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The use of competitive displacement agents to resolve albumin binding problems observed during the development of a radioimmunoassay for ICI 215001

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Abstract: A radioimmunoassay has been developed for the analysis of ICI 215001, a carboxylic acid metabolite of ICI D7114. The level of binding and sensitivity of the assay were good in the absence of plasma. However, the addition of plasma to the incubation medium reduced the antibody binding of radiolabelled tracer (**I**) from 41 to 9%. This was attributed to the high (>99%) plasma albumin binding of ICI 215001. A series of compounds (DL-tryptophan, octanoic acid, bilirubin, ponalrestat, warfarin, phenylbutazone and salicylic acid) which all bind to plasma albumin, were examined for their effect on the tracer–antibody interaction. Warfarin and phenylbutazone were the only compounds to specifically displace the iodinated tracer from albumin; warfarin was the only compound to restore antibody binding to control levels. The warfarin concentration and the pH of the incubation medium also had a substantial effect on the magnitude of the displacement. The optimized method for the analysis of ICI 215001 in human plasma (20 μl) used phosphate buffer (pH 6.0, 0.1 M) containing warfarin (50 μg ml^{-1}), which gave an assay with the desired specificity, precision and sensitivity.

Keywords: *Radioimmunoassay; protein binding; specific displacement; warfarin.*

Introduction

(*S*)-4-[2-(2-Hydroxy-3-phenoxypropylamino)-ethoxy]-*N*-(2-methoxyethyl) phenoxyacetamide (ICI D7114; Fig. 1) was undergoing clinical investigation for the treatment of obesity and non-insulin dependent diabetes. ICI D7114 is rapidly converted *in vivo* to the carboxylic acid metabolite, (*S*)-4-[2-(2-hydroxy-3-phenoxypropylamino)ethoxy] phenoxyacetic acid (ICI 215001; Fig. 1). This metabolite, which is a selective and potent β3-adrenoreceptor stimulant, is the major drug related component found in the circulation of rats and dogs following oral administration of ICI D7114. Extrapolation of the data from these animal studies indicated that systemic ICI 215001 concentrations in man would require an assay with sub-nanogram sensitivity. Hence, a radioimmunoassay (RIA) was developed to monitor ICI 215001 concentrations in plasma samples from clinical trials.

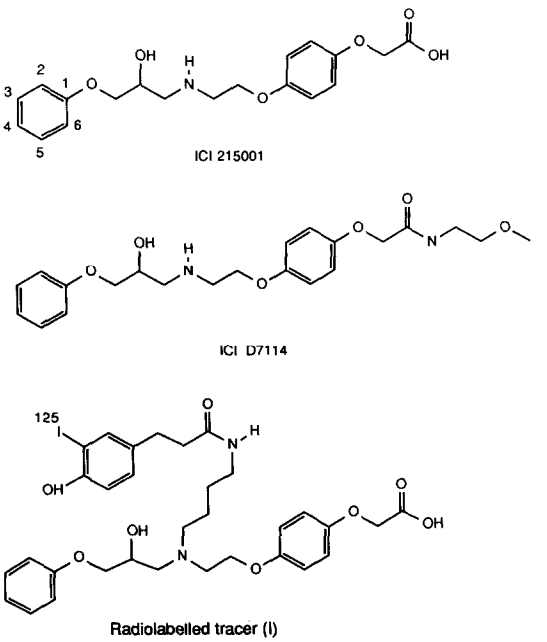

Figure 1
Structures of ICI 215001, ICI D7114 and radiolabelled tracer (**I**).

ICI 215001 and ICI D7114 have both been shown to be metabolized in animals and man by hydroxylation at the 4-position in the left hand ring (Fig. 1) to yield ICI 218346 and ICI 218347, respectively. Consequently, an antibody that was specific for ICI 215001 in the presence of D7114 and the hydroxy metabolites was required.

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During the development of the RIA it was observed that the presence of human plasma markedly decreased the binding of the iodinated tracer (Fig. 1) to antibody, resulting in an assay with poor sensitivity. This problem was thought to be due to the tracer binding to plasma proteins as well as to antibody.

