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A specific radioimmunoassay for the measurement of ICI 200,880, an elastase inhibitor, in human serum

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Abstract: ICI 200,880, 4-(4-chlorophenylsulphonylcarbamoyl)benzoyl-L-valyl-L-proline-1(*RS*)-(1-trifluoroacetyl-2-methylpropyl)amide (**I**), is a human neutrophil elastase inhibitor in development by ICI Pharmaceuticals Group. A specific and sensitive radioimmunoassay has been developed for this compound in human serum. An hydroxysuccinimide ester analogue of ICI 200,880 was coupled to the lysine residues of bovine serum albumin, characterized by gel electrophoresis and injected into rabbits to stimulate antibody formation; a highly specific, high titre antibody was obtained. An ^{125}I-diiodinated 4-hydroxy-phenyl derivative of ICI 200,880 was utilized as the radiolabelled antigen. Satisfactory zero and non-specific binding were achieved with a 2-h pre-incubation of ICI 200,880 and antiserum at 37°C, followed by the addition of radiolabelled antigen and a second 2-h incubation. Separation of bound and free radiolabel was achieved by employing PEG-goat anti-rabbit IgG separant. Sensitivity of 25 pg ml^{-1} ICI 200,880 in human serum was achieved (n = 12), with quantitative recovery of 106%, inter-assay precision of 6.3% and an average relative binding statistically different than that of pooled human serum. Serum quality control samples spiked at 600 and 100 pg ml^{-1} ICI 200,880 averaged 104% recovery over 6 validation days, with intra-assay precision of 12.8% RSD and inter-assay precision of 9.2% RSD (n = 24). Cross-reactivity of the ICI 200,880 antibody to three known metabolites and several analogues was negligible. Over 1200 clinical samples following aerosol administration of ICI 200,880 have been analysed by this procedure. After maximum exposure ranging from 0.3 to 32 mg ICI 200,880, mean peak levels from 0.08 to 20.5 ng ml^{-1} were measured, respectively, with compound detected generally up to 16 h post-dose.

Keywords: *ICI 200,880; radioimmunoassay; elastase inhibitor; aerosol delivery.*

Introduction

ICI 200,880 is a selective, competitive, potent and reversible synthetic trifluoromethyl ketone tripeptide inhibitor of human neutrophil elastase that is under development by ICI Pharmaceuticals Group. It is designated chemically as 4-(4-chlorophenylsulphonylcarbamoyl)benzoyl-L-valyl-L-proline-1(*RS*)-(1-trifluoroacetyl-2-methylpropyl)amide (Fig. 1, **I**). The compound has undergone a clinical trial in subjects with chronic obstructive pulmonary disease, and studies in other disease states involving release of elastase by neutrophils are planned. ICI 200,880 exists as two epimers, one of which is active. In addition, at least eight metabolites have been identified in rat bile.

ICI 200,880 is administered either intravenously or as an inhaled aerosol. The compound is not systemically available following oral administration. Due to the apparently long residence time of ICI 200,880 in the lung, as well as its rapid elimination from the blood, circulating drug levels following aerosol inhalation are low. In order to support pharmacokinetic and clinical evaluation of the drug, a sensitive analytical technique for the determination of ICI 200,880 in serum was required, with a quantitation limit of 0.5 ng ml^{-1} or less. This paper describes the synthesis of immunogens, ^{125}I-labelled material and the subsequent development and validation of a radioimmunoassay (RIA) to measure ICI 200,880 concentrations in human serum following acrosol administration of the drug.

Experimental

Materials

ICI 200,880 (**I**) and compounds **II–IX** (Fig. 1) are products of ICI Americas, Inc., ICI Pharmaceuticals Group (Wilmington, DE, USA). Bovine serum albumin, chloramine-T, Freund's adjuvant (complete and incomplete), thimerosal and polyethylene glycol (molecular weight 8000) were obtained from Sigma (St Louis, MO, USA). Water soluble carbodiimide and polyoxyethylene sorbitan monolaurate (Tween-20) were obtained from

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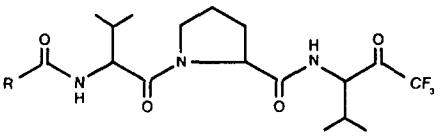


