Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 2/3, pp. 235–239, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00

Short Communication

The validation of a radioimmunoassay for the quantification of eflumast in clinical samples

M. SURTEES,† D.M. GOTT,† Y. YEANG,† K.W. CLOW† and M.J. DENNIS*‡

†*Rhône-Poulenc Ltd, Dagenham, Essex RM10 7XS, UK*
‡*Smith-Kline Beecham Pharma, Postfach 100231, D-4040 NEUSS-1, Germany*

Keywords: *Radioimmunoassay; eflumast; clinical pharmacokinetics.*

Introduction

Eflumast (RP42068; *N*-(2-hydroxy-3-acetyl-5-fluorophenyl)-5-carboxamido-1*H* tetrazole) is under development as an orally active anti-allergic agent. The clinical results from some single and repeat dose tolerance studies have already been reported [1]. During these studies, the principal analytical method that was employed for the assay of this compound was a radioimmunoassay (RIA) and this has now been used to produce some preliminary kinetic data from the same tolerance trials [2].

When applying RIA to the quantification of compounds in biological fluids, it is necessary to demonstrate the specificity of the method. This is especially so in the case of drugs, due to the possibility of metabolic products of the drug, that may have an affinity for the antibody employed in the RIA.

The purpose of this paper is to present some additional evidence for the specificity of the RIA for eflumast with respect to analyses performed on samples from humans. In addition, some further human kinetic data are presented.

Experimental

Antibody production

An immunogen was prepared by conjugating carboxymethyl eflumast to keyhold limpet haemocyamin (KLH; Sigma Chemical Co., Poole, Dorset) using ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, Sigma Chemical Co.).

Carboxymethyl eflumast (50 mg) was dissolved in 10 ml disodium hydrogen phosphate buffer (0.1 M, approx. pH 8) together with KLH (50 mg) and EDCI (30 mg). The solution was gently mixed for 4 days at 4°C. The immunogen was purified by using Sephadex G25 (Sigma Chemical Co.) gel filtration chromatography and eluting with disodium hydrogen phosphate buffer (0.1 M). The protein fraction was collected and shown to contain eflumast by the presence of an extra ultraviolet absorption band with a maximum at about 430 nm. Aliquots (1 ml) estimated to contain about 1 mg of protein, were stored at −30°C until required.

Two Soay sheep were immunized with this immunogen as follows. Initially, a total of about 125 μg of immunogen in an emulsion with Freund's complete adjuvant (FCA, Sigma Chemical Co.) was injected subcutaneously into four axillary sites. The emulsion was prepared with 3 volumes FCA to 1 volume immunogen solution plus a drop of Tween 80 (Sigma Chemical Co.). Booster injections of about 60 μg were given 7 weeks and 8 months later in the same sites. The booster injections were prepared by mixing Freund's incomplete adjuvant (Sigma Chemical Co.) with the immunogen solution in a ratio of 3:1 (v/v), respectively, with a drop of Tween 80. One week after the last booster injection, the sheep were bled via the jugular

*Author to whom correspondence should be addressed.

vein. The blood was left to clot at room temperature for 2 h. Serum was removed after centrifugation at 3500*g* for 30 min at 4°C.

Radiolabelled eflumast derivative

The tyramide derivative, carboxymethyl eflumast (4.32 mg, 10 μmol), in dimethylformamide (100 μl) was added to sodium [^{125}I] iodide (1 mCi, 2.2 nmol) in aqueous solution (pH 7–11, 50 μl) with vigorous mixing. A solution of chloramine-T (0.85 mg, 3 μmol) in water (50 μl) was added and the vigorously mixed solution allowed to stand for 1 h. The reaction mixture was evaporated to dryness under reduced pressure and the residue dissolved in a small volume of methanol. The product was purified by TLC on a 20 × 20 cm, 0.2 mm thick, silica gel plate using chloroform–methanol–acetic acid (86:10:4, v/v/v) as the solvent. The product zone was located with the aid of a marker containing a mixture of the starting tyramide, and the required mono-iodo and di-iodo compounds, and removed from the plate. The product was extracted from the silica with spectroscopically pure ethanol and the solution was diluted to 25 ml.