The two basic approaches to solving this problem are either to denature or eliminate the protein prior to the RIA procedure or to displace the analyte from the particular albumin binding site. In the former case, the additional analysis steps involved (protein precipitation or heat treatment, for example) would significantly increase the analysis time for each sample with a consequent reduction in sample throughput. Since a large clinical programme was envisaged, the displacement approach which involves no additional steps to a conventional RIA procedure, except to include the displacing agent in the assay buffer, was adopted here.

Various methods for displacing analytes from plasma proteins have been reported [1–3]. One method that has proved successful is to include 8-anilino-1-naphthalene-sulphonic acid (ANS) in the incubation medium [1]. ANS works by displacing the analyte from the plasma albumin binding site, restoring binding to that of control experiments. However, it also has a detrimental effect on the specific antibody–antigen interaction, rapidly decreasing binding as ANS concentrations are increased (B. Law, unpublished data).

An alternative, in which analogues of the analyte are used as specific displacement agents, has been successfully employed in a number of steroid immunoassays [2, 3]. However, this method is of limited utility owing to problems associated with the cross-reactivity and availability of a suitable analogue.

A novel approach used here is to displace the analyte with a compound known to bind to plasma albumin but which is structurally unrelated to the analyte. This method avoids the problems associated with non-specific agents (ANS) and the possibility of cross-reaction when a close analogue of the analyte is used.

Experimental

Materials

All compounds were either of analytical reagent grade or used as received, unless otherwise stated. ICI D7114, ICI 215001, ICI 227246, ICI 218346, ICI 218347 and ponalrestat (3-[(4-bromo-2-fluorophenyl) methyl]-3,4-dihydro-4-oxo-1-phthalazineacetic acid) were obtained from ICI Pharmaceuticals. Warfarin (98%) was obtained from Aldrich Chemical Company Ltd (Gillingham, UK). Phenylbutazone, DL-tryptophan, bilirubin, salicylic acid, octanoic acid, bovine gamma globulins (BGG) (purity approximately 99% prepared from Cohn Fraction II, III) and keyhole limpet haemocyanin (KLH) were obtained from Sigma Chemical Company Ltd (Poole, UK). Bolton and Hunter reagent was obtained from Amersham International plc (Aylesbury, UK). Polyethylene glycol (PEG, mol. wt 6000), ammonium acetate, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate, boric acid and sodium hydroxide (GPR) were obtained from BDH Chemical Company Ltd (Liverpool, UK).

Phosphate buffers (pH 5.0, 6.0 and 7.4, 0.1 M) were prepared from a mixture of potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate and contained sodium azide (0.1%, w/v) and BGG (0.2%, w/v). Borate buffer (pH 8.6, 0.1 M) was prepared by dissolving boric acid (6.2 g) and sodium hydroxide (0.96 g) in de-ionized water (1 l).

Blood from volunteers was transferred into oxalated tubes and centrifuged to obtain plasma which was stored at −20°C.

Synthesis of immunogen and production of antisera

ICI 227246, the *N*-(4-aminobutyl) derivative of ICI 215001, was used to link to KLH and to synthesize a radiolabelled tracer. Linking through the secondary amine on ICI 215001 should allow discrimination between ICI 215001 and the related parent compound and metabolites. ICI 227246 was conjugated to KLH using glutaraldehyde, employing the method of Reichlin *et al.* [4], and the resulting conjugate was purified by gel permeation chromatography and pressure dialysis. The incorporation ratio of derivative to KLH was not determined.

Six sheep were immunized with the conjugate and all animals responded well, giving antibody titres of >1:70,000. The antibody titre was determined as the working dilution of antiserum which bound 50% of added radiolabelled tracer. This experiment was conducted

as described in the method section with reagents prepared in phosphate buffer (pH 7.4). The antisera were assessed for their specificity and sensitivity to ICI 215001 using solutions prepared in buffer. The antiserum chosen (S24-3.2) had a high titre (>1:100,000) and showed minimal cross-reactivity with ICI D7114 and the 4-hydroxy metabolites.