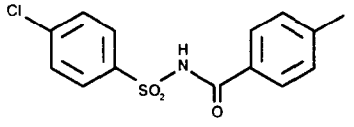
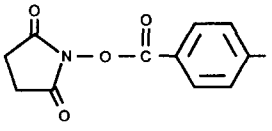
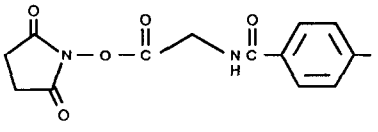
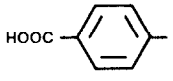
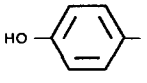
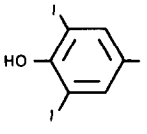
	R	% Cross Reactivity
(I)		100
(II)		–
(III)		–
(IV)		100
(V)		100
(VI)		100

Figure 1
Structure of ICI 200,880 (I) and compounds used in the radioimmunoassay.

Aldrich (Milwaukee, WI, USA). Carrier-free sodium [^{125}I]iodide was purchased from New England Nuclear Research Products (Boston, MA, USA). Rabbit gamma globulin was acquired from Calibiochem Corporation (San Diego, CA, USA). Goat anti-rabbit IgG antiserum was obtained from Cambridge Medical Diagnostics (Billerica, MA, USA). Dulbecco's phosphate buffered saline was purchased from Biofluids (Rockville, MD, USA). Acetonitrile (HPLC grade) was from J.T. Baker (Phillipsburg, NJ, USA).

Equipment

Disposable conical polystyrene incubations tubes (12 × 75 mm) were purchased from Sarstedt (Newton, NC, USA). Becton–Dickinson plain glass Vacutainer tubes (13 × 100 mm) were obtained from VWR Scientific (Philadelphia, PA, USA). An Eppendorf repeating pipettor and Combitips were purchased from Brinkman Instruments (Westbury, NY, USA). A Micromedic 10/200 plus MACC gamma counter is a product of ICN Biomedicals (Huntsville, AL, USA). The

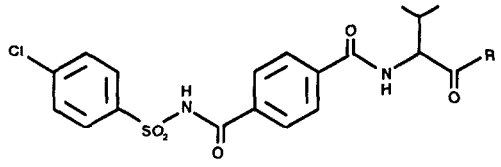
SpeedVac Concentrator used was purchased from Savant Instruments (Farmingdale, NY, USA). A water bath and multi-tube vortexer were obtained from local scientific suppliers.

Methods

Preparation of the bovine serum albumin immunogens. A solution of recrystallized BSA (250 mg, 3.79 μmol) and activated ester **II** (68.2 mg, 0.114 mmol) in distilled water (3 ml) and DMF (30 drops) was stirred overnight at ambient temperature. TLC showed that no **II** remained. The mixture was lyophilized to give 320 mg of impure product. The conjugate was repeatedly washed with methanol until no UV-absorbing materials and no *N*-hydroxy succinimide could be observed by TLC in the washes. After drying *in vacuo*, 207 mg of conjugate was obtained. Analysis by gel electrophoresis and by difference UV spectrophotometry showed that approximately 13 moles of hapten were incorporated per mole of BSA.

A solution of recrystallized BSA (150 mg, 2.27 μM) and activated ester **III** (45.5 mg, 0.068 mmol) in water (3 ml) was stirred at ambient temperature until the reaction was complete (2 h) to give 200 mg of impure product. Purification as described above gave 142 mg of conjugate. Analysis by gel electrophoresis and by difference UV spectrophotometry showed that approximately 10 moles of hapten were incorporated per mole of BSA.

It was decided to conjugate an analogue of ICI 200,880 on the end of the molecule opposite the trifluoromethyl ketone (TFMK) because in animals the major metabolites result from reduction of the TFMK to a mixture of the corresponding diastereomeric alcohols (Fig. 2, **VII**, **VIII**). Conjugate formation away from the TFMK would be expected to maximize the chances for production of antisera which would not recognize these metabolites. Additionally, conjugate connection that allowed the TFMK portion to



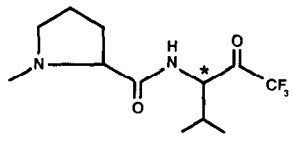
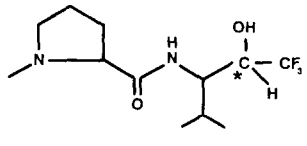
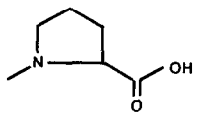
	*	R	% Cross Reactivity
(Ia)	SSS		41.5
(Ia)	SSR		138.5
(VIIa)	SSSR		0.20
(VIIb)	SSRS		
(VIIIa)	SSSS		0.57
(VIIIb)	SSRR		
(IX)			0.025