TLC examination of the product was used to confirm radiochemical purity and identity by comparison of R_f values with an authentic sample. Typical radiochemical yields were in the order of 15%.

Assay conditions

Phosphate buffered saline (PBS) was prepared by dissolving 49.7 g of anhydrous disodium hydrogen phosphate, 23.5 g of sodium dihydrogen phosphate, 45 g of sodium chloride and 0.5 g of sodium azide (all supplied by M&B Laboratory Chemicals, Dagenham, Essex, UK) in HPLC-grade water (M&B Laboratory Chemicals) and diluting to 5 l with HPLC-grade water.

Assay buffer was prepared by adding Tween 80 (Sigma) to give a final concentration of 0.1% (v/v). Donkey anti-sheep (DAS) second antibody was purchased from Benenden Chest Hospital, Cranbrook, Kent, and diluted with PBS, usually 1 + 14 but 1 + 19 was used with the higher dilutions of antiserum. The radiolabelled eflumast derivative was diluted in assay buffer to give about 15 000 cpm in 100 μl. Antiserum was diluted in PBS 1 + 399 or 1 + 599 depending on the sensitivity required.

Stock standards of eflumast were prepared by dissolving known amounts of a reference batch of eflumast in HPLC-grade methanol (M & B Laboratory Chemicals) and these were used to prepare assay standards of eflumast in stock human plasma. The range of standards prepared depended on the sensitivity of the assay that was required.

Plasma samples were assayed against standards in plasma after both had been diluted to the same extent with assay buffer. Standards were prepared in plasma as it was found that inaccurate results occurred if buffer standards were used to assay eflumast in plasma. In addition plasma was diluted at least 10-fold in assay buffer to eliminate excessive protein binding. Dilutions of 1 + 24 to 1 + 99 were generally used for plasma samples obtained during the early clinical trials of eflumast.

Plasma samples were diluted appropriately and duplicate aliquots (usually 50 μl) of each sample or standard, were added to 3.5 ml polystyrene assay tubes (Sarstedt Ltd). To this was added 600 μl of an assay cocktail that consisted of all the other reagents, i.e. antiserum, radiolabelled eflumast derivative and DAS at the appropriate dilutions, pre-mixed in a ratio of 2:1:1, respectively, and equilibrated at room temperature for 1 h. The solutions were vortex mixed and incubated for 1 h at room temperature and then overnight at 4°C. The performance of the assay was monitored by the inclusion of three quality control (QC) samples that were prepared at concentrations to cover a range appropriate to the sensitivity of each assay run. The QC samples were treated in exactly the same way as the standards and samples.

After the overnight incubation the assay tubes were centrifuged at 3000*g* for 20 min and the supernatant solutions aspirated. The bound radioactivity in the precipitate was quantified by using a LKB 1275 Minigamma Counter (Pharmacia Ltd, Milton Keynes, Bucks, UK). This was linked to an IBM XT personal computer with an on-line data reduction program (Riacalc 2, Pharmacia, Ltd), that was used to fit a spline function standard curve and calculate the concentrations of eflumast in the samples.

Assessment of assay specificity

The specificity of the assay was confirmed using urine obtained from a healthy volunteer 4–8 h after oral dosing with eflumast (400 mg).

Additionally, urine, collected from a rabbit 8–24 h after an oral dose of [^{14}C] labelled eflumast, that was known to contain at least 2 metabolites as well as unchanged eflumast, was used to provide further evidence of specificity. Aliquots (100 μl) of urine were injected onto a HPLC gradient system with a 25 cm × 0.46 cm i.d. μBondapak (Waters Ltd, Watford, Herts, UK) C18 column and eluted using a water–methanol linear gradient (from 0 to 100% methanol in 30 min: flow rate 1 ml min^{-1}) and 0.5-ml fractions were collected. The elution time for eflumast in this system was known to be between 12 and 14 min. Aliquots of the fractions were diluted 1 + 24 in assay buffer and 100 μl aliquots were analysed by RIA using a standard curve (ranging from 0.78 to 200 ng ml^{-1}) in assay buffer.

