Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 685–689, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00

Development of a radioimmunoassay for idazoxan hydrochloride*

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Abstract: To facilitate the measurement of idazoxan in clinical trials an immunoassay capable of detecting low ng ml^{-1} concentrations was required. A stable derivative was prepared which, after linking to keyhole limpet hemocyanin (KLH), was subsequently immunized into sheep. Early assay development was carried out with both fluorescent (1-FITC) and iodinated (I-125) labels. The assay methodology was the same in both cases, using magnetizable solid-phase particles to which the antibody was linked. The sensitivity of the assay was such that a large sample volume was required which, in turn, led to problems of increased protein interference. The use of pepsin to digest the protein was used effectively after several blocking agents were unsuccessfully used. The limit of detection was in the region of 3 ng ml^{-1}. Cross-reactivity studies showed that the antibody was specific for idazoxan. Intra- and inter-assay precision was 7 and 12%, respectively. Correlation with the analytical GC–MSD method was in the order of 0.90.

Keywords: *RIA; idazoxan hydrochloride.*

Introduction

Idazoxan hydrochloride, (2-[2-(1,4-benzodioxanyl)]-2-imidazoline hydrochloride, Fig. 1), an α_2-adreno-receptor antagonist [1], has been in development for a number of years. Up to the present time idazoxan has been measured by either a ligand binding assay [2] or, more recently, by gas chromatography with mass selective detection (GC–MSD) [3]. The aim of this study was to develop an immunoassay for idazoxan hydrochloride in serum or plasma at low ng ml^{-1} concentrations.

Development of an immunoassay for a drug normally involves the preparation of an immunogen, immunization of the species of choice, and preparation of a suitable label, i.e. fluorescent or iodinated (I-125), to determine the free and bound moieties. Idazoxan, in common with most drugs, is a hapten, and must be linked to a larger carrier protein to stimulate an immunogenic response. Keyhole limpet hemocyanin (KLH) was found to be a suitable carrier for idazoxan.

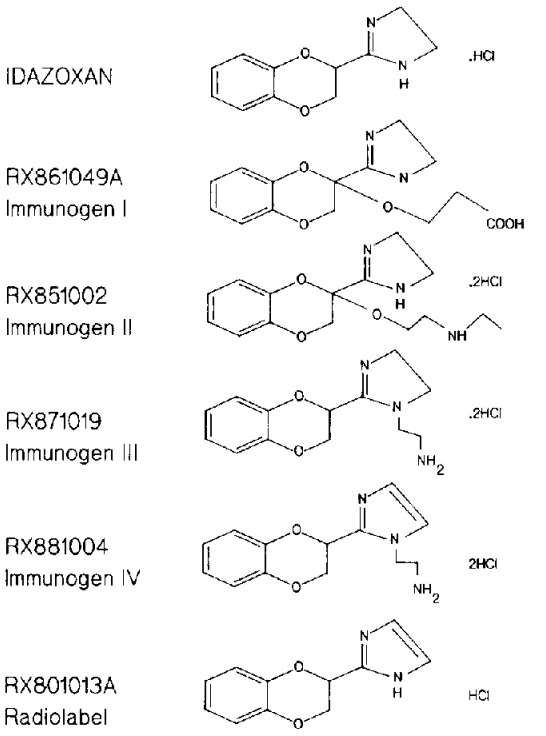

Figure 1
Some of the derivatives used in the conjugation studies. Also shown is the compound from which the radiolabel was prepared.

Experimental

Chemicals and reagents

Compounds for the conjugation and cross-

*Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

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reactivity studies were obtained from the Chemical Services Department, Reckitt and Colman Pharmaceuticals (Hull, England). Ovine antisera were raised under contract to Polyclonal Antibodies Limited (Blaenwaun Farm, Llandysul, UK). Pooled, normal serum was from ILS Limited (London, UK). KLH was from Calbiochem-Behring, Hawick, Cambridge, UK. The carbodiimide, FITC and solvents were obtained from Sigma (Poole, Dorset, UK). Sodium iodide (I-125) and the Bolton-Hunter reagent were obtained from Amersham International (Bucks, UK). Adjuvants were obtained from Difco Laboratories (Detroit, USA), and the magnetic separators were from the Unipath Laboratory (Bedford, UK).