Figure 2
ICI 200,880 (**I**) epimer and metabolite (**VII–IX**) cross-reactivity.

be as far from the BSA molecule as possible also maximized the chance for specificity in recognizing the trifluoromethyl ketone portion rather than the peptide *per se*, as there is no natural source of CF_3 in the body. Since it was known that the SSS and SSR diastereomers of ICI 200,880 interconvert in aqueous solution to approximately 50% of each at equilibrium, diasteromeric mixtures of the activated esters **II** and **III** were used for BSA conjugation. Attempts to react acid **IV** with BSA failed. Interestingly, the material recovered after reaction and overnight dialysis of **IV** with BSA showed enhanced UV absorption at 254 nm suggesting that conjugation occurred. However, gel electrophoresis showed that the product had a molecular weight similar to BSA, and fluorine NMR did not show the expected resonance broadening of the fluorine peaks. Thus, it was concluded that the enhanced UV absorption observed was due to non-covalently bound hapten. However, use of activated esters **II** and **III** did give conjugates with the desired covalent incorporation of hapten (approximately 13 and 10 molecules, respectively, for esters **II** and **III**). The expected fluorine NMR peaks were broadened in the fluorine NMR spectra of the conjugates.

Production of the anti-ICI 200,880 antiserum. The **II**- and **III**-bovine serum albumin immunogens (0.25 mg) were solubilized in sterile isotonic saline (0.125 ml) and homogenized with Freund's complete adjuvant (0.375 ml) to obtain an emulsion. The mixture of each conjugate (0.5 ml) was injected subcutaneously into five or six sites on the rear flank of New Zealand white rabbits. Rabbits were similarly boosted 1 month later with conjugate (0.125 mg) in Freund's incomplete adjuvant (0.375 ml) and sterile isotonic saline (0.125 ml), and were bled 6 days later from the central ear artery, using a small amount of xylene as a vasodilator. This boosting and bleeding schedule was continued for 3 months.

Preparation of the radiolabel. A solution of **V** [4-hydroxybenzoyl-L-valyl-L-prolyl-(*R*,*S*) valyl trifluoromethyl ketone] (100 mg, 0.206 mmol) in absolute ethanol (50 ml) was treated at ambient temperature with sodium [^{125}I]-iodide (300 mg, 2.0 mmol) dissolved in phosphate buffer (0.25 M, pH 7.4, 100 ml). The radioiodination was initiated by the addition of a phosphate buffer solution (0.25 M, pH 7.4) of chloramine-T (40 mg ml^{-1}, 4 ml). The mixture was stirred for 1 h at ambient temperature. An aliquot (50 μl) was removed and treated with a phosphate buffered solution (0.25 M, pH 7.4) of sodium metabisulphite (40 mg ml^{-1}, 0.21 mmolar) to a colourless endpoint. This was then shaken with chloroform (0.25 ml), and the chloroform layer was removed by pipette and evaporated under a stream of dried nitrogen. The residue was analysed by HPLC (Zorbax ODS, 4.6 mm × 250 mm, 1.0 ml min^{-1}, 215 nm, H_2O–AcCN–THF–TFA, 55:35:15:0.1) and the second peak (^{125}I-**VI**) collected (Fig. 3). The reaction was essentially complete; thus, the reaction mixture was quenched with sodium metabisulphite solution as described above. The colourless mixture was diluted with deionized water (30 ml) and twice extracted with chloroform (100 ml). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford 130 mg of crude product. This was purified by flash chromatography on Merck neutral silica gel (15 g), eluting with ether–hexane (50:50) until the toluene sulphonamide had washed off the column. The desired diastereomeric product was eluted with diethyl ether (100%) to afford 107.2 mg (70.5%) of product. The elemental analysis calculated for $C_{23}H_{28}F_3I_2N_2O_5$ was C 37.47, H 3.83 and N 5.70; found was C 37.19, H 4.09 and N 5.55. By NMR (DMSO-d_6) δ 0.79–0.99 (m, 12H), 1.77–2.19 (m, 6H), 3.56–3.65 (m, 1H), 3.80–3.88 (m, 1/2H), 7.35–7.45 (dd, 1H, J = 7.5 Hz), 8.31 (s, 4H), 8.60–8.63 (m, 2H), 10.07 (s, 1H); ms (CI, CH_4) M + 1 at *m/e* 738.

