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A radioimmunoassay for the determination of tipredane in human plasma

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Abstract

A sensitive method for the determination of tipredane in human plasma has been developed. Prior to radioimmunoassay, proteins are removed from plasma by precipitation with acetonitrile. A [^{125}I]iodohistamide derivative of tipredane is used as a heterogeneous radioligand. The primary antiserum is raised in sheep against a tipredane (*O*-carboxymethyl) oxime–bovine serum albumin conjugate. Donkey anti-sheep IgG antiserum is used as the secondary antiserum in a double antibody separation procedure. The limit of quantification of the method is 1 ng ml^{-1} for 500 μl of plasma. There is no significant interference from either established or putative metabolites of tipredane, endogenous steroids or a wide range of other drugs. The assay was used to determine the concentrations of tipredane in plasma samples collected in pilot clinical studies.

Keywords: Radioimmunoassay; Tipredane; ^{125}I-labelled radioligand

1. Introduction

Tipredane [(11β,17α)-17-(ethylthio)-9α-fluoro-11β-hydroxy-17-(methylthio)androsta-1,4-dien-3-one, **I**, Fig. 1] is a potent, topically active, synthetic glucocorticoid developed by E.R. Squibb & Sons for the treatment of inflammatory skin diseases [1]. The compound was developed by Fisons as a treatment for bronchial asthma and related disorders. In order to support pharmacokinetic and clinical evaluation of the drug, a sensitive method for its determination in human plasma was required. As tipredane was administered in low doses and undergoes rapid metabolism in man [2,3], the peak concentration of the parent drug in plasma was expected to be around or below 10 ng ml^{-1}.

Radioimmunoassay has been successfully used in the trace analysis of many steroids [4–7]. Conjugation of a protein such as bovine serum albumin (BSA) is required to produce an immune response from a small molecule such as a steroid. The 3-*O*-carboxymethoxime bridging group was used in the preparation of both the immunogen and the radioligand. A high specific activity ^{125}I-

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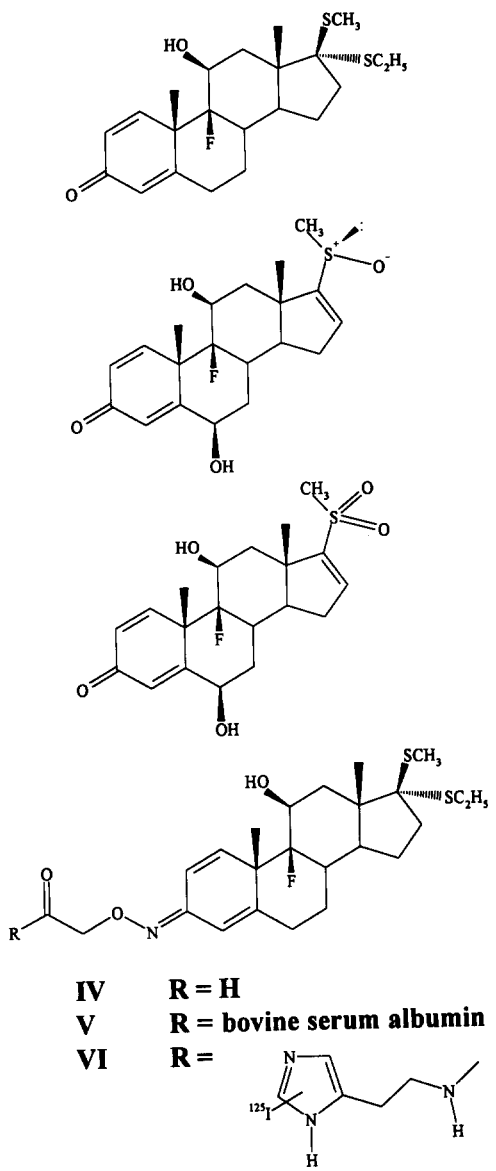


Fig. 1. Structure of tipredane (**I**), major human metabolites (**II** and **III**) and other derivatives.

labelled radioligand was synthesized to obtain the maximum sensitivity.

Major variations between blank plasma samples from different subjects were observed during initial studies on a direct radioimmunoassay. A purification step was therefore incorporated into the assay using acetonitrile to precipitate plasma proteins.

The specificity shown by the antiserum towards the two major human urinary metabolites [8], FPL 66365XX and FPL 66366XX (**II** and **III**, Fig. 1) and a range of putative metabolites was determined.

