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THE ASSAY OF A NOVEL HISTAMINE H_2-RECEPTOR ANTAGONIST, SK&F 93479, IN HUMAN PLASMA BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective assay of a new histamine H_2-receptor antagonist, SK&F 93479, in human plasma has been developed. The method uses liquid—liquid extraction from the biological sample and analysis of the resulting extract by normal-phase high-performance liquid chromatography with UV detection for quantitation of the drug and an added standard. The assay is sufficiently accurate and precise to determine the compound at concentrations as low as 0.025 mg l^{-1}. The coefficient of variation of the assay averages 5.7% at concentrations between 0.1 and 2.0 mg l^{-1}, but increases to 21.8% at 0.02 mg l^{-1}. SK&F 93479 can be determined in spiked plasma samples, at concentrations between 0.05 and 0.80 mg l^{-1} with a bias of between —7.5 and +3.6%, but at 0.02 mg l^{-1} concentrations were underestimated by 15% on average. The assay has been used for pharmacokinetic and bioavailability studies: after a single 0.5 mg kg^{-1} oral dose in man, plasma concentrations can be monitored for up to 70 h after dosing.

INTRODUCTION

SK&F 93479, 2-{2-[5-(dimethylaminomethyl)furan-2-ylmethylthio]ethylamino}-5-(6-methylpyrid-3-ylmethyl)-4-pyrimidin-4-one (I in Fig. 1), is a novel, non-imidazole antagonist of histamine at H_2-receptor sites. This compound was considerably more potent than cimetidine (Tagamet®) as an inhibitor of histamine stimulated gastric acid output in experimental animals [1]. In clinical studies with healthy male subjects [2] SK&F 93479 was found to be up to 20 times more potent than cimetidine, on a molar basis, in reducing gastric acid output stimulated by a test meal. In addition, the maximum antisecretory effect of SK&F 93479 was maintained for longer periods than were seen with cimetidine.

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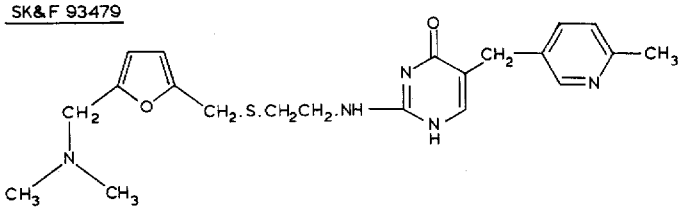
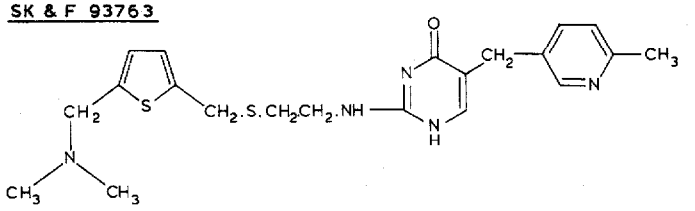
SK&F 93479



I

SK & F 93763



II

Fig. 1. Structures of SK&F 93479 and SK&F 93763 (internal standard).

Potent drugs, such as SK&F 93479, require a sensitive and specific assay to enable investigators to follow the kinetics over the time-course of pharmacological activity, which in turn permits an optimum dosage regimen to be devised.

Previous chromatography-based assays for histamine H_2-receptor antagonists have used a variety of organic solvents for extraction of the drugs and metabolites from biological fluids. Cimetidine has been extracted using ethyl acetate [3, 4], methylene chloride [5, 6] and octanol [7, 8], which has also been used to extract ranitidine [9] and oxmetidine [10]. The resulting extracts have then been subjected to high-performance liquid chromatography (HPLC) for separation of drug, metabolites and added (internal) standard. Normal-phase silica chromatography has been used for cimetidine assay [6—8] and oxmetidine assay [10]. Reversed-phase (octadecylsilyl-bonded silica) chromatography has been used in the analysis of cimetidine [3, 4] ranitidine [9] and oxmetidine [10] and cyano-bonded silica has been employed in a recently published assay for cimetidine [5].

This paper presents an analytical method for the determination of SK&F 93479 in human plasma by UV absorption after normal-phase HPLC of an extract. The extraction procedure, which precedes chromatography, is similar to that described by Lee and Osborne [7] for cimetidine. The validated method was used to assay plasma samples taken from healthy male subjects to whom the drug was administered.