Methods

Immunogen preparation. Immunogens **I**, **II** and **III** were coupled to KLH and immunized into sheep. A further analogue, immunogen **IV** (RX881004, a stable aminoethyl imidazole derivative of idazoxan), was linked to KLH, as follows. RX881004 hydrochloride (0.11 mmol) was dissolved with 50 mg of KLH in 4 ml of water to which 200 μl of pyridine was added. The conjugation reagent, 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC), was added over 7 h. The mixture was dialysed and lyophilized to give 45 mg of immunogen.

Preparation of labelled derivatives. The fluorescent label, an isomer of fluorescein isothiocyanate (FITC), was linked to the idazoxan derivatives used in the immunogen studies. Further studies were carried out with an iodinated radiolabel (I-125).

Fluorescent label (Immunogen III, RX871019). The derivative (RX871019, 3 mg) was dissolved in methanol (300 μl) and triethylamine (50 μl). The FITC isomer (2 mg, 5 μmol) was added and the reaction stirred at room temperature for 30 min. Volatile substances were removed under vacuum and the residue azeotroped with methanol (3 × 1 ml). The product was isolated by TLC to give the pure idazoxan label.

Radiolabel preparation (RX801013). RX801013 (2 mg) was dissolved in ethanol (2 ml) from which a solution was prepared containing 1 ng μl^{-1} in phosphate (pH 8, 0.5 M) buffer. An aliquot (5 μl) was transferred to the iodination vial. Phosphate buffer (pH 8, 0.5 M, 10 μl) was added, followed by a solution of sodium iodide (I-125, 5 μl, 0.5 mCi) and chloramine-T (10 μl, 2.5 mg ml^{-1}). The reaction was vortexed for 1 min at ambient temperature, followed by the addition of sodium metabisulphite (10 μl, 30 mg ml^{-1}) to terminate the reaction. The mixture was diluted with buffer (200 μl) and extracted with ethyl acetate (400 μl). The product was isolated by TLC.

Production of antibody. Groups of mature ewes were immunized with immunogen **IV**, both subcutaneously (s.c.) and intramuscularly (i.m.), at multiple sites, including all four limbs and both sides of the neck. The primary immunization was carried out with 4 mg of the immunogen in 4 ml of an emulsion of saline and complete Freund's adjuvant. Four weeks later, and each subsequent 4 weeks, the sheep were re-immunized with the immunogen, 2 mg per sheep, using incomplete Freund's adjuvant. Two weeks following each booster injection, samples of blood were taken for antibody assessment.

Antibody assessment. Preliminary assessment of the antisera was carried out by polarization fluorescence (PFIA) which proved to be a rapid and simple technique to evaluate the antibody response [4–6]. Essentially, the assay was based on the reaction between a fluorescent-labelled antigen (F-Ag) and a specific antibody (Ab). A change in the size of the labelled antigen (by binding with antibody, F-Ag:Ab) and its subsequent retardation in the Brownian motion, was measured and compared with the free label. On excitation by polarized light the emitted fluorescent light was related to the total effective molecular size, the small free label having a low polarization signal compared with the larger, antibody bound component which possessed a higher signal.

Magnetizable solid-phase preparation. Ovine anti-idazoxan antisera (2 ml) were coupled to magnetizable cellulose particles (1 g) using cyanogen bromide following established procedures [7, 8]. The particles were washed in assay-diluent buffer (phosphate, 0.1 M, pH 7.4, with Triton X-100 (0.1%) and preservative (sodium azide, 0.1%) and resuspended to 50 g l^{-1}, the particles being stored at 4°C until required.

Radioimmunoassay. Standard or serum (100 μl) was added to 100 μl of the labelled idazoxan, the radiolabel being diluted to give 10,000 cpm, followed by 100 μl of the anti-idazoxan solid-phase (2 g l^{-1}). The tubes were incubated for 2 h at ambient temperature with constant mixing after which diluent buffer (1 ml) was added. The solid-phase was sedimented on the magnetic separators for approximately 2 min. The supernatant containing the free fraction was discarded to waste, and the bound fraction counted on a multi-detector gamma counter.