2. Experimental

2.1. Materials

Tipredane was supplied by Bristol Myers Squibb (Sword Laboratories, Dublin, Ireland). [11-^{3}H]Tipredane and the metabolites of tipredane were synthesised at Fisons, Pharmaceutical Division (Loughborough, Leics., UK). Hydrocortisone, corticosterone, cortisone, testosterone, progesterone, estradiol, estrone, estriol, acetylsalicylic acid, salicylic acid, caffeine, ibuprofen, paracetamol, saccharin, aspartame and bovine serum albumin were purchased from Sigma Chemical (Poole, Dorset, UK). All other chemicals and solvents were purchased from Fisons Scientific Equipment (Loughborough, Leics., UK) and were of analytical grade.

Donkey anti-sheep IgG antiserum was purchased from the RAST Allergy Unit, Benenden Chest Hospital (Cranbrook, Beds., UK). Non-ulcerative Freunds incomplete adjuvant (Morris) was purchased from Guildhay Antisera (Guildford, Surrey, UK) and BCG vaccine was purchased from Glaxo (Greenford, Middlesex, UK).

Blank human plasma was obtained by the centrifugation of lithium heparin-anticoagulated blood (100 ml) collected, using Microflex infusion sets and disposable syringes (50 ml), from an "in-house" panel of healthy human donors.

2.2. Equipment

The Tecan Robotic Sample Processor (RSP, Model 5052) was obtained from Tecan (UK) (Goring, Oxon, UK). The gamma counter (Cobra, Model 5005) was purchased from Packard Canberra (Pangbourne, Berks, UK). Preparative HPLC was performed on a system consisting of

two Waters Model 510EF HPLC pumps coupled to a Waters Model 680 automated gradient controller, all of which were obtained from Millipore, Waters Product Division (Harrow, Middlesex, UK).

The ultraviolet absorbance detector (Gilson Holochrome UV) was equipped with a 10 mm, 11 μl analytical flow cell and was purchased from Anachem (Luton, Beds, UK). Scanning of radioactivity on TLC plates was carried out using a Berthold LB284/500HR linear analyser obtained from Berthold Instruments (St. Albans, Herts., UK). Mass spectra were recorded on either a VG Model 70-250S mass spectrometer purchased from VG Analytical (Withenshaw, Manchester, UK) or a TSQ 700 mass spectrometer obtained from Finnegan-MAT (Hemel Hempstead, Herts, UK). NMR spectra were recorded on a Bruker AM360 spectrometer supplied by Bruker Spectrospin (Coventry, Warwickshire, UK).

2.3. Synthesis of the hapten (3-carboxymethyloxime derivative of tipredane, IV, Fig. 1)

Tipredane (1 g) and carboxymethoxyamine hemihydrochloride (1 g) were dissolved in a mixture of ethanol (45 ml) and aqueous sodium hydroxide (2 mol l^{-1}, 4.4 ml). The reaction mixture was refluxed for 4 h, the solvent was removed in vacuo and the crude product was dissolved in water (25 ml). The pH of the aqueous solution was adjusted to 1–2 by the addition of hydrochloric acid (10%, v/v) and then extracted with ethyl acetate (2 × 25 ml). The organic extracts were combined, washed with water (25 ml) and the solvent removed in vacuo to afford a glassy white solid. The crude product was purified by flash chromatography on silica using chloroform–methanol (19:1 v/v) as the eluent. Fractions (ca. 20 ml) were collected and examined by TLC using toluene–propan-2-ol–acetic acid (20:2:1, v/v/v) as the mobile phase. Those fractions containing the desired material were combined and the solvent was removed in vacuo to yield a sticky, white solid. Trituration with hexane–diethyl ether (1:1, v/v) afforded, after drying in vacuo, a white crystalline solid [**II**, 604.7 mg; MS (thermospray), m/z 484 (M + H)]. The structure of the hapten was confirmed by ^{1}H NMR spectroscopy and the final product found to be a mixture of the *syn* and *anti* isomers at the 3-position.

2.4. Preparation of the immunogen (tipredane–bovine serum albumin conjugate, V, Fig. 1)

The hapten (**IV**, Fig. 1; 111 mg) and 1,1-carbonyldiimidazole (56 mg) were placed in a round-bottomed flask and purged with a stream of dry nitrogen. Dry tetrahydrofuran (5 ml) was added and the mixture stirred at room temperature for 2.5 h. An aliquot (100 μl) of the reaction mixture was removed and diluted with ethanol (5 ml), then analysed by TLC on silica (Whatman AL SILG/UV, chloroform). This revealed a reaction product that was less polar than the starting material (**II**). Consequently, the remainder of this initial reaction mixture was injected into the vortex of a rapidly stirred solution of BSA (200 mg) in aqueous sodium hydrogencarbonate (5%, w/v, 10 ml). After this reaction mixture had been vigorously stirred for a further 1 h, examination of an aliquot of its contents by TLC on silica [Whatman AL SILG/UV, chloroform–diethyl ether–formic acid (7:2:1, v/v/v)] revealed the presence of a significant amount of UV-absorbing material which was strongly retained whilst the unreacted oximes were weakly retained.

