Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 7, pp. 557–561, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

A sensitive radioimmunoassay, incorporating solid-phase extraction, for fluticasone 17-propionate in plasma

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Abstract: Antibodies were produced in rabbits immunized with fluticasone 17-propionate (FP) conjugated to bovine thyroglobulin via its 3-carboxymethoxime with isobutylchloroformate. The antibodies were used to develop a sensitive and specific radioimmunoassay (RIA) for FP in human plasma. The limit of quantitation of the RIA is 50 pg per assay tube. This translates to 50 pg ml^{-1} plasma when a solid-phase extraction preceded the RIA. The interassay and intraassay relative standard deviations were <15% in the centre of the assay concentration range rising to <25% at the lower and upper limits (50 and 250 pg ml^{-1}). No appreciable binding was seen between the antibodies and the metabolite of FP that has been identified in man. The RIA was used to study the time course of plasma concentrations in man following inhalation of FP.

Keywords: *Fluticasone 17-propionate; radioimmunoassay; solid-phase extraction.*

Introduction

Fluticasone 17-propionate (FP) is a new anti-inflammatory steroid currently being developed for the treatment of asthma [1–3] and rhinitis [4–6]. FP, once absorbed, is rapidly cleared by hepatic metabolism in animals [7] and man [8–10], and very low drug concentrations are achieved in the plasma. Consequently a highly sensitive method of assay was required for estimating systemic exposure of the drug. Whilst conventional RIA is very sensitive it was clear from early work with FP that the performance would not be adequate for clinical monitoring. Consequently a sample concentration step, utilizing solid-phase extraction, was incorporated to increase the sensitivity 10-fold. This paper describes the final method adopted and also presents some data to illustrate the plasma concentrations produced by the administration of FP by inhalation.

Experimental

Chemicals

Fluticasone 17-propionate (FP), potential metabolites and related compounds were synthesized by Glaxo Group Research Ltd. [Propionyl-^{3}H] fluticasone 17-propionate (tritiated FP) 57 Ci $mmol^{-1}$ (purity >98%) was custom synthesized by Amersham International. Other compounds used in this study were obtained readily from commercial sources.

Preparation of reagents

Tricine buffered saline (TBS) contained 0.9% w/v NaCl, 1.79% w/v tricine and 0.1% w/v sodium azide with the pH adjusted to 7.2–7.4 with NaOH.

Dextran-coated charcoal suspension contained Dextran T70 (0.5 g) and Norrit GSX (5.0 g) in 1.0 l distilled water.

Preparation of the immunogens

FP was conjugated to bovine thyroglobulin by first forming the 3-carboxymethoxime derivative which was then coupled to the protein. The detailed procedure for the preparation of immunogen is given below.

(a) *Preparation of FP 3-carboxymethoxime.* FP (1.93 mmol) and carboxymethoxylamine hemihydrochloride (3.86 mmol) were dissolved in pyridine (10.0 ml) by stirring for 25 min and the reaction was allowed to continue at 21°C for 24 h (Fig. 1). The mixture was then poured into 0.5 M sulphuric acid (600 ml) and

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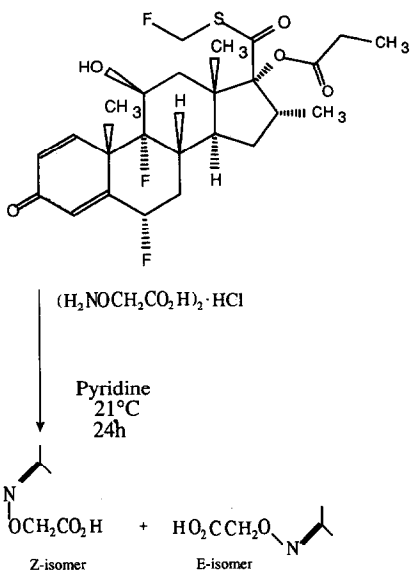

Figure 1
Procedure for the preparation of the FP 3-carboxymethoxime.