EXPERIMENTAL

Materials

Analytical grade chemicals were used throughout this study with the following exceptions: 1-octanol (Koch-Light, Colnbrook, Great Britain; puriss), methanol HPLC grade and acetonitrile HPLC grade S from Rathburn (Walkerburn, Great Britain) and 0.88 sp. gr. ammonium hydroxide solution

(May and Baker, Dagenham, Great Britain; reagent grade). The water used was purified by deionisation, then distilled in an all-glass apparatus and stored in glass containers.

All solvents and solutions for HPLC were filtered through 0.5-μm membrane filters (Millipore type HA and FH for aqueous and organic solvents, respectively); prior to use the components of the solvent system, except the ammonia, were mixed and degassed by the application of reduced pressure. The ammonium hydroxide solution was then added.

Solutions of 1 mol/l carbonate buffer (pH 9) were prepared as follows; to 5 l of 1 mol/l sodium bicarbonate was added sufficient 1 mol/l sodium carbonate to adjust the pH value of the solution to 9.0.

The polypropylene centrifuge tubes (12 ml) and stoppers (Type 300 PP and 301 PT, respectively) were obtained from Henleys Medical Supplies (London, Great Britain).

A standard solution of SK&F 93479 (I, Fig. 1) was made by dissolving 1.263 mg of the trihydrochloride salt (equivalent to 1.000 mg base) in 100 ml ethanol; a solution containing 1.000 mg of SK&F 93763 (II, Fig. 1), 2-{2-[5-(dimethylaminomethyl)thien-2-ylmethylthio] ethylamino}-5-(6-methylpyrid-3-ylmethyl)-pyrimidin-4-one, dissolved in 100 ml ethanol was used as an internal standard. Both ethanolic solutions were stored at −20° C until used and were found to be stable for at least six months under these conditions.

Plasma samples

Blood from subjects who were receiving the drug was withdrawn by cannula or syringe into heparinised containers, mixed, centrifuged and the plasma obtained transferred to plain tubes, which were quick frozen over solid carbon dioxide and then kept at −20° C pending analysis.

Extraction of plasma samples

To 2 ml plasma in a polypropylene centrifuge tube (12 ml) were added 1 ml of 1 mol/l (pH 9) carbonate buffer, 50 μl ethanol containing 500 ng SK&F 93763 as an internal standard, and 5 ml 1-octanol. The tubes were stoppered, placed on a blood cell suspension mixer for 15 min, then centrifuged at 2500 *g* for 5 min, after which 4.5 ml of the organic layer were transferred to another tube containing 3 ml of 0.02 mol/l hydrochloric acid.

After stoppering, the tubes were re-extracted by the same technique and centrifuged to separate the two phases; the octanol was removed by aspiration and 2.5 ml of the acid layer were transferred to another centrifuge tube to which 200 μl acetonitrile were added.

The contents of each tube were mixed by vortex; about 5 g of anhydrous potassium carbonate were added and the contents were again mixed by vortex. The effect of this saturation with potassium carbonate was to salt out the acetonitrile, which was then separated cleanly by centrifugation for 2 min at 2500 *g* and transferred to glass vials, which were stored at −20° C to await chromatographic analysis.

Standard curve

The standard curve was prepared using 2-ml aliquots of drug-free human plasma which had been spiked in duplicate, with 0, 10, 20, 50, 100, 150 and

200 μl of the standard ethanolic solution of SK&F 93479, corresponding to 0, 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 mg l^{-1} of SK&F 93479 in plasma. These tubes were vortex mixed, allowed to equilibrate for 5 min and then extracted at the same time and by the same procedure as the test samples.

High-performance liquid chromatography

The chromatograph consisted of a Perkin-Elmer Series 3B pump and an LC-75 variable-wavelength UV detector with the wavelength and absorbance set at 220 nm and 0.02 a.u.f.s. respectively. The separation of analytes was achieved by a stainless-steel column 250 mm × 4.6 mm I.D. packed with 5-μm silica (Ultrasphere from Altex). The mobile phase consisted of acetonitrile–methanol–water–ammonium hydroxide 0.88 sp. gr. (200:80:10:1.5, v/v) and was pumped at a flow-rate of 2 ml min^{-1}.