Synthesis of radiolabelled tracer

The radiolabelled tracer was prepared by conjugation of ICI 227246 using the primary amino-group of the butyl side-chain with Bolton and Hunter reagent [5]. ICI 227246 was dissolved in borate buffer (0.1 M, pH 8.6) at a concentration of 1 mg ml^{-1}. An aliquot (50 μl) of this solution was added to dried Bolton and Hunter reagent (0.5 mCi, 0.225 nmol) and left at room temperature for 15 min. The mixture was purified by reversed-phase HPLC using a 100 × 4.6 mm i.d. column packed with 5-μm Hypersil ODS and a mobile phase of methanol–water (50:50, v/v) containing ammonium acetate (0.1 M). The products were detected using a Beckman 170 radioactivity monitor. The radiolabelled (^{125}I) tracer (**I**), which eluted at a capacity factor of 13, was collected in a 2-ml fraction and had a specific activity of 2200 Ci $mmol^{-1}$.

Method

The antisera and radiolabelled tracer were diluted in the appropriate phosphate buffer (pH 5.0, 6.0 or 7.4). The working dilution of antisera was typically 1:100,000 and the radiochemical concentration of the tracer was approximately 40 kcpm/0.1 ml. ICI 215001 standards were prepared in either phosphate buffer or human plasma.

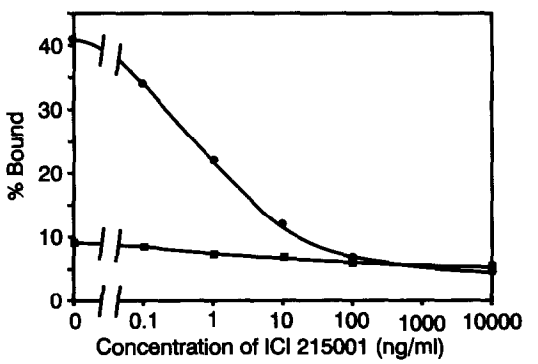

Figure 2
Comparison of calibration curves for ICI 215001 in phosphate buffer (●, 50 μl, pH 7.4, 0.1 M) and human plasma (■, 50 μl).

A typical assay involved mixing ICI 215001 standard solutions (20–100 μl), tracer (100 μl) and antiserum (100 μl). This mixture was then incubated at room temperature overnight. The bound and free tracer were separated by addition of PEG 6000 (0.5 ml, 27.5%, w/v), vortex mixing, then centrifugation (2800**g**, 15 min, 20–22°C) and finally aspiration of the supernatant. The precipitates were counted in a gamma-counter (NE 1600, 75% counting efficiency for ^{125}I) for a minimum of 60 s.

Either phosphate buffer or human plasma was used to determine non-specific binding, as appropriate.

Results and Discussion

As is common practice in RIA, initial development work on the assay was carried out with standards, radiolabelled tracer and antisera prepared in phosphate buffer (pH 7.4), i.e. without plasma. Under these conditions one antiserum (S24-3.2) was chosen for its high binding at zero ICI 215001 concentration (41%), high antibody titre (>1:10^5) and low cross-reactivity with ICI D7114 and the hydroxy metabolites. However, when the standards were prepared in human plasma the binding at zero concentration fell to 9% (Fig. 2). Consequently, the assay required optimization to regain the sensitivity required for clinical analysis.

The plasma protein binding of ICI 215001 (measured in human plasma by equilibrium dialysis) had previously been determined to be >99% for concentrations of ICI 215001 of 0.02–10 μg ml^{-1}. The radiolabelled tracer is assumed to have similar albumin binding properties since it also contains the carboxylic acid group which is often an important factor in drug–albumin binding. It was postulated, therefore, that inclusion of plasma in the incubation medium resulted in the tracer preferentially binding to plasma albumin rather than to the antibody.

The decrease in binding was not observed in the original assessment of the sheep antisera when the antibody dilution curves were carried out and the concentration of sheep proteins would have been high. This is probably due to the sheep antisera containing a high concentration of antibody relative to albumin since the titres were relatively high, typically >10^4. Consequently, the binding of tracer to antibody in the presence of albumin is determined

by a combination of the relative albumin–antibody concentration and the affinity of the antibody for the tracer. Alternatively, sheep serum albumin might be sufficiently different from human plasma albumin such that it does not bind the tracer to any great extent.

The effect of competitive binding agents

There are at least two specific drug binding sites on human plasma albumin [6]. It has been shown that drugs binding to these specific sites can be displaced by the use of an appropriate competitor [6]. Therefore, a series of compounds known to bind avidly to plasma albumin were evaluated to determine whether they could competitively displace the tracer from plasma albumin and consequently return the tracer–antibody binding back to control levels. The compounds initially selected were: DL-tryptophan, octanoic acid, bilirubin, ponalrestat, warfarin and salicylic acid.