extracted into ethyl acetate (2 × 100 ml). The combined extracts were washed with water (4 × 50 ml), dried ($MgSO_4$) and the solvent was removed *in vacuo*. Recrystallization from acetone gave solvated colourless crystals (569 mg, 0.96 mmol) of the title carboxymethoxime, m.p. 246–249°C (decomp), $[\alpha]_D$ + 53°C (*c* 1.07, dioxan), λ_{max} (ethanol) 247 nm (ε14,200), 274.5 nm (ε16,800), (Found: C, 56.5; H, 6.0; F, 10.1, 9.7, 9.7; N, 2.3; S, 5.5, 5.5. $C_{27}H_{34}F_3NO_7S.0.2Me_2CO$ requires C, 56.6; H, 6.1; F, 9.7; N, 2.4; S, 5.5%). Proton NMR spectroscopy (in $(CD_3)_2$ SO) showed the oxime to be a mixture of *E*- and *Z*-isomers (3:1), δ 3.45 (d, 10 Hz), 3.10 (broad d, 10 Hz), 3.96 (m) (*E*-isomer); 3.59 (d, 10 Hz), 3.75 (broad d, 10 Hz), 3.20 (m) (*Z*-isomer).

(b) *Conjugation of FP 3-carboxymethoxime with bovine thyroglobulin.* Tributylamine (250 μl) was added to a solution of FP 3-carboxymethoxime (0.17 mmol) in dioxan (10 ml) and the mixture was cooled to 4°C. Isobutylchloroformate (50 μl) at 4°C was added and the mixture was stirred for 30 min at 10°C. In a separate vessel 280 mg of bovine thyroglobulin (0.0004 mmol) was dissolved in distilled water (13 ml) and made alkaline by the addition of 1 M NaOH (100 μl). The solution was stirred for 5 min and then dioxan (9 ml) was added. Twenty millilitres of this thyroglobulin solution was added to the FP 3-carboxymethoxime solution, stirred for 4 h at 10°C and then dialysed against running tap water at room temperature for 48 h. The solution was then freeze dried. The number of steroid molecules attached to the bovine thyroglobulin could not be determined because of the poor solubility of the conjugate in Tris/HCl, Tricine buffer saline or phosphate buffer.

Immunization procedures

Three male and one female New Zealand white rabbits were immunized. The immunogen was emulsified in a mixture of equal volumes of 0.9% NaCl, 1% Tween 80, and Freund's Complete Adjuvant. Each rabbit received 4 mg of immunogen. One millilitre of emulsion was administered intramuscularly in each hind limb and a further 1.0 ml was administered intradermally in multiple dorsal sites. Booster injections were administered at monthly intervals. These were prepared using Freund's incomplete adjuvant, and only half of the amount of immunogen used in the primary immunization. Antiserum was harvested 10–14 days after each booster injection.

Sample extraction procedure

The required sensitivity was achieved by an extraction stage prior to RIA. Plasma (1.0 ml) was applied to a 3 ml C-18 solid-phase extraction cartridge (Bond Elut, Analytichem) that had been conditioned with methanol (1.0 ml) and TBS (1.0 ml). The cartridge was then washed with TBS (1.0 ml) and water (1.0 ml). FP was eluted with methanol (1.0 ml), the solvent was removed, and the sample reconstituted in horse plasma (0.1 ml) prior to RIA. If a 10-fold reduction in sensitivity could be tolerated, plasma (0.1 ml) could be assayed directly without the initial concentration step.

Radioimmunoassay procedure

RIAs without the pre-extraction stage were performed in duplicate, whereas samples assayed after extraction were usually assayed singly because of the larger sample volume required. Calibrants were, however, always assayed in duplicate. Plasma samples (0.1 ml) were mixed with 5% ethanol in TBS (0.1 ml) and antibody in TBS (1:10,000, 0.1 ml). The mixture was incubated at 2–8°C for 30 min. Tritiated FP in TBS (6000 dpm, 0.1 ml) was added and the mixture incubated for a further 30 min at 2–8°C prior to separation of free and bound radiolabel using dextran-coated char-

coal (1.0 ml). Calibration standards in the range 25–250 pg per tube were used for quantitation. This was achieved by fitting a smoothed spline function to a plot of bound radiolabel against concentration (Riacalc, LKB Wallac).

Specificity of the antibodies

The specificity of the antibodies was determined by assaying known amounts of potential metabolites, endogenous steroids and other related compounds against an FP calibration curve.

Stability of FP in plasma

Selected samples were assayed fresh, and after up to 10 months storage at −20°C. In addition selected samples were assayed after 24 h storage at 4°C.