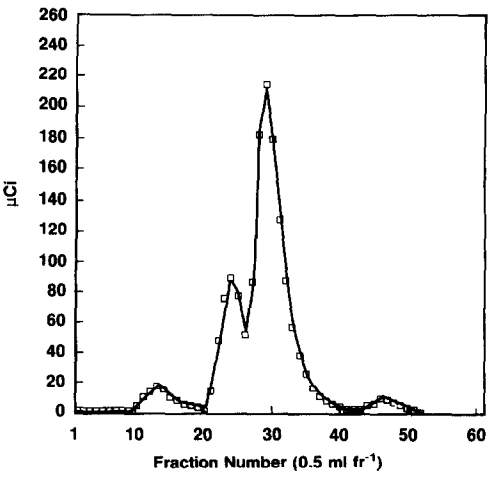


Figure 3
HPLC trace of purified, radiolabelled **VI**.

Preparation of the buffer used in the radioimmunoassay. The Dulbecco's phosphate-buffered saline (PBS) used in the radioimmunoassay was prepared by adding Tween-20 (0.25 ml) and thimerosal (0.05 g) to 500 ml of commercially obtained Dulbecco's PBS. PBS with carrier was prepared by adding rabbit gamma-globulin (20.0 mg) to 100 ml of the PBS prepared above. Both buffers were stored at 4°C.

Preparation of standard solutions of ICI 200,880 and standard and quality control serum samples. Anhydrous ICI 200,880 (10.0 mg) was dissolved in acetonitrile (10.0 ml) and sonicated to obtain a stock standard solution with a concentration of 1.0 mg ml^{-1} and a near 50:50 ratio of the SSS:SSR epimers (Fig. 2). A working standard solution with a concentration of 1.0 μg ml^{-1} was prepared by diluting stock standard solution (10.0 μl) with PBS (10.0 ml). The highest concentration serum standard (20,000 pg ml^{-1}) was prepared by adding working standard solution (40 μl) to pooled blank human serum (1.96 ml). Additional standard serum samples at concentrations of 10,000, 5000, 2500, 1250, 625, 312, 156, 78.1, 39.1, 19.5 and 9.80 pg ml^{-1} were obtained by serially diluting the highest concentration serum standard with pooled blank human serum (1.0 ml). Quality control serum samples at concentrations of 600 and 100 pg ml^{-1} were prepared in a similar fashion. All serum samples were aliquotted (125 μl) into disposable conical polystyrene incubation tubes, capped and stored at −20°C until analysis.

Preparation of the solutions used in the radioimmunoassay. The anti-ICI 200,880 antiserum solution was prepared by the addition of an appropriate volume of rabbit anti-ICI 200,880 serum to PBS with carrier to obtain a final dilution of 1/300,000. This required several dilutions, and an intermediate dilution (1/1000) has been successfully stored at 4°C for up to 12 months. The working radioligand solution was prepared by diluting stock ^{125}**I-VI** (10 μl) with PBS with carrier (990 μl). An aliquot (10 μl) of this solution was counted, and the dilution required to yield approximately 10,000 cpm/100 μl was calculated and an appropriate amount of working radioligand solution was diluted with PBS with carrier. The dilution of this solution was adjusted as the material decays to maintain approximately 10,000 cpm/100 μl. The radioligand has been shown to be stable for up to 90 days when stored at 4°C. Goat anti-rabbit serum was diluted (1:4) with PBS. Polyethylene glycol (PEG) was prepared as a 12% solution (w/v) using deionized water.

Protein precipitation of serum samples. Test serum samples were thawed in a lukewarm water bath and vortexed prior to sampling. An aliquot (125 μl) was pipetted into duplicate disposable conical polystyrene incubation tubes. Serum standard and quality control samples were previously aliquotted. Blank pooled human serum was pipetted into 10 disposable conical polystyrene incubation tubes for use in the radioimmunoassay as non-specific and maximum binding tubes. Acetonitrile (250 μl) was pipetted into each tube, using an Eppendorf repeating pipettor. The tubes were vortexed (20 s) and allowed to remain at room temperature (30 min). The tubes were centrifuged (15 min, 3200 rpm, 22°C), and the supernatant (300 μl) transferred to clean disposable conical polystyrene incubation tubes. The tubes were evaporated using a SpeedVac concentrator, and the dried residues stored at −20°C until analysis, or reconstituted with PBS (200 μl) for immediate analysis.