Aliquots of the sample extracts (40 μl) were introduced into the chromatograph via either a Rheodyne 7125 valve or Waters WISP automatic injector. Under these conditions the retention times of SK&F 93479 and the internal standard were 3.2 and 2.4 min, respectively.

Quantitation of SK&F 93479 concentrations

The detector output was fed into a Perkin-Elmer Sigma 10 data station which integrated the areas of the peaks corresponding to SK&F 93479 and the internal standard. A plot of the SK&F 93479/internal standard peak area ratios versus drug concentration was drawn for the spiked standards by a program on a Hewlett-Packard 9825 desk top computer. A regression line was drawn through the points; using this regression another program calculated the concentration of SK&F 93479 in the test samples using the peak area ratios.

Radiochemical studies

The efficiency of the extraction process, for various concentrations of SK&F 93479 in plasma, was assessed by the use of [^{14}C]SK&F 93479, labelled in the C-2 position of the pyrimidone ring (Fig. 1) (radiochemical purity: 96.4%, specific activity: 97.1 μCi mg^{-1}). This material was used to spike drug-free human plasma over a concentration range of 0.025–1.00 mg l^{-1}. A 100-μl aliquot of the spiked plasma and the recovered acetonitrile layer from each sample were put into separate plastic scintillation vials together with 5 ml Picofluor scintillant and counted in a Searle Mk. III counter. From the radioactivity data obtained, the overall recovery of the method was calculated.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms observed after injection of extracts of human plasma are shown in Fig. 2. No endogenous compounds with retention times corresponding to that of SK&F 93479 have been encountered in the predose samples of volunteers who received the drug, nor in the drug-free human plasma used for validation of the assay. The unknown endogenous compounds, giving peaks at 5 min after injection, do not interfere with the automated assay if samples are injected at 4-min intervals.

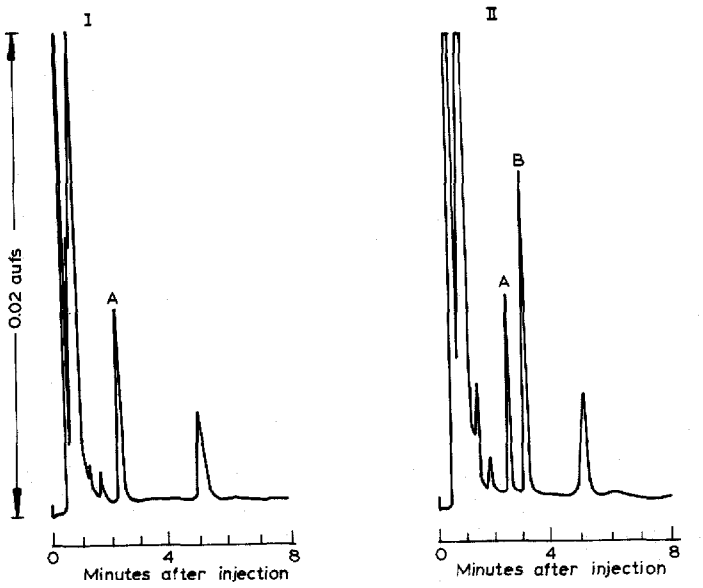


Fig. 2. Normal-phase chromatograms of extracted human plasma taken (I) before and (II) after the administration of SK&F 93479 to the subject. Peaks: A = internal standard, SK&F 93763; B = SK&F 93479.

Co-extraction of putative metabolites such as the N-oxide and S-oxide is unlikely to interfere with the assay since these compounds are considerably more polar than SK&F 93479 and thus would have longer retention times in the normal-phase chromatographic system used. However, if present, such metabolites would restrict the throughput of samples.

Recovery of SK&F 93479 from plasma

The results showing the recovery of [^{14}C]SK&F 93479 are presented in Table I; as can be seen the recovery of the drug in the range 0.025—1.00 mg l^{-1} averaged 29.1%. This fell to 18.6% at 0.025 mg l^{-1}. These low recoveries were partly the result of taking less than total volumes of the octanol and hydrochloric acid extracts;this reduced the possible recovery to 75% of maximum, so that losses due to adsorption or inefficient partition were about 60%. This contrasts with losses for oxmetidine by a similar process [10] of only 25%.

