Journal of Chromatography, 572 (1991) 227–238
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6066

Sensitive high-performance liquid chromatographic determination of famotidine in plasma

Application to pharmacokinetic study

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(First received March 6th, 1991; revised manuscript received July 18th, 1991)

ABSTRACT

A sensitive high-performance liquid chromatographic (HPLC) method for the quantitation of famotidine in human plasma is described. Clopamide was used as the internal standard. Plasma samples were extracted with diethyl ether to eliminate endogenous interferences. Plasma samples were then extracted at alkaline pH with ethyl acetate. Famotidine and the internal standard were readily extracted into the organic solvent. After evaporation of ethyl acetate, the residue was analysed by HPLC. The chromatographic separation was accomplished with an isocratic mobile phase consisting of acetonitrile–water (12:88, v/v) containing 20 m*M* disodium hydrogenphosphate and 50 m*M* sodium dodecyl sulphate, adjusted to pH 3. The HPLC microbore column was packed with 5 μm ODS Hypersil. Using ultraviolet detection at 267 nm, the detection limit for plasma famotidine was 5 ng/ml. The calibration curve was linear over the concentration range 5–500 ng/ml. The inter- and intra-assay coefficients of variation were found to be less than 10%. Applicability of the method was demonstrated by a bioavailability/pharmacokinetic study in normal volunteers who received 80 mg famotidine orally.

INTRODUCTION

Famotidine is a relatively new histamine H_2-receptor antagonist having a structure related to that of cimetidine and ranitidine. On a weight basis famotidine is twenty times more potent than cimetidine in inhibiting basal and pentagastrin-simulated gastric acid secretion in humans [1]. Famotidine is now widely used for the treatment of duodenal ulcers, benign gastric ulcer and hyper-acid secretory conditions such as Zollinger-Ellison syndrome.

As therapeutic doses of famotidine recommended in patients are low (40 mg daily) and these doses produce very low therapeutic concentrations in plasma (*e.g.* 78 ng/ml after a 40-mg oral dose) [1–3], a sensitive analytical method is

0378-4347/91/$03.50

required in order to determine famotidine concentrations in samples from clinical studies. Only a few high-performance liquid chromatographic (HPLC) procedures have been reported for the quantitation of famotidine in plasma [4–6]. Two methods [4,5] employed solid-phase extraction (SPE) techniques as a means of sample preparation followed by a reversed-phase HPLC analysis. The other HPLC method [6] involved an extraction of plasma samples with diethyl ether followed by extraction with ethyl acetate before chromatographic separation. We tried all the published methods [4–6], but no suitable assay for use in our laboratory was found. This was due to either endogenous peaks coeluting with famotidine or the non-elution of the drug from the SPE column. Therefore, we have developed a sensitive and selective HPLC procedure for the determination of famotidine in human plasma.

EXPERIMENTAL

Materials

All chemicals were of analytical grade. Famotidine was kindly supplied by Douglas Pharmaceuticals (Auckland, New Zealand). The internal standard (clopamide) was a gift from Pacific Pharmaceuticals (Auckland, New Zealand). HPLC-grade acetonitrile, diethyl ether, ethyl acetate, $NaHCO_3$, Na_2CO_3 and sodium dodecylsulphate (SDS) were obtained from BDH (Poole, UK). Water was double glass-distilled and Milli-Q-filtered. All glassware was cleaned and silanized with 0.5% Aquasil (Pierce, Rockford, IL, USA) before use.

Equipment

The HPLC system consisted of a Model 250 Perkin Elmer LC pump (Perkin Elmer, Norwalk, CT, USA) equipped with a WISP 712 Waters autoinjector (Waters Assoc., Milford, MA, USA). A variable-wavelength ultraviolet detector (Spectroflow 757, Kratos Analytical Instruments, Ramsey, NJ, USA) was used. The detector was set at 267 nm and 0.01 a.u.f.s. The chromatographic response was recorded by a Hitachi D2500 integrator (Hitachi, Tokyo, Japan), set at an attenuation of 3 (*i.e.* 8 mV).

Chromatographic conditions

The analytical column used was a microbore HPLC column (100 mm × 2 mm I.D.) packed with reversed-phase C_{18} material, 5 μm ODS Hypersil (Shandon, London, UK). Analysis of the samples of famotidine was performed using a mobile phase consisting of acetonitrile–water (12:88, v/v) containing 20 m*M* Na_2HPO_4 and 50 m*M* SDS and adjusted to pH 3 with orthophosphoric acid. The mobile phase was filtered and degassed for 10 min by using an ultrasonic waterbath. It was then passed through the HPLC column at a flow-rate of 0.5 ml/min. Chromatographic separations were performed at room temperature.