The immunogen was purified by size-exclusion chromatography on a Sephadex G-25 column, with water as the eluent and methyl orange as a low molecular weight reference standard. The solution containing the protein conjugate was applied to the column and fractions (ca. 20 ml) collected. A small aliquot was taken from each fraction and tested using Bradford's protein reagent to detect the presence of the protein conjugate. The fractions found to contain the protein conjugate were combined and lyophilized to yield a fluffy white solid.

The UV absorbance of BSA, the hapten (**IV**) and the immunogen (**V**) were measured at 273 nm and indicated that ca. 21 mol of hapten were incorporated per mole of BSA.

2.5. Production of the anti-tipredane antiserum

A solution of the immunogen (**V**, 1.2 mg) in sterile isotonic saline (4 ml) was homogenized with non-ulcerative Freunds incomplete adjuvant (8 ml) and aqueous BCG solution (0.2 ml) prepared as for intradermal injection, to obtain an emulsion. The mixture was cooled on ice whilst preparing the emulsion. The emulsion (0.5 ml) was injected intramuscularly into each of the limbs of three Suffolk-cross sheep. Subsequent boosting immunizations, from which the BCG solution was omitted, were performed on days 25, 56 and 180 after the primary immunization. The sheep were bled, in general, on the tenth day after each boosting immunization.

2.6. Preparation of the radioligand (*VI*, Fig. 1)

A sample of the hapten (**IV**, 2.3 mg, 4.76 μmol) was dissolved in dry tetrahydrofuran (500 μl) and cooled to − 10°C in an ice–salt bath. Tri-*n*-butylamine in tetrahydrofuran (1:6, v/v; 6 μl) and isobutyl chloroformate in tetrahydrofuran (1:10, v/v; 6.7 μl) were added to the hapten solution and the reaction mixture shaken vigorously and then left to stand at − 10°C. An aliquot of this mixed anhydride (50 μl) was removed and added to a solution of unlabelled iodohistamine (2 μg ml^{-1}, 1 ml) in ethanol–water (1:1, v/v). After 20 min the reaction mixture was allowed to warm to room temperature and a portion (100 μl) analysed by reversed-phase HPLC on a Nova-Pak C_{18} column (150 × 3.9 mm i.d.) using a mobile phase of aqueous ammonium acetate (0.5%, w/v)–acetonitrile in the gradient mode and UV detection at 254 nm, to confirm the presence of the desired product. A further aliquot (50 μl) of the solution containing the mixed anhydride was removed and added to a solution of [^{125}I]iodohistamine (1 ml, 18.5 MBq, 81.4 TBq $mmol^{-1}$, 0.227 nmol ml^{-1}) in ethanol–water (1:1, v/v) at − 10°C. The reaction mixture was shaken well and after 20 min allowed to warm to room temperature.

The crude radioligand was purified by reversed-phase HPLC using the conditions described for analysis of the reaction product. As there was insufficient radiolabel to allow detection by UV absorbance, in-line radiochemical detection was used to permit manual collection of the eluate fractions containing the radiolabel. The fractions were pooled to give the final product. The radiochemical yield was determined by gamma counting to be approximately between 10 and 20%. The radiochemical purity was determined to be typically > 90% by reversed-phase TLC [Whatman KC18F, acetonitrile–aqueous ammonium acetate (0.5%, w/v) (7:3, v/v)] with radioactivity scanning.

2.7. Preparation of the buffer used in the radioimmunoassay

The sodium phosphate buffer (0.1 M) used in the assay was prepared by the adjustment to pH 6.0 of an aqueous solution of sodium dihydrogenorthophosphate dihydrate.

2.8. Preparation of standard solutions of tipredane

Tipredane (ca. 0.7 mg) was dissolved in methanol to obtain a stock solution at a concentration of 100 μg ml^{-1}. This stock solution was diluted to 10 μl ml^{-1} with methanol and the absorbance measured at 238 nm, and compared with the reference value of 0.405. Two stock solutions were prepared independently, one for standard samples and the other for quality contol samples. Working standard solutions were prepared at concentrations of tipredane of 50, 100, 200, 500 and 1000 ng ml^{-1} by dilution of the methanolic stock solutions, using diluent buffer.

2.9. Preparation of standard and quality control plasma samples

The plasma standard solutions at concentrations of 0, 0.5, 1.0, 2.0, 5.0 and 10.0 ng ml^{-1} were prepared by dilution of the appropriate working standard solution (100 μl) with pooled plasma (9.9 ml) obtained from human blood collected over lithium heparin. Quality control samples at concentrations of 2.0 and 5.0 ng ml^{-1} were prepared in a similar manner using larger volumes of pooled human plasma. Aliquots (0.5 ml) of the prepared plasma samples were dispensed into

polypropylene tubes (Sarstedt, 12 × 75 mm) and stored at − 20°C or below.