The potential competitors were dissolved in phosphate buffer (pH 7.4, 0.1 M) at a concentration of 1 mg ml^{-1}, with the exception of bilirubin where a saturated solution (concentration <1 mg ml^{-1}) was used. Aliquots of these solutions (10 μl, bilirubin 20 μl) and of a control using phosphate buffer (10 μl, pH 7.4) without competitor were added to individual assay tubes to determine total binding in the presence or absence of plasma (50 μl) for each competitor. Table 1 shows that warfarin is the only compound to exhibit an appreciable restoration of specific binding and suggests that warfarin and the tracer bind to the same site on human albumin. By implication the other compounds must either bind to different sites on albumin or bind much less avidly than the tracer. In later experiments (data not shown) phenylbutazone, which binds to a different region of the warfarin binding site on human albumin [6], was evaluated. Phenylbutazone was found to be less potent than warfarin and consequently it was not studied further.

Additional experiments showed that increasing the warfarin concentration in the assay medium from 0.4 to 400 μg ml^{-1} led to a substantial improvement in tracer–antibody binding. When only 50 μl of plasma was used, a warfarin concentration of 400 μg ml^{-1} was sufficient to raise binding to the level observed in the absence of plasma. However, with 100 μl of plasma the same concentration of warfarin (400 μg ml^{-1}) was less effective (Fig. 3).

The effect of increasing warfarin concentration on the binding of tracer to antisera, in the absence of plasma, was evaluated in a control experiment (Fig. 3). Binding decreased slightly as the warfarin concentration increased, which indicates that the effect of warfarin is not as specific as originally assumed. Surprisingly, at the highest warfarin concentration used (400 μg ml^{-1}) tracer–antibody binding was higher with 50 μl of plasma than in the control experiment.

One possible explanation for these phenomena relates to the free or active concentration of warfarin. In the presence of plasma, a substantial proportion of warfarin will be bound to the albumin, leaving only a small free fraction to effect any non-specific interactions. In the control experiment, with no plasma present, the free concentration of warfarin will be approximately equal to the total warfarin

Table 1
The effect of displacing reagents on the binding of radiolabelled tracer (I) to an ICI 215001 antiserum in the presence and absence of plasma (50 μl)

Compound*	% Binding at zero concentration	
	Without plasma	With plasma
Control	44.4	13.7
DL-Tryptophan	49.4	14.2
Octanoic acid	43.1	14.2
Bilirubin	52.5	14.3
Ponalrestat	45.9	15.3
Warfarin	45.8	27.2
Salicylic acid	46.5	14.9

*The concentration in the assay tube was approximately 2.5 μg ml^{-1}.

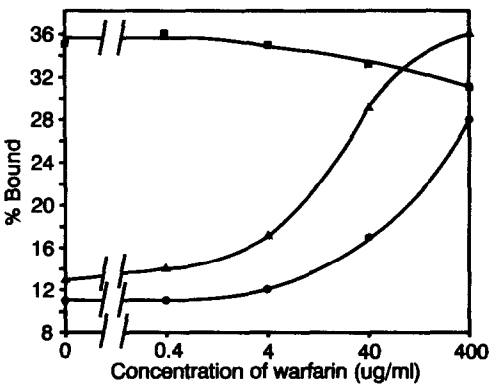

Figure 3
The effect of warfarin concentration on the binding of radiolabelled tracer to antibody at zero concentration of ICI 215001 in phosphate buffer (■, 50 μl, pH 7.4, 0.1 M) and plasma (▲, 50 μl; and ●, 100 μl).

concentration and the potential for non-specific interactions will be at their greatest.

The decrease in binding observed with warfarin concentrations greater than 400 μg ml^{-1} could have implications for the quantitation of samples originating from subjects on multiple treatments which may include warfarin. However, the therapeutic range of warfarin is tightly controlled between 1 and 3 μg ml^{-1} [9] and this will have a negligible effect on the total concentration of warfarin in the incubation mixture.

It is also interesting that for a two-fold increase in plasma concentration, an approximately 10-fold increase in warfarin concentration was required to give a similar level of antibody binding.