The remainder of the fractions from the rabbit urine sample were added to liquid scintillation fluid (Luma Gel, Lumac, Landgraav, The Netherlands) and the amount of [^{14}C] in terms of Bequerels was determined using a Packard, Tri-Carb 460 CD liquid scintillation counter. Some of the fractions obtained from the human urine (that contained large amounts of eflumast) were reassayed at dilutions of 1 + 99 and 1 + 199 so that the total amount of eflumast could be calculated.

Clinical studies

Single dose tolerance studies of orally administered eflumast have been performed after receiving approval from independent ethics committees. Oral doses of up to 400 mg were administered to healthy male volunteers who had fasted for approximately 8 h prior to a light breakfast 1.5 h before ingesting the capsules containing eflumast. Consumption of tea, coffee and alcohol was not allowed during the study. Blood samples were taken at intervals for up to 48 h post-dose. Plasma was separated immediately and frozen at −20°C until assayed for eflumast. The plasma samples were diluted appropriately in assay buffer and assayed against standard concentrations of eflumast in plasma. In any assay run the dilutions were the same for standards, plasma samples and QC samples.

The following kinetic parameters were estimated from the eflumast concentration–time data for each volunteer: the maximum plasma concentration (C_{max}); the time to maximum plasma concentration (T_{max}); the terminal elimination half-life ($T_{½\beta}$) and the area under the plasma concentration–time curve, extrapolated to infinity ($AUC_{0-\infty}$). The area under the plasma concentration–time curve up to the last sampling point (AUC_t) was calculated by the trapezoidal rule. The value obtained by dividing the concentration at the last time point by the final $T_{½\beta}$ was added to AUC_t to obtain the $AUC_{0-\infty}$. $T_{½\beta}$ was estimated by fitting a line to the terminal linear phase of an exponential plot of the data.

Results

Assay sensitivity

The sensitivity of the assay was determined by the equation:

$$cpm_L = cpm_0 - 3 \times \delta\ cpm_0,$$

where cpm_L is the counts per minute equal to the minimum detectable concentration (MDC); cpm_0 is the counts per minute of the bound fraction obtained from the zero standard and $\delta\ cpm_0$ is the standard deviation of the cpm_0 measurement. By using this assay with a plasma dilution of 1 + 9, the MDC was found to be 3.3 ng ml^{-1} of sample.

Assay precision

During the assay of eflumast in clinical samples, the assay precision was monitored by the inclusion of QC samples at eflumast concentrations of 1, 3, 5, 10, 15 and 25 μg ml^{-1}. The within-assay relative standard deviations (RSD) at these concentrations were 6.4, 4.0, 4.3, 5.3, 1.0 and 4.50%, respectively. The between-assay RSD at concentrations of 1, 3, 5 and 10 μg ml^{-1} were 9.9, 3.0, 12.4 and 11.8%, respectively. The between-assay RSD for the higher concentrations are not available as only one assay run was performed in the concentration range requiring those QC samples.

Assay accuracy

The accuracy of the assay was assessed from the results obtained for the QC samples expressed in terms of the mean percentage recovery of the nominal QC values. Thus, the assay accuracy was 96, 92, 103, 105, 98 and 105% at nominal concentrations of 1, 3, 5, 10, 15 and 25 μg ml^{-1}, respectively.

