA method. This paper describes the development and validation of a DELFIA method for the specific determination of AR-C68397XX in human plasma samples.

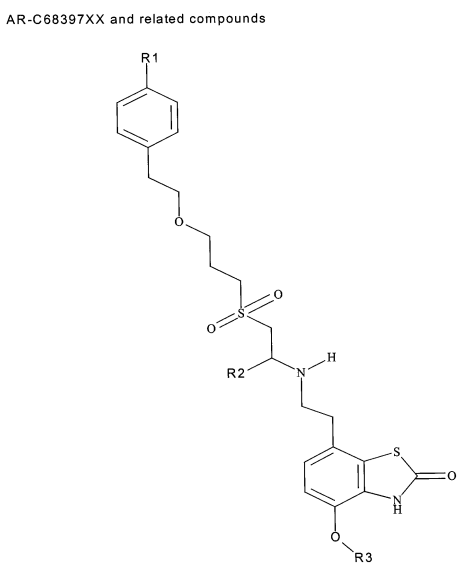

Fig. 1. Chemical structure of AR-C68397XX and related compounds.

2. Experimental

2.1. Chemicals

Chemicals were obtained from several sources. Buffer salts (Analytical grade) and Diethylene triamine penta acetic acid (DTPA) were purchased from Sigma UK and Fisher Scientific; methanol (HPLC grade) was supplied by Fisher Scientific. Bovine Serum Albumin (BSA, RIA grade) and Tween 40 were purchased from Sigma UK. AR-C68397AA and the Eu^{3+} containing DTPA derivative of AR-C68397XX, and the β-glucuronide conjugate of AR-C68397AA, were synthesised in the Department of Medicinal Chemistry, AstraZeneca R&D Charnwood, Loughborough, UK. A specific anti-AR-C68397 antiserum was raised in sheep in these laboratories, as described previously [5]. DELFIA wash buffer, DELFIA enhancement solution and Goat anti-Sheep IgG-coated microwell plates (CC33-105) were obtained from Wallac. Deionised water (>18 Mohm cm^{-1}) was obtained using a Milli-Q system.

2.2. Preparation of immunoassay working solutions

The immunoassay buffer consisted of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.05% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Tween 40 and 1 mM DTPA. This solution was stored for up to 1 month at a nominal 4°C.

Working solutions of the AR-C68397XX DELFIA tracer and antiserum (described below) were prepared on each day of analysis. The tracer working solution was prepared by dilution of a 10 μg ml^{-1} stock to a final concentration of 125 pg ml^{-1} in immunoassay buffer. Antiserum working solution was prepared by diluting the neat antiserum 250 000-fold in immunoassay buffer. 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 achieved by perform-

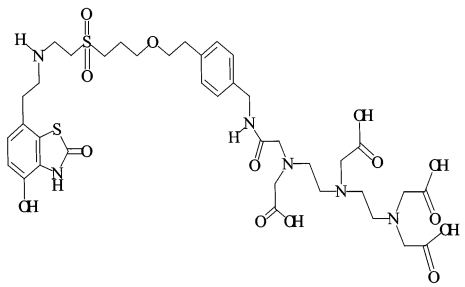

Fig. 2. Chemical structure of AR-C68397-DTPA conjugate.

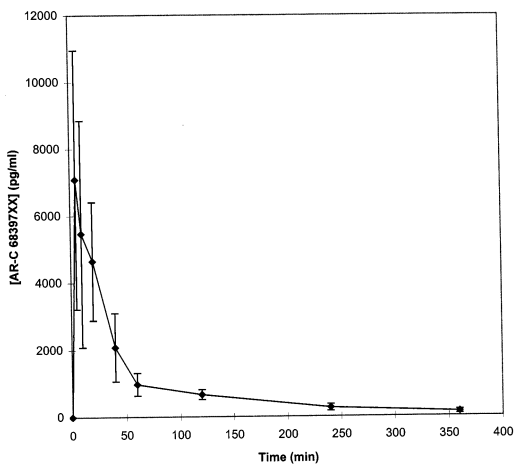

Fig. 3. Concentrations of AR-C68397XX in human plasma samples from an ascending multiple dose clinical safety, tolerability and pharmacokinetic study (CP 9022) using the metered dose inhaler formulation. (error bars plotted at 1 SD level).

ing the immunoassay incubation in 96-well format, second antibody pre-coated microwell strips, and decanting the supernatant to waste after completion of the reaction.