The probable reason for the less efficient extraction of SK&F 93479 at pH 9.0 is the pK_a value of 8.53 associated with this molecule, indicating a substantial ionisation at the selected pH. However, the other pK_a values for SK&F 93479 are 3.03, 6.11 and 10.20 which makes difficult the selection of an appropriate pH for extraction into octanol.

Under the conditions employed (2 ml plasma sample and injection of 40 μl of the final acetonitrile extract) the useful sensitivity of the assay was considered to be 0.025 mg l^{-1}.

TABLE I

RECOVERY OF [^{14}C]SK&F 93479 FROM SPIKED HUMAN PLASMA

In all cases $n = 10$.

Concentration of SK&F 93479 (mg l^{-1})	Percent mean recovery*	Standard deviation
0.025	18.6	1.67
0.050	19.5	2.19
0.10	23.0	3.41
0.25	27.7	2.16
0.50	31.1	4.34
1.00	28.5	2.95

*Calculated from $\frac{\text{d.p.m. in total recovered acetonitrile} \times 100}{\text{d.p.m. in plasma sample extracted}}$. Having regard to the volumes of octanol and hydrochloric acid taken, the maximum possible recovery was 75%.

TABLE II

BETWEEN-DAY PRECISION OF SK&F 93479 PLASMA ASSAY

Concentration of SK&F 93479 (mg l^{-1})	Mean drug/I.S. ratio*	Standard deviation	Number of determinations	Coefficient of variation (%)
0.020	0.11	0.02	10	21.8
0.050	0.16	0.02	20	11.1
0.10	0.31	0.02	20	7.4
0.25	0.75	0.04	19	5.9
0.50	1.56	0.08	20	4.9
0.75	2.64	0.13	10	4.9
1.00	3.17	0.22	20	6.8
1.50	4.92	0.25	10	5.1

*I.S. = Internal standard.

Precision and accuracy of the assay

The precision and accuracy of the assay were assessed by spiking 2-ml volumes of drug-free plasma with various concentrations of SK&F 93479. The precision of the assay is presented in Table II and is expressed as the coefficient of variation (C.V.). In the range 0.10—2.00 mg l^{-1} the average C.V. was 5.7%, however at lower concentrations the assay became less precise, viz. 11.1% C.V. at 0.05 mg l^{-1} and 21.8% C.V. at 0.02 mg l^{-1}.

The assessment of the accuracy of the method is presented in Table III and shows that the average deviation in the range 0.05—0.80 mg l^{-1} was 4.0% but at 0.02 mg l^{-1} the mean calculated concentration was 15% below the known concentration.

TABLE III

ACCURACY OF SK&F 93479 PLASMA ASSAY

Concentration of SK&F 93479 (mg l^{-1})	Mean calculated concentration	Standard deviation	Number of determinations	Percent deviation of mean from known value
0.02	17	7	10	−15.0
0.05	49	5	10	−2.0
0.10	103	6	8	+3.0
0.20	185	6	9	−7.5
0.40	384	13	9	−4.0
0.80	829	62	8	+3.6

These results supported the decision to set the limit of sensitivity of the assay at 0.025 mg l^{-1}.

Stability of SK&F 93479 in plasma

In order to assess whether there were any potential problems of stability of the drug in deep-frozen plasma samples awaiting analysis, sufficient 2-ml aliquots of drug-free plasma were spiked at 0.10 and 0.50 mg l^{-1} to permit the assay of ten tubes for each concentration at 0, 1, 2, 4, 6 and 10 weeks after freezing and storage at −20°C. When thawed the samples were extracted as described above and freshly spiked plasma samples were also analysed to produce a calibration curve.

The results of the study are presented in Table IV and show that the drug in plasma was stable for approximately four weeks; thereafter there was a 20% fall in drug concentration between weeks 4 and 10. Therefore, it is essential that plasma samples are stored for no longer than four weeks before analysis.

The stability of SK&F 93479 in plasma during the immediate post-sampling period was also determined: forty tubes, each containing 2 ml human plasma spiked at 0.10 mg l^{-1} and a second set containing 0.50 mg l^{-1} were allowed to equilibrate for 10 min after vortex mixing.