2.10. Preparation of the reagents used in the radioimmunoassay

The following figures are typical values as the volumes of primary antiserum and radioligand solution were optimized to provide a binding response of ca. 50% for the tipredane-free standard samples and radioactivity of ca. 10 000 cpm in each tube. The anti-tipredane antiserum reagent was prepared by the addition of sheep anti-tipredane antiserum (17.5 μl) and control sheep serum (150 μl) to buffer (100 ml). The radioligand reagent was prepared by the addition of donkey anti-sheep igG antiserum (5.0 ml) and stock radioligand solution (260 μl) to buffer (95 ml). These solutions were prepared freshly on the day of use.

2.11. Processing of plasma samples

Reagents were dispensed automatically by the Tecan RSP 5052. Test plasma samples were obtained from blood collected over lithium heparin. Plasma samples and extracts were dispensed manually with Eppendorf pipettes. Plasma samples were removed from storage, thawed and centrifuged (1900*g*, 5 min). Aliquots (2 × 0.5 ml) of each test plasma sample were dispensed into the polypropylene tubes (12 × 75 mm). Analysis batches were prepared with the samples in the following order: quadruplicate standard samples, duplicate quality control samples, test samples and duplicate quality control samples. Acetonitrile (0.5 ml) was added to tubes containing plasma samples (0.5 ml). The tubes were then vortex mixed briefly, capped and centrifuged (1900*g*, 10 min). The radioligand reagent was dispensed (0.5 ml) into Pyrex tubes (12 × 75 mm). The first four tubes were discarded and the next four, the "totals", removed, but not discarded. To the remaining tubes, dublicate aliquots (0.3 ml) of the plasma supernate were added manually, followed by anti-tipredane antiserum reagent (0.5 ml). After overnight incubation (16–20 h) at 37°C, the tubes were centrifuged (1900*g*, 15 min) to separate antibody-bound and free radioligand, the supernates were then decanted into a sink and any residual liquid was wiped from the tube rims. The precipitates containing bound radioligand were counted for 3 min so that at least 10 000 counts were accumulated for the tipredane-free standard plasma samples. Binding values were calculated for each sample tube by comparison (cpm) with four "totals" tubes which contained only the radioligand reagent. The binding values (%) obtained from the standard plasma tubes were used to prepare a calibration curve based on either a spline or four-parameter fit. The results for quality control and test samples were obtained by interpolation of this curve.

2.12. Processing of aqueous samples

Solutions containing tipredane at concentrations of 2 and 50 ng ml^{-1} were prepared by dilution of the methanolic stock solution in buffer. Solutions containing concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 ng ml^{-1} in buffer were then prepared using the Tecan RSP 5052. The radioimmunoassay was then performed as for plasma samples except that aqueous solutions (100 μl) containing tipredane or other compounds of interest were dispensed into the Pyrex tubes.

3. Results

3.1. Anti-tipredane antiserum production

After the first boosting, sera from all three sheep were found to bind [^{3}H]tipredane. Sera obtained from the subsequent boosting immunizations were assessed on titre and the displacement of bound ^{125}I-labelled radioligand by a small amount of unlabelled tipredane. Antiserum obtained from one sheep after the third boost (reference 48-041289) was found to be the most suitable for the radioimmunoassay. The titre of this antiserum using the ^{125}I-labelled radioligand was approximately 1 in 40 000.

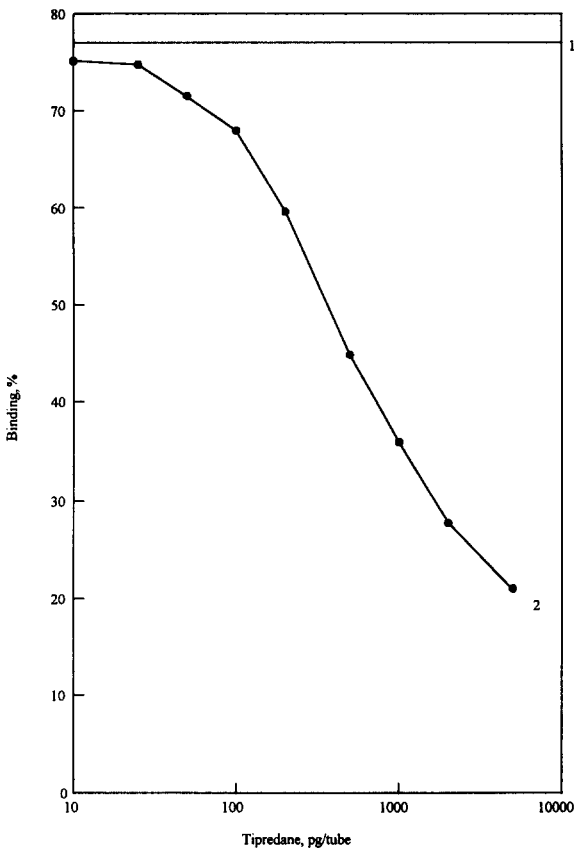


Fig. 2. Calibration curve for tipredane in aqueous samples. 1, binding for blank samples; 2, tipredane standards.