Pepsin digestion radioimmunoassay. Aliquots (300 μl) of sample and/or standard (prepared in drug free serum) were incubated with an equal volume of pepsin solution at a concentration of 125 mg l^{-1} (in 100 mmol HCl) for 1 h at 37°C. Aliquots (200 μl) of the digested extract were then added to 100 μl of the I-125 idazoxan-radiolabel followed by 100 μl of the anti-idazoxan solid-phase. After 30-min incubation at room temperature, diluent buffer (1 ml) was added. The contents of the tubes were sedimented with the aid of the magnetic separators, with the bound fractions counted as before.

Results

A lack of response with immunogen I was probably due to the conjugation reaction and molecular cyclization may have offered the best reaction route. Early antisera activity was assessed by the PFIA method using fluorescein labels prepared from immunogens **II** and **III** (Fig. 1).

Titres obtained from immunogen **II** were very low, however, increases in titre were obtained after further booster injections in the sheep to 1/136 and 1/272. This enabled a displacement curve, albeit flat, to be established. There was, however, little displacement with idazoxan, even at 1 mg ml^{-1}, whereas higher displacement with RX851002 (immunogen **II**), demonstrated that bridge binding was apparent.

Immunogen **III** (RX871019) produced antibodies to both open and closed ring species. This was shown by a base hydrolysis of the standards, which showed a similar profile to the normal standards. The titre obtained from the immunogen **III** immunized sheep, as measured by PFIA, was in the region of 1/200 and 1/480.

Immunogen **IV** (RX881004) displayed the highest antibody responses (due to the greater stability of the immunogen) with titres in the region of 1/700 to 1/1200, using the standard immunization protocol. Furthermore, the titres were increased by immunizing the sheep through a combination of injection sites (s.c., i.m. and i.p.). The maximum titres improved to 1/4800 over several further injections. The sera were assayed with a label prepared from immunogen **III**. A label prepared from immunogen **IV** did demonstrate a higher titre (thus showing some bridge binding), but the label was not displaced by idazoxan itself.

Initial attempts to radiolabel the idazoxan derivatives were carried out using the Bolton–Hunter technique [9], but this gave rise to high non-specific binding, and was abandoned. Later studies used RX801013, which was iodinated according to the Chloramine-T method [10].

Higher non-specific binding problems were encountered when the sample volume was increased to improve the sensitivity of the assay. All attempts to remove the effect of binding of the label to the serum proteins were unsuccessful. The blocking agents, salicylate, aminonaphtholsulphonic acid (ANS) and thiomersal, were not effective. Alteration of the pH and the addition of trichloroacetic acid (TCA) to precipitate some of the protein also proved ineffective. The use of pepsin to digest the protein effectively removed the interference of the serum proteins. A standard curve (Fig. 2) was established for idazoxan (200 μl of

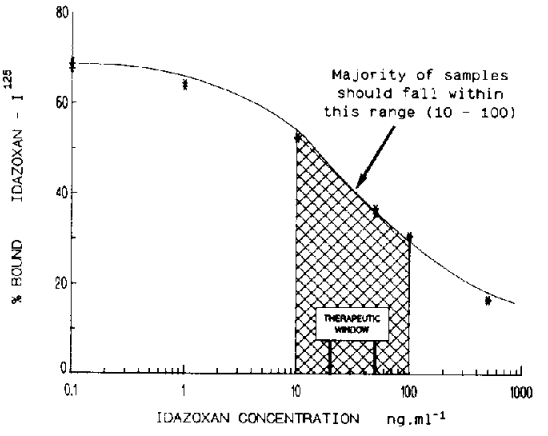

Figure 2
Standard curve for the pepsin digestion radioimmunoassay, the hatched region denoting the area in which most samples will fall. Also shown is the therapeutic range for idazoxan (20–50 ng ml^{-1}).

the digested extract assayed) over the range 0–500 ng ml^{-1}. The zero dose displayed a level of binding (% B/T) of approximately 70%.

Assay validation

Precision and accuracy. Serum samples containing 19.4, 75.5 and 145.7 ng ml^{-1} of idazoxan were each assayed 20 times in one assay and in duplicate in each of 10 separate assays. The within-assay precision (RSD) were 6.0, 8.1 and 8.2%, respectively, for the three idazoxan levels. The between-assay variations (RSD) were 10.4, 10.4 and 15.4% for the same three serum pools. The accuracy was determined by recovery of idazoxan from sera spiked with known concentrations (0, 14, 46 and 75 ng ml^{-1}) of the drug. The recoveries (±SD, $n = 4$) were 89.3 ± 7.4, 101.7 ± 4.9, 101.2 ± 1.2 and 93.9 ± 9.2%, respectively.