The radioimmunoassay. The experimental design consisted of total count tubes (2), non-specific binding tubes (2), maximum binding tubes (4), 12 concentrations of serum standards (24), three concentrations of serum quality control samples (6), test samples (2 each) and blank pooled human serum (2). PBS (100 μl) was added to the non-specific binding tubes. Diluted anti-ICI 200,880 solution (100 μl) was added to all radioimmunoassay tubes except the total count and non-specific binding tubes. The radioimmunoassay tubes were covered with parafilm and vortexed (20 s) on a multi-tube vortexer. They were incubated (2 h) in a preheated water bath (37°C). Diluted ^{125}**I-VI** (100 μl) was then added to all tubes. The samples were vortexed (20 s) on a multi-tube vortexer, and returned to the water bath (37°C, 2 h). Diluted goat anti-rabbit IgG (50 μl) was added to all except total count tubes. The radioimmunoassay tubes were covered with parafilm and vortexed (20 s) on a multi-tube vortexer. PEG solution (1 ml) was

added to all except total count tubes. The radioimmunoassay tubes were centrifuged (15 min, 3200 rpm, 22°C) to fractionally precipitate and separate the bound ^{125}I-**VI**. The supernatant was aspirated to waste, leaving a small, white button in the bottom of the conical tubes. The bound radioligand was counted (2 min), and the counts obtained for the standard tubes fitted to a four-parameter logistic equation [1]. The concentrations for the remaining tubes were determined from this equation, using the duplicate counts for each sample.

Results

Anti-ICI 200,880 antiserum production

Following several immunizations, sera from all 12 rabbits were found to bind ^{125}I-**VI**. Rabbits receiving **III**-bovine serum albumin immunogens showed generally higher titres, due in part to the presence of a glycine spacer which allowed increased recognition of the hapten. A production bleed from one rabbit (Rb788059B6) was chosen because of its high titre (approximately 1/300,000), specificity and sensitivity, although bleeds from other rabbits showed similar sensitivities with lower titres. The two epimers of ICI 200,880 (Fig. 2. **I**) did not cross-react equally with the antiserum, which was approximately three times more sensitive to the inactive SSR epimer than to the active SSS epimer. Cross-reactivity experiments showed that the antisera produced discriminated well against available analogues containing modifications of the TFMK group. The antisera also did not cross-react with proline or with valine. As expected, substituent changes on the benzene ring of the sulphonamide had little, if any, effect on antibody recognition. For example, the *p*-bromo (not shown), *p*-carboxylic acid, *p*-hydroxy and 3,5-diiodo-5-hydroxy analogues cross-reacted 100% (Fig. 1).

Properties of the radioimmunoassay

Optimization. Studies of the first incubation period were made at 37°C, room temperature and 4°C over a period of 48 h. Little difference was observed at any temperature in the rate of increase in binding with time; however, at least 16 h of incubation was required for any equilibration of binding to occur at 4°C or room temperature. Only a 2-h incubation was required to achieve suitable binding of tracer at 37°C. A 2-h pre-incubation of unlabelled antigen present in spiked or test samples and antibody at 37°C resulted in improved sensitivity by establishing a non-equilibrium competition for antibody binding sites. Binding of anti-ICI 200,880 antibody to goat anti-rabbit IgG was studied at room temperature after incubations from 0 to 45 min. No significant difference in specific or non-specific binding was observed at any time, suggesting that separation was rapid and complete.

Standard calibration. The concentration–response curve for ICI 200,880 in human serum was linear from approximately 50 to 1000 pg ml^{-1} as shown in Fig. 4, generated using a four-parameter logistic fit [1]. The estimate of slope (Parameter B) averaged 1.0 ± 0.06 (n = 6), while the estimated dose at 50% binding averaged 178 ± 18 pg ml^{-1} (n = 6). Serum samples with concentrations exceeding approximately 1 ng ml^{-1} were diluted with blank pooled serum to bring them into the linear range, extending the applicable range to at least 50 ng ml^{-1}.