3.2. Properties of the assay in a plasma-free system

A typical calibration curve obtained using aqueous standards in a plasma-free system is shown in Fig. 2. This demonstrates the detection limit of the assay in the absence of plasma to be in the region of 10 pg per tube.

The susceptibility of the assay to organic co-solvents, methanol and acetonitrile, is demonstrated in Figs. 3 and 4. Increasing the amount of organic modifier decreases both the binding in the blank sample and the binding range across the concentrations, thus decreasing the sensitivity of the assay. The use of either 50% (v/v) methanol or 25% (v/v) acetonitrile destroys the assay at room temperature, but incubation at 37°C in 25% (v/v) acetonitrile produces a discernible calibration curve.

Using the method described by Abraham [9], the cross-reactions were determined in the plasma-free system for the 17-epimer of tipredane (**VII**, Fig. 5) and a range of putative metabolites (**VIII–XII**, Fig. 5). The cross-reactivities of all these metabolites were insignificant.

3.3. Sensitivity, accuracy and precision of the assay in plasma

A typical plasma calibration curve in shown in Fig. 6. The limit of detection of the assay is 0.8 ng ml^{-1}, according to the definition of Feldman and Rodbard [10], as calculated from the variation in

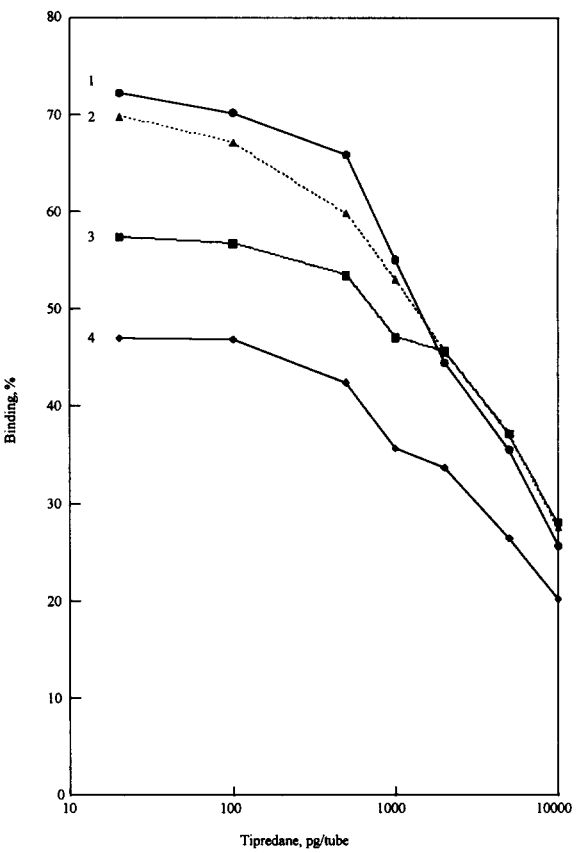


Fig. 3. Calibration curves obtained for aqueous samples in various media by incubation at room temperature. 1, sodium phosphate (pH 6, 0.1 M); 2, methanol–sodium phosphate (pH 6, 0.1 M) (10:90, v/v); 3, methanol–sodium phosphate (pH 6, 0.1 M) (25:75, v/v); 4, acetonitrile–sodium phosphate (pH 6, 0.1 M) (10:90, v/v).