Limit of detection. The limit of detection was calculated from the results of 20 replicate assays of the zero dose. The minimum detectable level was found to be 3 ng ml^{-1} with 95% confidence limits.

Correlation. A number of samples were obtained from a clinical study which had been assayed previously by the analytical GC–MSD method [3]. These same samples were also assayed according to the pepsin digest RIA protocol. Analysis of the data showed that there was a correlation coefficient of 0.90, with the RIA displaying the slight high bias (Fig. 3).

Cross-reactivity. The cross-reactivity of some of the major metabolites and several closely related analogues was determined using the pepsin digest RIA. Derivatives were assayed in the absence and presence of idazoxan at a concentration of 10 μg ml^{-1} in drug free serum. The major metabolites in man, the 6-OH and 7-OH benzene hydroxylation products (42% of degradation) showed a cross-reactivity of 3.2 and 1.0%, respectively. Since these metabolites constitute the major metabolic pathway of idazoxan degradation, the other cross-reactivities were considered to be negligible.

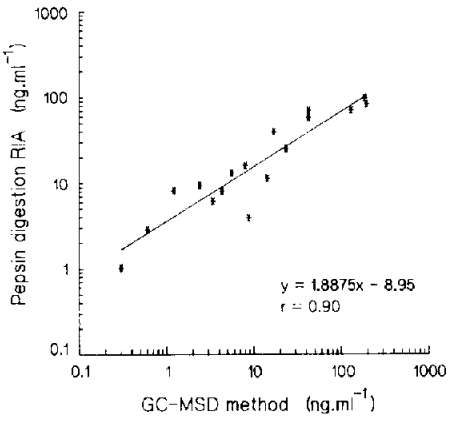

Figure 3
A comparison of the GC–MSD and pepsin digestion radioimmunoassay data. Samples were assayed in duplicate. Also shown is the fitted regression line.

Discussion

Over the described study several immunogens were produced and immunized into sheep. The integrity of the early immunogens was suspect, due to compound stability, which was borne out by the associated lack of antibody responses. It was not unusual to find no antibody response as was obtained for immunogen **I**, and this was interpreted as a failure to couple the drug to KLH.

Early immunogens exhibited various levels of bridge binding and exhibited higher levels of non-specific binding. The bridge was large and displayed many structural features. Also, the imidazoline ring nitrogen may have participated in unwanted side reactions during the coupling procedure. Instability of the imidazoline ring was the greatest problem in obtaining an immunogen suitable for further study. This may be the reason why the antibody population obtained from immunogen **III** was of mixed species. The product from a base hydrolysis of the immunogen was compared with the normal standards and showed similar displacement, which suggested that the antibody population was derived from both open and closed ring species.

Further stability of the structure, as with immunogen **IV**, gave rise to the best antibody responses, the maximum titre being of the order of 1/5000. All studies relating to the cross-reactivity and precision were carried out with this antibody. The anti-idazoxan solid-phase technique proved to be a reliable and rapid method for the evaluation of the antibody responses.

Assays were performed to assess the sensitivity, precision and accuracy of the method after the problem of high non-specific binding had been eliminated. Blocking agents were not successful; however, the incubation of the

serum sample with pepsin proved effective, and resulted in a satisfactory assay. The pepsin digest RIA was used to analyse samples previously assayed by GC–MSD, with a resultant correlation of 0.90. Cross-reactivity of closely related analogues and metabolites did not cause a problem, and showed that the anti-idazoxan antibody was specific.

Conclusions

An RIA was established that could detect idazoxan in serum to a level of 3 ng ml^{-1}. The best antibodies (immunogen **IV**) obtained from a rigorous conjugation programme were linked to a solid-phase, allowing the assay to be carried out quickly and reproducibly. The immunoassay thus established should replace the analytical GC–MSD assay as the assay of choice for the measurement of idazoxan in plasma and, therefore, help to facilitate development of the drug.

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[Received for review 5 April 1990; revised manuscript received 19 April 1990]