Limit of quantitation. The binding of ^{125}I-**VI** in precipitated, pooled blank human serum was 23.7%, with non-specific binding at 1.3% across a 6-day validation period. The estimated dose at 80% binding (ED_{80}) averaged 42 ± 6 pg ml^{-1} (n = 6). The relative binding (%B B_{max}^{-1}) of precipitated, blank serum from 20 different individuals averaged 107% ± 7%, while the relative binding of 12 precipitated serum samples spiked at 25 pg ml^{-1} averaged 88% ± 2%. These two sets of samples were statistically different by the unequal variance *t*-

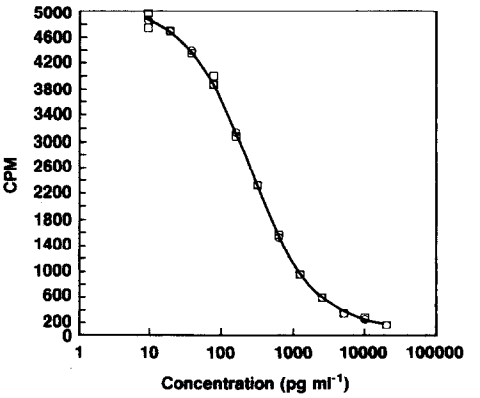


Figure 4
Serum standard curve for the determination of ICI 200,880.

test at a significance level of 0.0001. Thus, based on statistical difference from blank serum, and accuracy and precision data discussed below, a quantitation limit of 25 pg ml^{-1} ICI 200,880 in human serum was chosen.

Accuracy, precision and recovery. The intra- and inter-assay variation was calculated from data obtained with serum pools spiked with different levels of ICI 200,880 and analysed in duplicate on 6 different days. The relative standard deviation calculated from these data are shown in Table 1. Serum quality control samples spiked at 600 and 100 pg ml^{-1} averaged 104% recovery, with 12.8% RSD within-day and 9.2% RSD between-day. By observing a between-day precision which is smaller than the within-day, one can conclude that the variance of the measurements about the mean each day is somewhat large due to determinate errors (change in curve shape, for example), but the resulting mean values on each day agree rather closely. Accuracy and recovery were assessed by analysing pooled human serum samples spiked with ICI 200,880 covering the entire validated range of the assay (Table 2). Accuracy, as determined by absolute per cent difference from theory, averaged 9.8% from 25.0 pg ml^{-1} to 50.0 ng ml^{-1} across the 6 validation days (12.6% RSD). Recovery averaged 107% (±10%) over the same concentration range.

Specificity. Because of substantial binding of ICI 200,880 to human serum albumin, an acetonitrile protein precipitation step was incorporated to minimize the protein binding of both labelled and unlabelled antigen. If serum proteins were not removed prior to analysis, falsely high or low ICI 200,880 concentrations could result depending on the individual serum albumin concentration. Blank serum from 20 human volunteers free from medication were analysed and contained no detectable levels of ICI 200,880, with a mean $\%B.B_{max}^{-1}$ of 107% (±7%). The method was specific against aspirin, acetaminophen, ibuprofen, caffeine, nicotine, heparin and any metabolites formed within 1 h of taking the above drugs. The available metabolites (Fig. 2) all cross-reacted less than 1% with the antiserum; their concentrations in humans is not known at this time.

Applicability of the radioimmunoassay to the analysis of serum samples from subjects who had received a single, aerosolized dose of ICI 200,880. The radioimmunoassay was used to determine the serum concentrations of ICI 200,880 in samples taken from normal subjects receiving a single dose of aerosolized ICI 200,880. Twenty-four subjects were distributed among four study groups. The total aerosolized exposure to active drug for the four groups was 0.30, 3.0, 16 or 32 mg ICI 200,880, administered in a rising fashion. The serum concentration versus time curves obtained are illustrated in Fig. 5. Serum drug concentrations

Table 1
Assay precision

Pool	*n*	Mean (pg ml^{-1})	RSD_{WD}* (%)	RSD_{BD}† (%)	RSD_T‡ (%)
QC 600	12	561	9.6	12.2	14.2
QC 100	12	114	16.1	6.1	14.9

*Within-day precision.
†Between-day precision.
‡Total precision.