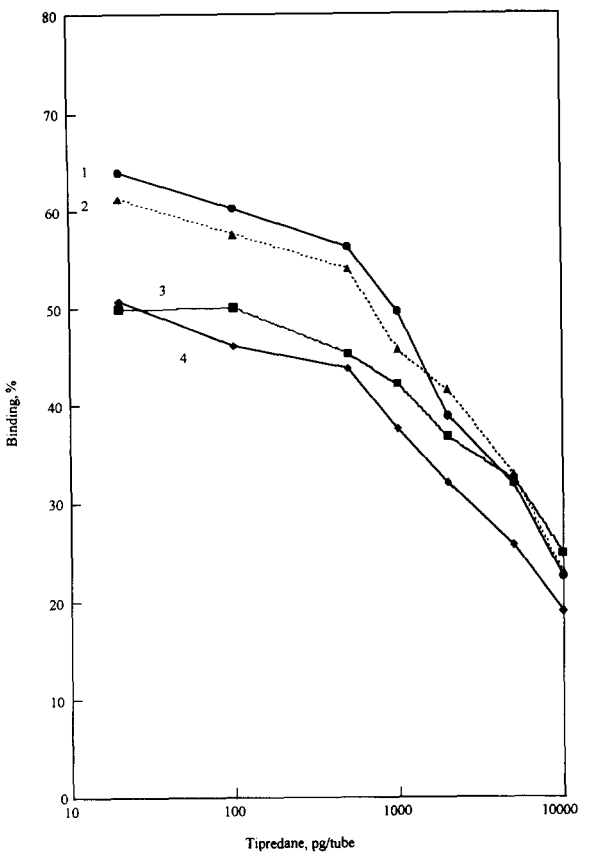


Fig. 4. Calibration curves obtained for aqueous samples in various media by incubation at 37°C. 1, sodium phosphate (pH 6, 1.0 M); 2, methanol–sodium phosphate (pH 6, 0.1 M) (10:90, v/v); 3, methanol–sodium phosphate (pH 6, 0.1 M) (25:75, v/v); 4, acetonitrile–sodium phosphate (pH 6, 0.1 M) (10:90, v/v).

response between blank samples from 10 individuals. The mean accuracy and precision data for the method are given in Table 1. The intra-assay mean accuracy and precision were determined by repeated analyses of calibration standard samples containing tipredane at 0.5, 1.0, 2.0, 5.0 and 10.0 ng ml^{-1}, in one batch. The intra-assay mean accuracy and precision were determined from the analysis of quality control samples at concentrations of 2.0 and 5.0 ng ml^{-1} in several batches.

The lower limit of quantification of the method, the lowest concentration at which the accuracy lies between 80 and 120% and the precision below 20%, is 1 ng ml^{-1}.

3.4. Specificity of the assay in plasma with respect to other drugs and the major metabolites of tipredane

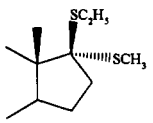
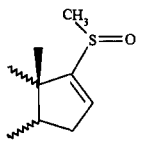
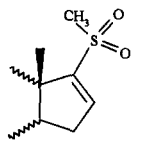
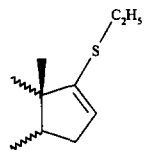
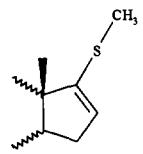
The cross-reaction of the major human metabolites of tipredane, FPL 66365XX and FPL 66366XX (Table 2), was negligible. The cross-reactions of a range of steroids and other drugs were determined (Table 2) at and above levels representative of physiological or therapeutic concentrations. None of these compounds exhibited significant cross-reactivity.

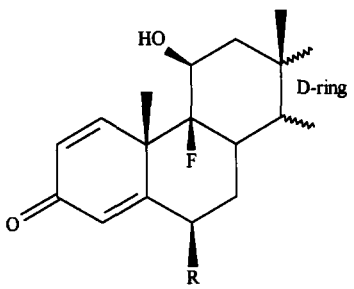
3.5. Application of the radioimmunoassay to the analysis of plasma samples from subjects taking part in clinical studies

The radioimmunoassay was used to determine concentrations of tipredane in plasma samples from four subjects taking part in two independent pilot clinical studies. In the first study, tipredane was delivered as a single dose of 2 mg by inhalation. In the second study, a dose of 1.6 mg of the drug was administered intranasally. Representative results from two subjects in each study are illustrated in Figs. 7 and 8. Even though the concentrations of tipredane were detectable in most of the plasma samples taken after dosing, the majority of the results were below the limit of quantification of the method of 1 ng ml^{-1}.

4. Discussion

The combination of the anti-tipredane antiserum and the ^{125}I-labelled radioligand results in a highly specific assay for tipredane. In a plasma-free system, even those putative metabolites which lack the 6β-hydroxy group (**VIII–X**, Fig. 5) exhibit cross-reactivities of 1.3% or less. The stereospecificity directed towards the steroid D-ring is exemplified by the 20-fold reduction in the cross-reactivity produced by epimerization of tipredane at the 17-position (**VII**, Fig. 5). It was not practicable to test a complete range of endogenous steroids and their metabolites. However, the structural selectivity of the assay,

Compound D-ring		R	Cross-reaction relative to tipredane ‡, %
VIII		H	5†
VIII		H	1.3
IX		H	0.2
X		H	<0.05
XI		H	<0..05
XII		OH	<0.05



† 17-S epimer of tipredane
‡ Determined at concentrations of 1, 100 and 1000 ng. per RIA tube

Fig. 5. Cross-reactivities of various metabolites and derivatives of tipredane in an aqueous, plasma-free system.

especially with regard to the steroid D-ring, indicates that there should be no significant interference from endogenous steroids at their physiological concentrations (Table 2). Even the relatively high concentrations of progesterone encountered in pregnant females should cause minimal interference. None of the other potential co-medicants had significant cross-reactivities at their therapeutic concentrations (Table 2).