Assay specificity

The results obtained from both the RIA of the HPLC fractions obtained after chromatog-

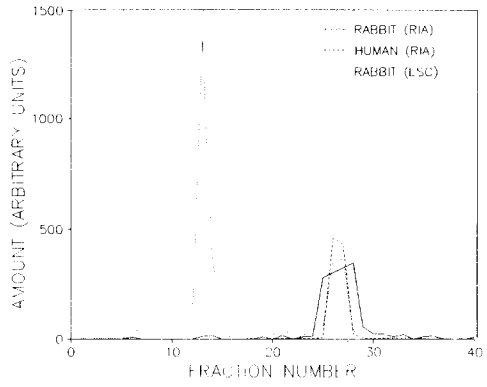

Figure 1
Graphical representation of the amounts (arbitrary units) of metabolites and eflumast detected in HPLC fractions obtained after chromatography of human and rabbit urine collected after oral administration of unlabelled or radiolabelled eflumast, respectively. The results are presented as amounts of eflumast or its metabolites detected by liquid scintillation counting [Rabbit (LSC)] or radioimmunoassay [Rabbit (RIA)] of fractions obtained from rabbit urine and radioimmunoassay of fractions obtained from human urine [Human (RIA)].

raphy of the rabbit and human urine and the liquid scintillation counting of fractions obtained after chromatography of the rabbit urine are shown in Fig. 1. It can be clearly seen from the results of the liquid scintillation counting that unchanged eflumast was only a minor component in rabbit urine, and that the most of the radioactivity derived from at least two metabolites. In contrast, the results for the RIA of both the rabbit and the human urine fractions revealed only one main component, which accounted for 96.5% of the immunoreactivity in the case of the human urine and more than 98% of the immunoreactivity in the case of the rabbit urine. In both cases the elution time of the major component, in terms of eflumast immunoreactivity, corresponded to that of eflumast.

Plasma kinetics of eflumast

The average estimates of C_{max}, T_{max}, $AUC_{0-\infty}$ and $T_{½\beta}$ for all doses are shown in Table 1. The C_{max} and AUC values were normalized to a dose of 50 mg and examined statistically to determine whether or not there was any departure from pharmacokinetic linearity. The data were examined by carrying out one-way analysis of variance followed by Bonferroni simultaneous confidence interval analysis for all comparisons [3]. Thus, although the analysis of variance of the AUC data indicated that there was a difference between the groups, the Bonferroni test shows that there were no differences large enough to be statistically significant. However, the analysis of variance of the C_{max} data followed by calculation of Bonferroni's confidence intervals revealed that the normalized C_{max} values from the 150 mg dose group were statistically significantly different from the group mean. The majority of the plasma concentration profiles from these studies displayed a biphasic elimination pattern when followed to 48 h post-dose (see Fig. 2 for an example).

Discussion

Antibodies to eflumast have been produced using an immunogen prepared with keyhole limpet haemocyanin (KLH). Previous attempts with an immunogen based on bovine serum albumin appeared to be unsuccessful, thus providing some support to the claims that KLH is to be prefered for the production of antibodies to naturally non-immunogenic haptens. In this case, it was possible to prepare an

Table 1
Mean (±SD) values for C_{max}, $AUC_{0-\infty}$ and $T_{½\beta}$ and median values for T_{max} for groups of eight volunteers. $T_{½\beta}$ was not calculated for the 50, 100 and 150 mg groups because data were available for up to 24 h post dose only. If averages were calculated from less than eight values the number used is shown. Only seven subjects were used from the 150 mg group because samples were not available from one of the subjects. Calculations of mean AUC and $T_{½\beta}$ for the 225 mg group were carried out on seven subjects because one had an anomalous $T_{½\beta}$ of around 90 h