Table 2
Accuracy and recovery

Amount added (pg ml^{-1})	*n*	Amount found (pg ml^{-1})	% Difference	% Recovery	% RSD_T*
5.00×10^4	6	4.22×10^4	−15.7	84.3	9.8
2.50×10^4	6	2.22×10^4	−11.0	89.0	8.9
1.00×10^4	10	1.34×10^4	13.6	114	16.8
5.00×10^3	10	5.52×10^3	10.4	110	11.2
1.00×10^3	12	1.06×10^3	5.8	106	9.9
5.00×10^2	12	5.25×10^2	5.1	105	13.0
2.00×10^2	12	2.13×10^2	6.6	107	12.8
1.00×10^2	12	1.00×10^2	7.0	107	9.6
5.00×10^1	12	5.90×10^1	17.5	118	19.3
4.00×10^1	12	4.50×10^1	13.1	113	12.9
2.50×10^1	12	2.70×10^1	6.5	106	12.1
			|9.8|	107	12.6

*Total precision.

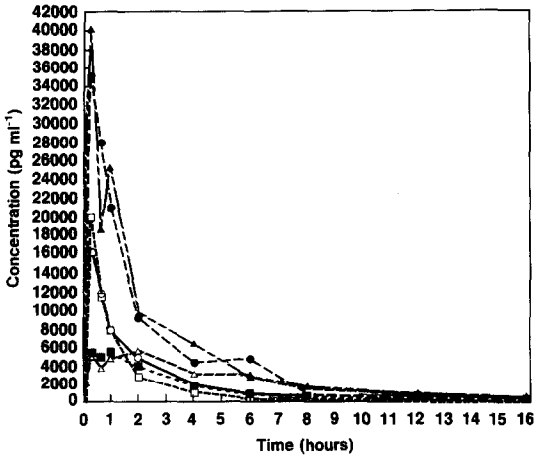


Figure 5
Serum concentration versus time curves following aerosol administration of ICI 200,880 (32 mg) to six volunteers.

remained quantifiable for up to 4 h after the administration of the 0.30 mg dose, and up to 16 h post-dose following the administration of the other three doses. The mean C_{max} for the 32-mg dose was 20.5 ± 6.0 ng ml^{-1}. C_{max}s and AUCs showed linear relationships to doses. The mean T_{max} for all doses was 0.86 h.

Discussion

Because ICI 200,880 is not active following oral dosing, aerosol administration into the alveoli of the lung was seen as vital for therapeutic success. The chance that an antiserum could be produced which would discriminate between the two epimers of ICI 200,880 was not deemed possible because of the facile aqueous interconversion of the two epimers. However, since the purpose of this work was to demonstrate clinical efficacy and patient compliance following aerosol dosing of ICI 200,880, this inability was not seen as an obstacle in meeting this goal. As shown in Fig. 2, the known metabolism of ICI 200,880 occurred in the VAL-TFMK region of the molecule; thus the antigenic response was directed toward the TFMK group. A series of analogues of ICI 200,880 were analysed to help determine the epitope of the antiserum and gain useful information regarding potential cross-reactivity of the antiserum with known and as yet unknown metabolites. By systematically replacing subunits of the ICI 200,880 molecule, it was determined that the VAL-PRO-VAL-TFMK portion of the compound was required for significant binding to occur. As expected, the two epimers of ICI 200,880 (SSS and SSR) were not recognized equally by the antibody mixture produced. While the diastereomeric mixtures of the activated esters **II** and **III** used for conjugation may have approximated 50%, the individual epimers being tested may have, in fact, epimerized during the length of the assay incubation period.

Two steps were taken to maximize the sensitivity of this assay. First, the assay was run as a 1-day assay at 37°C, with a 2-h preincubation of spiked and test serum samples containing ICI 200,880 and antiserum, followed by the addition of ^{125}**I-VI** and a second 2-h incubation. This non-equilibrium condition permitted additional unlabelled antigen to compete for antibody binding sites, thus improving detection limits. Secondly, because of substantial binding of ICI 200,880 to human serum albumin, an acetonitrile protein precipitation step, removing more than 99% of serum proteins [2], was incorporated to minimize the protein binding of both labelled and unlabelled antigen. This served to increase sensitivity by improving precision at low concentration levels by reducing inter-subject blank variation.

The radioimmunoassay has been successfully extended to other human fluids, including plasma, urine, bronchoalveolar lavage fluid and sputum, with only minor modifications. The serum radioimmunoassay has been used for a period of more than 4 years to support clinical safety and pharmacokinetic studies of single and multiple aerosol doses in volunteers and patients with chronic obstructive pulmonary disease and cystic fibrosis.

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