Acetonitrile is a more efficient precipitating agent than methanol for plasma proteins [11]. However, it has a much more adverse effect than methanol on the immunoassay (Fig. 4), which can be reduced by increasing the immune reaction temperature to 37°C (Fig. 5). A compromise was reached between deproteinizing efficiency and the amount of organic solvent present during the immune reaction. After deproteinizing 0.5 ml of plasma with 0.5 ml of acetonitrile, only 0.3 ml of the supernate is analysed. Hence a plasma volume

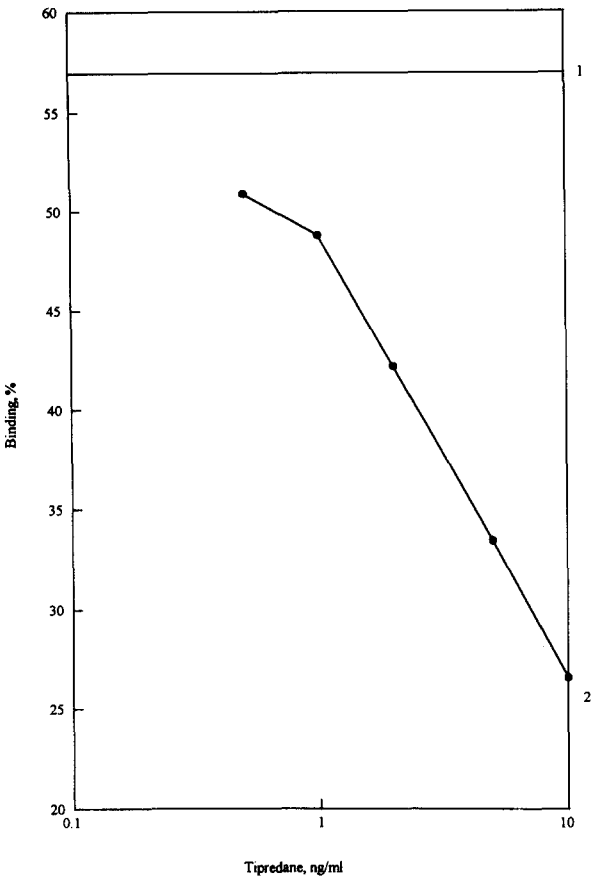


Fig. 6. Calibration curve for the determination of tipredane in human plasma. 1, binding for blank plasma samples; 2, tipredane standards.

of 0.15 ml is analysed in each radioimmunoassay tube which also contained 10% (v/v) acetonitrile during the immune reaction. The representative calibration curve in plasma (Fig. 6) is very similar to that obtained at 37°C in a aqueous, plasma-free system, containing 10% (v/v) acetonitrile (curve 4, Fig. 4). Hence the effects of plasma constituents have been minimized and the organic modifier is mainly responsible for the limit of detection in plasma, 0.8 ng ml^{-1}, or 120 pg per tube, being much worse than that for the aqueous, plasma-free system, of 10 pg per tube.

Table 2
Specificity of the assay in plasma with respect to various other drugs and the major metabolites of tipredane

Acetylsalicylic acid Compound	Concentration Concentration range[a] ($\mu g\ ml^{-1}$)	Cross-reaction Cross-reaction relative to tipredane (%)
Tipredane	–	100
Corticosterone	2	
Cortisone	2	
Estriol	2	
Estrone	2	<0.4
Estradiol	2	
Testosterone	2	
Progesterone	1–10	<0.08
Paracetamol	5–50	<0.02
II	1–100	
IIII	1–100	<0.008
Cortisol	1–100	
Caffeine	200	
Saccharin	200	<0.003
Aspartame	200	
Ibuprofen	240	<0.002
Acetylsalicylic acid	400	
Sodium salicylate	400	<0.001

[a] Where a single concentration was tested, the method described by Abraham [9] cannot be used, so a direct ratio of the "apparent concentration of tipredane" against the actual concentration of the compound was used to assess cross-reaction.

Table 1
Mean accuracy and precision data

Property	Added concentration of tipredane ($ng\ ml^{-1}$)				
	0.5	1.0	2.0	5.0	10.0
Intra-assay mean accuracy (%)[a]	138	100	103	99.7	104
Intra-assay precision (%)[a]	17.0	8.2	4.1	5.0	4.3
Inter-assay mean accuracy (%)[b]	–	–	108	101	–
Inter-assay precision (%)[b]	–	–	8.1	6.8	–

[a] From the analysis of eight calibration standard samples at each concentration in one analysis batch.
[b] From the analysis of duplicate samples in eleven analysis batches.