2.3. Apparatus

Time-resolved dissociation-enhanced lathanide fluorescence counting was performed by means of a Wallac Victor 1420 multi-label fluorescence counter. The counter was linked to a PC with the Wallac MULTICALC™ data reduction software installed. The four parameter logistic (4PL) or smoothed spline algorithms were employed to convert sample fluorescence measurements, expressed as counts (delayed fluorescence measured over 1 s at 613 nm following excitation at 340 nm), into concentration values (pg ml^{-1}). Microwell strips were aspirated and washed using a DELFIA 1296-026 Plate washer, and DELFIA enhancement solution was dispensed using a DELFIA 1296-041 Plate dispenser.

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 the 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 [5] have shown that AR-C68397XX is stable for up to 6 months.

2.5. Methods

2.5.1. Synthesis of Eu^{3+}-containing DTPA derivative of AR-C68397

For DELFIA methodology, a lanthanide chelate conjugated to the compound was required. The cyano-derivative of the compound was synthesised initially. Specific reduction of the cyano group with sodium trifluoroacetylborohydride gave the corresponding amine. Conjugation with an excess of diethylenetriaminepentaacetic dianhydride gave a mixture of products from which the mono-conjugated product (Fig. 2) was isolated by reversed phase (C-18) HPLC. Reaction with europium trichloride gave the required DELFIA label. The reaction scheme is shown in Fig. 5.

2.5.2. DELFIA method

2.5.2.1. Sample preparation. Plasma samples were centrifuged, as required, to remove fibrin clots and other particulate material, before being diluted 5-fold with assay buffer to eliminate non-specific matrix interference and prevent removal

of bound europium by the anticoagulant from the chelate in the subsequent immunoassay incubation. Samples expected to contain concentrations of AR-C68397XX above the working range of the method were further diluted as appropriate before analysis.

2.5.2.2. DELFIA. Calibration standards were prepared over the range 10–2000 pg ml^{-1} AR-C68397XX in pooled human plasma. A zero plasma calibration standard, and plasma quality control samples at three different levels were also analysed. All plasma samples were prepared as described under Section 2.5.2.1 prior to DELFIA incubation. Analysis of the diluted samples was carried out in duplicate using adjacent wells within the same microwell strip, for all calibration standards, quality controls and test samples. Following a preliminary wash cycle to prepare the coated wells (2×200 μl DELFIA wash buffer), 0.05 ml sample was added to both wells, followed by 0.1 ml of the antiserum working solution. After mixing briefly, the strips were incubated for 2 h at ambient temperature. To each well 0.1 ml of the tracer working solution was added, and the strips were then incubated overnight (at least 15 h) at a nominal 4°C. In each instance, the final DELFIA incubation contained 0.05 ml sample extract, 0.1 ml tracer working solution and 0.1 ml antiserum working solution. Non-specific binding (NSB) and total counts tubes were included with each batch of analyses.

After overnight incubation, the wells were allowed to reach ambient temperature, and unbound tracer and free ligand were then removed by removal of the liquid phase. This operation, and a subsequent wash step (6×200 μl, DELFIA wash buffer), were carried out using an automated plate washer. After removal of residual wash buffer, DELFIA enhancement solution (200 μl), containing a β-napthyl trifluoroacetone at low pH, was added to each well to release free Eu^{3+} from the AR-C68397-DTPA conjugate, and allow formation of a fluorescent Eu^{3+}–β-TFA complex. After vortex mixing each strip for 30 min, the wells were counted in time-resolved mode for 1 s with excitation and emission wavelengths of 340 and 615 nm, respectively. Calibration curve and sample data was processed using the Wallac Victor 1420 /MULTICALC combination described in Section 2.3. The DELFIA procedure was used for method validation studies, and to analyse human plasma samples from phase II clinical studies.

2.5.3. Validation of the SPE-RIA method

The DELFIA method 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. Since the antiserum used for this method was identical to that used in the RIA method, and was used under the same conditions, detailed stability and cross-reactivity experiments were not performed in this study. However, to ensure that the new method performed acceptably in a clinical support situation, a detailed comparison of a range of clinical samples analysed with both RIA and DELFIA methods was made, and confirmation was obtained that there was no significant cross-reaction with the principal circulating metabolite.

2.5.3.1. Specificity studies.

(1) *Interference by endogenous plasma components*

Control-plasma samples, taken from six different subjects, were analysed by the SPE-RIA to assess any interference by endogenous material. Aliquots of the same samples were analysed following addition of AR-C68397XX to a concentration of 100 pg ml^{-1}.