Human studies

Twenty four male volunteers received FP by inhalation of 1 mg from a metered dosed inhaler giving a dose of 250 mcg per puff. Plasma was taken for FP assay at 10, 20, 30 and 45 min and 1, 1.5, 2, 3, 4, 6 and 8 h after dosing. The plasma was stored frozen until assayed using FP RIA following solid-phase extraction.

Results

Antibodies of high titre were obtained in most rabbits that had been immunized. Optimal antibody production occurred after the third booster injection, when titres as high as 1 in 25,000 were obtained.

Radioimmunoassay

A typical calibration curve for FP in horse plasma is shown in Fig. 2. Horse and human plasma produced identical results and the former was routinely used for calibration for convenience. The limit of quantitation (LOQ) was 50 pg per tube giving overall assay sensitivity of 50 or 500 $pgml^{-1}$ with and without the extraction step, respectively. This value was based on the unacceptably high errors associated with the assay of 25 pg per tube (Table 1). Concentrations lower than 50 pg per tube could often be detected in samples, but interference by endogenous materials made those results unreliable. The non-specific binding was <5% of the total radioactivity in each tube. A recovery of 80% was obtained for the extraction step.

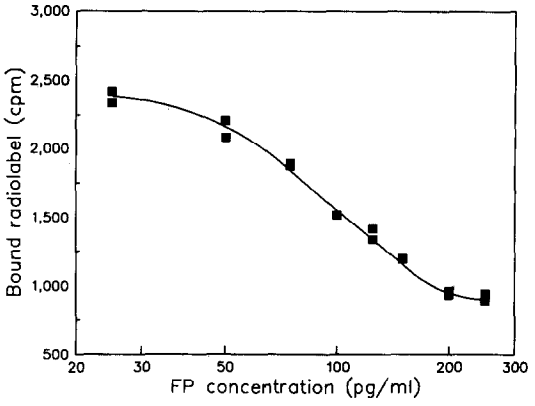

Figure 2
A typical standard curve of FP in horse plasma.

Assay performance was established by analysing replicate samples both in a single batch and in several batches. The intra- and inter-assay performance is presented in Table 1. For comparison, quality control data obtained from the RIA performed without extraction are presented in Table 2.

The cross-reactivity of antibodies with endo-

Table 1
Precision and accuracy of the FP RIA incorporating solid-phase extraction

Nominal conc. ($pg\ ml^{-1}$)	Intra-assay RSD* (%)	Intra-assay Bias (%)	Inter-assay RSD* (%)	Inter-assay Bias (%)
25	21	+20	80	−40
50	22	+10	13	−8
75	18	+10	18	−26
100	9	+10	10	−12
125	14	+10	10	−20
150	6†	+10†	14	−7
200	14	+10	17	−10
250	13	−4	23	−12

*Relative standard deviation.
†n = 5. For all other values, n = 6.

Table 2
Precision and accuracy of the FP RIA in the absence of an extraction step

Nominal conc. (ng ml^{-1})	Inter-assay RSD* (%)	Bias (%)
0.5	16	−2
1.0	9	0
1.5	10	4

* Relative standard deviation.
n = 31. Table compiled from QC data obtained over a 2 month period.

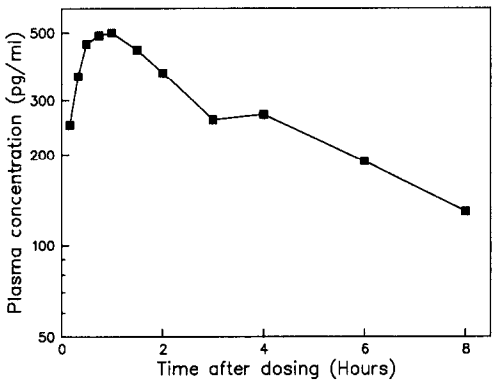

Figure 3
Plasma concentrations of FP after inhalation (1 mg) in a male volunteer.

genous steroids, potential FP metabolites and potential coadministered steroids is presented in Table 3. The cross-reactivity is expressed as the percentage ratio of FP IC_{50} to analogue IC_{50}. Only three compounds showed a significant degree of cross-reactivity, and these were compounds that differed from FP at the 11- or 6-positions only.