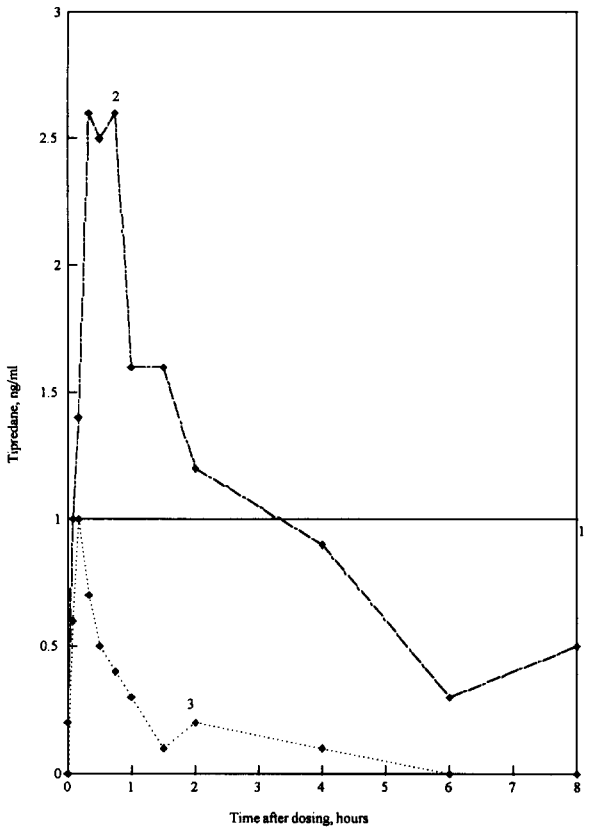


Fig. 7. Plasma concentrations found in plasma samples from two subjects who had received a 2 mg dose of tipredane via a metered dose inhaler. 1, limit of quantification for the method; 2, subject no. 1; 3, subject no. 2.

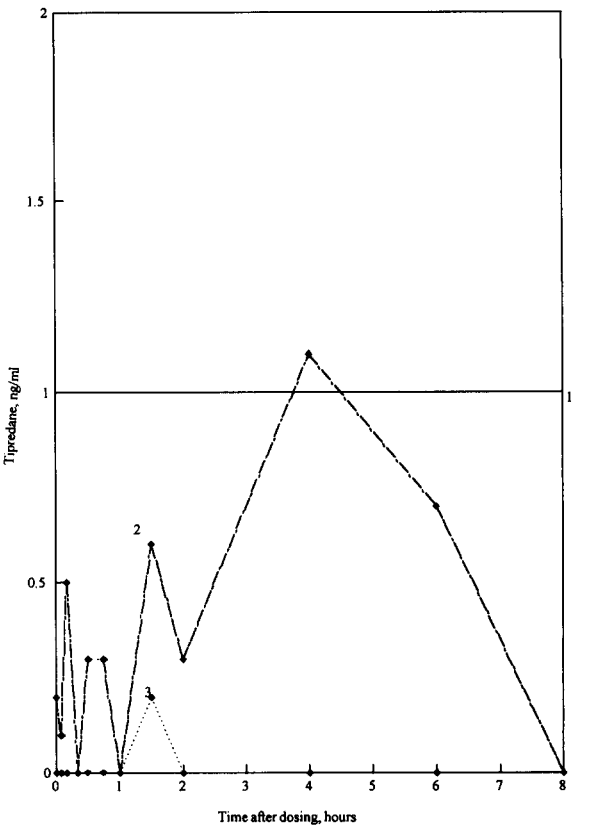


Fig. 8. Plasma concentrations found in plasma samples from two subjects who had received a 1.6 mg dose intranasally. 1, limit of quantification for the method; 2, subject no. 1; 3, subject no. 2.

Results from the analysis of samples from two pilot clinical studies indicated that the method was not sensitive enough to supply data suitable for the calculation of pharmacokinetic parameters. However, the intrinsic sensitivity of the radioimmunoassay in an aqueous, plasma-free system indicated that a more suitable clean-up of plasma could yield a more sensitive method. The development of such a method is reported elsewhere [12].

5. Conclusions

A robust, sensitive and highly specific method with a limit of quantification of 1 ng ml^{-1} has been developed for the determination of tipredane in human plasma. The removal of plasma protein with acetonitrile before the radioimmunoassay stage removes the bulk of the interfering constituents but reduces the intrinsic sensitivity of the immune reaction. Analysis of samples from two pilot clinical studies have shown the concentrations of tipredane to be 2 ng ml^{-1} or less in most cases and demonstrate the need for a more sensitive assay.

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