Dose	50 mg	100 mg	150 mg	225 mg	300 mg	400 mg
C_{max} ($\mu g\ ml^{-1}$)	1.8 ± 0.4	3.0 ± 0.5	8.3 ± 2.0 ($n = 7$)	10.2 ± 2.6	13.7 ± 3.9	14.6 ± 4.7
T_{max} (h)	2	2	2 ($n = 7$)	2	2	3
$AUC_{0-\infty}$ (h $\mu g\ ml^{-1}$)	17.0 ± 3.7	31.0 ± 5.2	67.8 ± 9.6 ($n = 7$)	117 ± 23 ($n = 7$)	120 ± 49	152 ± 21
$T_{½\beta}$ (h)	—	—	—	27 ± 9 ($n = 7$)	10 ± 7	16 ± 5

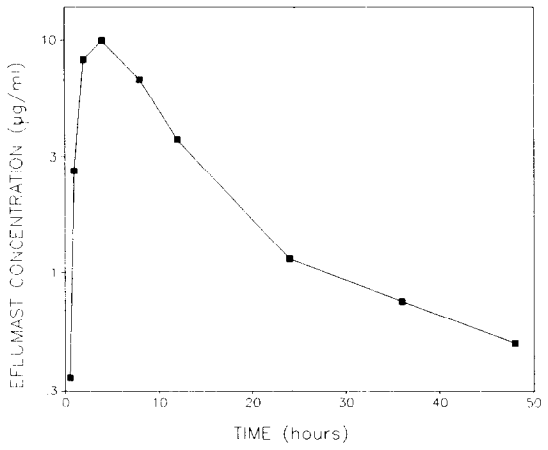

Figure 2
The plasma concentration–time curve for a volunteer administered (po) 225 mg of eflumast, demonstrating the biphasic plasma elimination of this compound.

antibody with a high degree of specificity to a drug molecule that is known to give rise to at least two urinary metabolites in rabbits and in man (unpublished data from the research laboratories of Rhône-Poulenc Ltd, Dagenham, Essex, UK).

The use of urine samples to demonstrate the specificity of the RIA represents a 'worse-case' situation, as the concentrations of these polar metabolites are likely to be lower in the plasma. Thus, the specificity of the RIA has been demonstrated for use with both plasma and urine samples. The quantitative data obtained from the analysis of samples obtained from early tolerance trials of eflumast can therefore be reported with some confidence.

These data have provided an indication of the kinetic behaviour of eflumast when administered orally to humans. Eflumast appears to be fairly rapidly absorbed with a median T_{max} of 2 h. The absolute bioavailability of eflumast has not been assessed, but the amounts detected in the plasma indicate that absorption of this compound is extensive. The plasma elimination of eflumast is biphasic in nature with a terminal elimination half-life in the region of 20 h. The kinetic behaviour, as assessed using normalized AUC values, appears to be linear over the oral dose range examined (i.e. up to 400 mg). However, there was a statistically significant departure from pharmacokinetic linearity when this was assessed using the normalized C_{max} value. This difference is, however, unlikely to represent a real departure from pharmacokinetic linearity because it was not observed in the analysis of the AUC values, with which one can be more confident, because it involved all the available data. In addition, it should be noted that the data presented here are from studies in groups of different volunteers and are not the results of a definitive pharmacokinetic study. Thus, within the limitations of these data it would appear that the pharmacokinetic behaviour of orally administered eflumast is linear, and the results obtained suggest that it will be suitable for twice or thrice daily administration.

In conclusion, the application of a specific RIA for eflumast has proved useful for the analysis of clinical samples and has provided an indication of the kinetic behaviour of this potential new drug.

Acknowledgements — The authors would like to thank K. Lord, M. Bath, L. Young and P. Wiebkin for their technical assistance.

References

[1] C. Walls, Y. Yeang, J. Walker and M. Dennis, *N. Engl. Reg. Allergy Proc.* **9**, 469 (1988).
[2] M. Surtees, V. Facchini, Y. Yeang, K. Clow and M. Dennis, *Br. J. Clin. Pharmac.* **27**, 111P (1988).
[3] R.G. Miller Jr, *Simultaneous Statistical Inference*, 2nd edn. Springer-Verlag, New York (1981).

[*Received for review 28 May 1991*]