(2) *Cross-comparison of DELFIA and SPE-RIA using plasma samples from dosed subjects*

Plasma samples taken from subjects dosed with AR-C68397AA via a pressurised metered dose inhaler (pMDI) formulation during a clinical tolerability and pharmacokinetic study (CP 9022) (Fig. 3) [6], and analysed by SPE-RIA, were reanalysed using the DELFIA method. This experiment was designed to ensure differences in methods did not give rise to any significant differences in the determined concentrations of the analyte under operational conditions.

(3) *Cross-reactivity of the assay with in vivo metabolites*

In vivo metabolism studies withAR-C68397AA have demonstrated that the only metabolite of this compound present at significant concentrations in plasma following dosing is the β-glucuronide (Fig. 1). Cross-reactivity was investigated by analysing samples of control human plasma spiked with the β-glucuronide at concentrations up to 5000 pg ml^{-1}.

2.5.3.2. 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 aliquotted and stored at −20°C prior to analysis. Intra-assay performance was assessed by six replicate determinations of the spiked control plasma validation samples.

2.5.3.3. Analysis of diluted samples. The concordance with nominal concentrations for high level plasma samples analysed over a range of different additional dilutions was investigated at dilution factors up to 250-fold, in order to extend the range of the method.

2.5.3.4. Automated DELFIA incubation setup. In order to facilitate rapid throughput using the DELFIA method in large scale clinical trial support, the DELFIA procedure was also automated using a suitable Robotic Sample Processor (RSP). The automated procedure was designed to mimic the order of additions used for manual incubation setup, including pre-incubation of primary antibody with unlabelled analyte prior to the addition of tracer. A single analysis batch involving the RSP was included in the validation procedure. Determined concentrations in validation samples between 50 and 2000 pg ml^{-1} are reported here.

3. Results and discussion

3.1. Validation of the DELFIA method

3.1.1. Specificity studies

3.1.1.1. Interference by endogenous plasma components. Control plasma samples from six different subjects were analysed. The results are presented in Table 1, 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.

3.1.1.2. Cross-comparison of DELFIA and SPE-RIA. The data obtained from this comparison is presented graphically in Fig. 4. The agreement between the two methods was acceptable, particularly in view of the potential for non-specific interference in direct immunoassay methods designed for ultra-trace analysis in the pg ml^{-1} range. The slight overall negative bias seen in the SPE-RIA data could reflect marginally lower recovery for some clinical samples during the extraction stage.

Table 1
Concentrations of AR-C68397XX determined in individual human plasma samples

	Determined concentration (pg ml^{-1})							
Sample details	1	2	3	4	5	6	Mean	CV (%)
Blank plasma	<10	<10	<10	<10	<10	<10	<10	–
200 pg ml^{-1} Spiked	181	204	182	209	216	204	194	7.5
Blank B/B_0 value (%)[a]	95.3	102	98	105	104	100	103	4.3

[a] Blank B/B_0 value is defined as the ratio of counts obtained for the blank plasma sample to the counts obtained in the zero plasma calibration standard, expressed as a percentage.

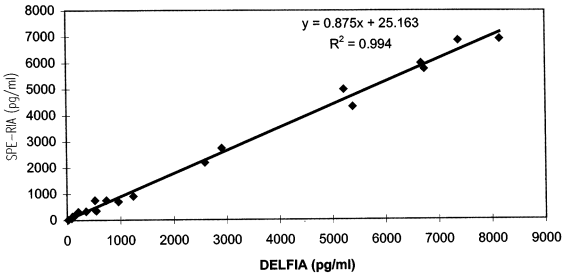

Fig. 4. Comparison of AR-C68397XX concentrations in human plasma samples from an ascending multiple dose clinical safety and tolerability study (CP 9022), determined by SPE-RIA and DELFIA methods.

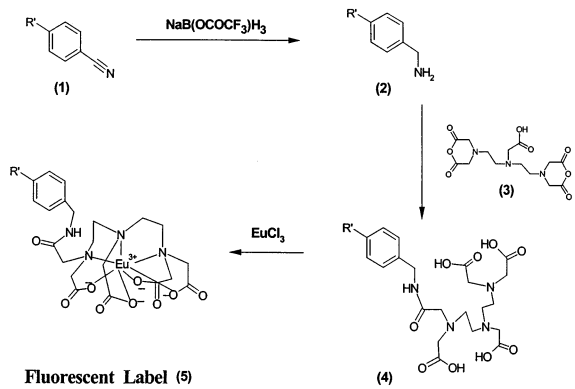

Fig. 5. Chemical synthesis of AR-C68397-DTPA-europium conjugate.