The effect of buffer pH on tracer–antibody binding

It is known that the state of ionization of a molecule can have a significant effect on drug protein binding [7, 8]. Therefore, the previous experiment was repeated using phosphate buffers at three different pH values (7.4, 6.0 and 5.0). The warfarin concentrations used were 40, 400 and 1000 μg ml^{-1}.

The levels of tracer–antibody binding were similar at pH values 5.0 and 6.0, and were substantially higher than at pH 7.4 for warfarin concentrations up to 1000 μg ml^{-1} (Fig. 4). An analogous pH effect was observed in the absence of warfarin. At the lower pH values of 5.0 and 6.0, the concentration of warfarin producing a maximal effect was approximately 10 times less than that seen previously at pH 7.4. These observations presumably reflect a change in the ionization of either the tracer, antibody or plasma albumin.

Optimal assay conditions

From the optimization work previously outlined, a buffer pH of 6.0 and a final warfarin concentration of 200 μg ml^{-1} would have been selected for the analysis of 100 μl of plasma. Ultimately, to achieve the required assay sensitivity, only 20 μl of plasma was required to be analysed. Consequently, the warfarin concentration in the incubation medium was adjusted to 50 μg ml^{-1}. In practice, this was achieved by dissolving warfarin at a concentration of 50 mg l^{-1} in the assay buffer used to dilute the antiserum and tracer.

A typical calibration curve for ICI 215001 under optimized conditions is shown in Fig. 5. It can be seen that there is a good level of binding at zero concentration and that the required sensitivity (lower limit of detection 0.5 ng ml^{-1}) has been achieved. The non-specific binding of the assay is typically 6%. In addition, the assay is also highly selective, showing cross-reactivity of 0.2, 0.001 and 0.1% with ICI D7114, and the hydroxy metabolites ICI 218346 and ICI 218347, respectively. The intra-assay precision was <10% over the range 2–64 ng ml^{-1} and the inter-assay precision was <15% over the range 2–80 ng ml^{-1}.

The accuracy of the assay was determined by spiking plasma at known ICI 215001 concentrations of 10–500 ng ml^{-1} and analysing the samples using the RIA and an existing HPLC procedure. The ICI 215001 plasma concentrations obtained by RIA were then compared with both the nominal concentrations and those obtained by HPLC, using linear regression analysis (Table 2). These data demon-

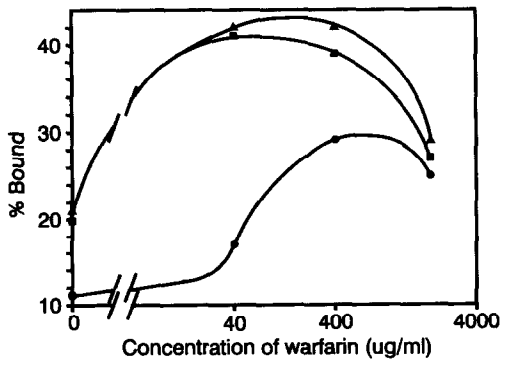

Figure 4
The effect of warfarin concentration on the binding of tracer to antisera at buffer pH 7.4 (●), 6.0 (▲) and 5.0 (■) in the presence of plasma and at zero concentration of ICI 215001.

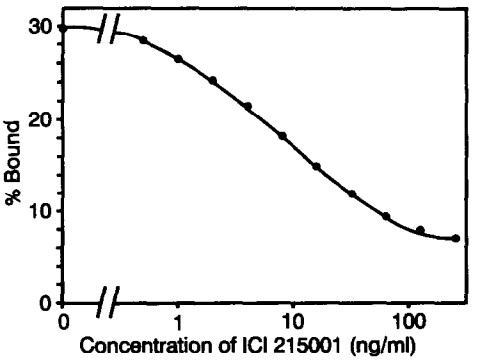

Figure 5
Typical calibration curve for ICI 215001 in human plasma using the optimized conditions.

Table 2
A comparison of ICI 215001 plasma concentrations determined by the RIA with the nominal concentrations and those obtained by an HPLC procedure. The results of the linear regression analysis are shown

Analysis parameter	Nominal value	HPLC value
Correlation coefficient	0.999	0.998
Slope	0.952	1.03
Intercept (ng ml^{-1})	0.910	2.02
n	16	16

strate an excellent correlation and minimal bias when the concentration determined by the RIA procedure is compared with either the nominal concentration or that determined by HPLC.

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