FP was stable in plasma at 4°C for at least 24 h. Samples stored frozen were stable for at least 10 months.

Use of the RIA to study absorption of inhaled FP in man

A graph of plasma FP concentrations against time following inhalation of 1 mg FP by a volunteer is shown in Fig. 3.

The C_{max} and T_{max} were 500 pg ml^{-1} and 1 h respectively. The terminal half-life, determined by log–linear regression, was 4 h. Similar profiles were obtained in all 24 volunteers.

Discussion

FP is a novel steroid being developed for inhalational [1–3] and intranasal [4–6] administration. As part of the safety component of the early administration to man it was necessary to monitor plasma levels of the administered substance by a specific and highly sensitive method. Radioimmunoassay was selected as the most appropriate method, and for this antibodies that were highly specific to FP were needed. The 3-carboxymethoxime derivative of FP was readily linked to thyroglobulin to yield an immunogen that elicited the production of antibodies with very high titre in rabbits. As might be expected, these showed almost no cross-reactivity either to analogues of FP that had changes to the molecule in and around the C-17 position or to endogenous steroids. However, the antibodies

Table 3
Cross-reactivity of potential metabolites, potential coadministered drugs, and endogenous steroids in FP RIA

Compound	Cross reaction (IC_{50}FP/IC_{50} analogue) × 100
Hydrocortisone	<0.2
Corticocosterone	<0.2
Progesterone	<0.2
Cholesterol	<0.2
Testosterone	<0.2
Oestriol	<0.2
Betamethasone	<0.2
Clobetasone 17-butyrate	<0.2
Clobetasol 17-propionate	0.6
FP 17-alcohol 17-carboxylic acid	<0.2
FP 17-alcohol 17-carbothioic acid	<0.2
FP 17-alcohol	<0.2
FP 17-carboxylic acid	<0.2
FP 17-carbothioic acid	<0.2
11-keto-FP	13.6
6β-hydroxy-6-desfluoro-FP	45
6-keto-6-desfluoro-FP	>100
6β-hydroxy-6-desfluoro-FP 17-carboxylic acid	<0.2

were insensitive to modifications to the FP molecule nearer to the point of attachment of the carboxymethoxime adduct. Thus compounds such as 11-keto-FP and, in particular, 6-keto-6-desfluoro-FP exhibited significant cross-reactivity. It is fortuitous that the only known metabolite found *in vivo* in man is FP 17-carboxylic acid [8, 10], and thus for practical purposes the antibodies are highly specific.

The sensitivity of the assay proved to be very good, with the limit of quantitation being 50 pg per tube. However, in conventional RIA techniques only a small aliquot (in this case 0.1 ml) of plasma is used, thus forfeiting sensitivity in return for sample throughput. The technique described here involves a very simple solid-phase extraction technique that increases the sensitivity of the method 10-fold to 50 pg ml^{-1} plasma, and this has proved adequate for the measurement of FP in plasma after all routes of administration.

The precision and bias of this assay were acceptable for the monitoring of levels of FP in clinical plasma samples. However, the performance is not as good as for many RIAs. The performance was similar for both the extraction and non-extraction methods, suggesting that the extraction process did not introduce significant errors into the assay. Various attempts were made to improve the assay by varying the incubation times, and replacing the charcoal separation step with one utilizing polyethylene glycol and precipitation by a second antibody. However, assay performance was not improved. In particular, the use of long incubation times which would lead to the establishment of equilibrium produced very flat calibration lines. FP is a poorly soluble compound, which is highly bound to plasma proteins (unpublished results), and it is possible that these properties may have an untoward effect on the assay performance.

Nevertheless the methodology was satisfactory for successfully establishing the pharmacokinetics of FP in man, following inhalation and intranasal dosing.

In this study we have demonstrated that the inhalation of FP leads to low but detectable levels in the plasma. These are unlikely to be due to absorption from the GI tract as FP undergoes almost total first pass hepatic metabolism when administered via that route [8–10]. Thus it is likely that some FP can be absorbed intact directly from the lung.

In conclusion, the methodology reported herein will provide a sound basis for monitoring plasma concentrations during the early use of FP in man.

Acknowledgement — The authors wish to thank Miss Portia Williams for secretarial services.

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[Received for review 24 August 1992; revised manuscript received 11 December 1992]