TABLE IV

STABILITY OF SK&F 93479 IN PLASMA STORED AT −20°C

Week No.	Calculated concentrations	
	0.100 mg l^{-1}	0.500 mg l^{-1}
	Observed concentrations (mg l^{-1})	
0	0.108 ± 0.003 (n = 9)	0.506 ± 0.021 (n = 10)
1	0.110 ± 0.006 (n = 10)	0.551 ± 0.019 (n = 9)
2	0.101 ± 0.008 (n = 9)	0.501 ± 0.018 (n = 9)
4	0.094 ± 0.008 (n = 10)	0.503 ± 0.026 (n = 8)
6	0.072 ± 0.007 (n = 9)	0.424 ± 0.030 (n = 10)
10	0.081 ± 0.001 (n = 10)	0.437 ± 0.019 (n = 10)

TABLE V

STABILITY OF SK&F 93479 IN PLASMA AT AMBIENT TEMPERATURES

Time (h)	Peak height ratio (SK&F 93479/SK&F 93763)			
	0.10 mg l^{-1} SK&F 93479	Percent of original	0.50 mg l^{-1} SK&F 93479	Percent of original
0	0.39 ± 0.01 (n = 8)	100	1.48 ± 0.06 (n = 10)	100
2	0.34 ± 0.04 (n = 10)	87	1.50 ± 0.06 (n = 10)	101
4	0.33 ± 0.01 (n = 8)	85	1.48 ± 0.10 (n = 10)	100
6	0.32 ± 0.02 (n = 9)	82	1.58 ± 0.04 (n = 10)	107

At time zero ten tubes of each concentration were extracted as described previously, this was repeated after 2, 4 and 6 h during which the tubes remained at ambient temperature. After HPLC analysis the peak height ratios of the drug to internal standard were calculated and the results are presented in Table V.

The drug was stable in plasma for at least 4 h at a concentration of 0.50 mg l^{-1}, but in the 0.10 mg l^{-1} samples there was a drop in concentration over the first 2 h. It is therefore recommended that all plasma samples are deep frozen as soon as possible after separation from blood cells.

Human studies

The assay was used to monitor the concentration of SK&F 93479 in plasma samples from a subject to whom the drug had been administered orally (0.5 mg kg^{-1}) and by intravenous infusion for 30 min (0.2 mg kg^{-1} h^{-1}). The results are presented in Fig. 3.

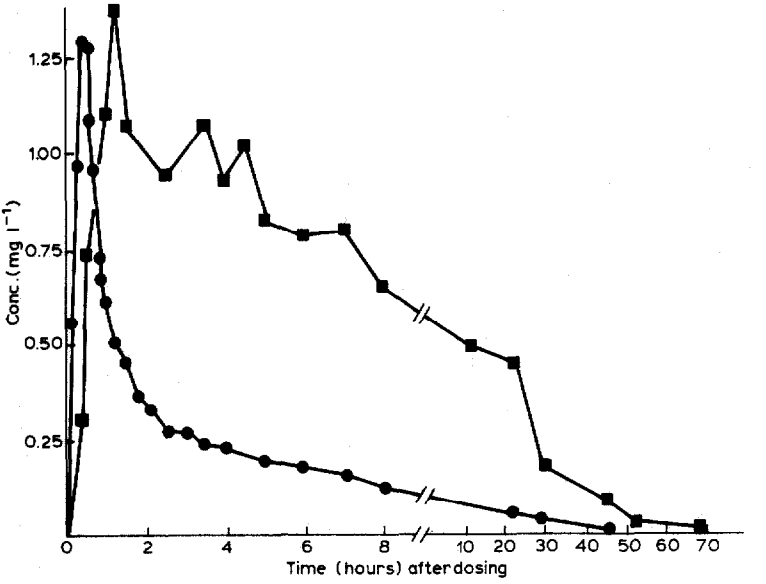


Fig. 3. Concentrations of SK&F 93479 in human plasma after oral or intravenous administration. ■, 0.5 mg kg^{-1}; ●, 0.2 mg kg^{-1} h^{-1} for 30 min.

Plasma concentrations were followed for 48 h after the infusion and for 70 h after the oral dose. Since the major phase of elimination of SK&F 93479 from the plasma has a half-life of about 5 h [11], this analytical method was sensitive enough to follow the elimination through several such half-lives. It may be used with confidence to assess the kinetic parameters applicable to SK&F 93479 in man and to predict the probable dosing regimen necessary to maintain control of gastric acid output.

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