3.1.1.3. Cross-reactivity. Data from in vivo metabolism experiments with AR-C68397AA confirm that the only metabolite present in significant quantities in plasma following dosing with the parent compound is the β-glucuronide (Fig. 1). Accordingly, metabolite cross-reactivity investigations with the DELFIA method were confined to this metabolite. Cross-reactivity of the method was tested by spiking control human plasma to concentrations up to 5000 pg ml^{-1} with the glucuronide. Determined concentrations in these samples were all below the LOQ of the method, confirming that the cross-reactivity was below the 1% level.

3.1.2. 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 2 and 3. The accuracy and precision data were used to define the lower limit of quantification (LOQ) of the method. The LOQ was defined as 50 pg ml^{-1} on the basis of acceptable inter- and intra-assay performance of the validation sample spiked at this concentration. The upper reporting limit for samples (1:5 diluted) was set at 2000 pg ml^{-1}. The range of inter-day precision and accuracy values over the working range of the DELFIA method (50–2000 pg ml^{-1}) were 90.6–104% (accuracy) and 4.9–12.8% (precision). These compare favourably with the corresponding ranges for the SPE-RIA method [5] (92–109% accuracy; 7.8–17.1% precision). The DELFIA method has a significant advantage in terms of ease of operation and throughput, due primarily to the absence of the SPE step. The only disadvantage is the higher LOQ (50 pg ml^{-1}); this has not proved to be a drawback to the use of the method, since plasma concentrations in patients receiving therapeutic doses of the compound can generally be followed to at least 6 h post dose.

3.1.3. Analysis of diluted sample extracts

The effect of sample dilution on AR-C68397XX concentrations determined in control plasma samples containing a range of different

Table 2
Inter-assay precision and accuracy of validation samples[a]

	Validation sample (pg ml^{-1})							
Sample	10	25	50	100	200	500	1000	2000
Accuracy (%)	184	106	102	101	104	101	90.6	97.5
Precision (%CV)	35.7	48.5	11.5	10.1	4.9	6.8	12.8	12.8

[a] $n = 10$ (two samples at each concentration analysed in each of five analysis batches).

Table 3
Intra-assay precision and accuracy of validation samples[a]

	Validation sample (pg ml^{-1})							
Sample	10	25	50	100	200	500	1000	2000
Accuracy (%)	277	151	105	100	100	97.8	91.4	89.8
Precision (%CV)	24.3	33.2	13.1	9.8	2.6	8.6	18.0	12.9

[a] $n = 6$ (six samples at each concentration analysed in a single analysis batch).

Table 4
Effect of sample dilution on measured AR-C68397XX concentration

	Mean determined AR-C68397XX concentration (pg ml^{-1})			
Dilution factor	10	25	50	250
Nominal concentration (pg ml$^{-1)}$	2000	5000	10 000	50 000
Determined concentration (pg ml^{-1})	2270	5280	11 000	54 500
% Nominal concentration	114	106	110	109

Table 5
Automated batch analysis using DELFIA methodology

	Validation sample (pg ml^{-1})							
Sample	10	25	50	100	200	500	1000	2000
Mean determined concentration (pg ml^{-1})	<10	<10	51	105	227	512	860	2530
Accuracy (%)	–	–	102	105	114	102	86.0	126

known AR-C68397XX concentrations was investigated. The data are presented in Table 4. Agreement with nominal concentrations up to 250-fold was within acceptable limits.

3.2. Automation of DELFIA methodology

The data shown in Table 5 indicate that acceptable curve shape and QC data are obtainable with the automated procedure. In particular, there is no evidence for any interference due to metal ions in the system and wash fluids used by the RSP equipment.

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

Samples were taken from healthy volunteers during a tolerability and pharmacokinetic inhalation study and analysed using the DELFIA method reported here. The data are summarised in Fig. 2, which represents the data for individual volunteers administered a single dose of 1.5 mg AR-C68397AA by inhalation. Preliminary PK analysis of the data suggests an initial elimination phase with a half-life of around 40 min followed by a longer terminal phase. Peak plasma concentrations reached approximately after 10 min after dosing.

4. Conclusions

A dissociation-enhanced lathanide fluorescence immunoassay (DELFIA) for the determination of AR-C68397XX in human plasma, has been developed and validated. The method is highly sensitive and specific, has levels of precision and accuracy acceptable for clinical analysis during compound development, and gives good agree-

ment with data obtained using a radioimmunoassay method reported previously [5]. The range of the method has been shown to be suitable for support to Phase II clinical pharmacokinetic studies, and its facility for increased automation, rapid throughput and robustness has facilitated bioanalytical support to the clinical trial programme.

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