Preparation of the stock solution and standards

A stock solution of famotidine with a concentration of 100 μg/ml was prepared by dissolving 10 mg of famotidine in 100 ml of methanol. The solution was kept at −20°C and brought to room temperature when required. Famotidine was found to be stable in methanol or at least one week at −20°C. Three standard solutions (0.1, 1 and 10 μg/ml) of famotidine in distilled water were made by further dilution of the stock solution of 100 μg/ml famotidine with appropriate volumes of water. These standard solutions of famotidine in water were used to prepare standard plasma solutions of famotidine. Blank plasma, *i.e.* drug-free plasma, was used to prepare the standard solutions. The plasma standard solutions had famotidine concentrations of 5, 10, 25, 50, 100, 200, 350 and 500 ng/ml.

The internal standard stock solution clopamide (50 μg/ml) was also prepared in methanol and stored at −20°C until required. This solution was found to be stable for at least one week. The internal standard, a clopamide solution with a concentration of 5 μg/ml, was prepared fresh each day of analysis by dilution of the stock solution with distilled water.

Preparation of plasma sample

Aliquots of plasma (1.5 ml) were pipetted into 15-ml silanized glass centrifuge tubes fitted with PTFE-lined screw caps. The plasma samples were acidified with 100 μl of 4 *M* HCl and the tubes were gently shaken to ensure mixing. The contents were then extracted with 8 ml of diethyl ether on a mechanical shaker for 15 min. After a brief centrifugation (1000 *g*, 4°C) for 5 min, the upper diethyl ether phase was carefully aspirated and discarded. To the residual aqueous phase, 1 ml of buffer solution (mixture of equal volumes of saturated solution of Na_2CO_3 and $NaHCO_3$) and 100 μl of the internal standard (5 μg/ml clopamide) were added. The contents were then extacted with 7 ml of ethyl acetate on a mechanical shaker for 15 min. After brief centrifugation (2000 *g*, 4°C) for 5 min, the upper organic phase was transferred to a clean tapered 10-ml centrifuge tube. The second extraction with ethyl acetate was repeated as above and the organic phase was transferred to the same tapered centrifuge tube. The combined ethyl acetate extract was then evaporated to dryness under nitrogen stream at 37°C (N-Evap; Organomation Assoc., Northborough, MA, USA). The dry residue was reconstituted with 150 μl of a mixture of acetonitrile–water (12:88, v/v) and thoroughly vortex-mixed. An aliquot of the resultant solution (90 μl) was injected onto the HPLC system.

Calibration curve for plasma

Standard plasma calibration curves of peak-height ratio (famotidine/internal standard) *versus* plasma famotidine concentration were constructed using standard plasma solutions in the concentration range 5–500 ng/ml. The standard plasma samples were processed as described above. The calibration curves were constructed by plotting plasma famotidine concentration (*x*-axis), expressed as

ng/ml, *versus* peak-height ratio (y-axis), using linear regression. Plasma concentrations of famotidine were calculated from the peak-height ratios and the regression equation of the calibration curve. Peak heights were measured using a digitizer and computer software. The operator selected the start, top and end of the chromatographic peak and digitized these points. From this information the software constructed a baseline and then calculated the peak height. In the clinical study, known concentrations of famotidine were also prepared as seed controls and frozen at −70°C. With each day's analyses, these quality control standards (seed controls) were assayed with the unknown plasma samples.

Recovery

The absolute recovery of famotidine from the extraction procedures was determined at different plasma concentrations (25 and 200 ng/ml) by comparing the peak heights of the drug obtained from extracted plasma samples with those obtained from direct injections of the pure famotidine standards in water of equivalent amounts. A similar procedure was used to evaluate the recovery of the internal standard at a concentration of 333 ng/ml (*i.e.* final concentration in the extracted plasma samples).

Clinical study: bioavailability of famotidine tablets

To demonstrate the reliability of this method for the study of famotidine pharmacokinetics, this assay was used to determine famotidine concentrations in plasma samples obtained from a bioavailability study of famotidine tablets. The study was designed to determine whether the new formulation of 20-mg famotidine tablets (Douglas Pharmaceuticals) is bioequivalent to an appropriate reference formulation (Pepcidine), which is currently marketed by Merck Sharp & Dohme.

A total of eighteen healthy male volunteers with a mean age of 21.6 years (range: 18–29 years) gave their written informed consent to participate in the study which was approved by the local Ethics Committee. The study was a two-way randomised cross-over with a one-week wash-out period. The subjects received a single 80-mg dose (*i.e.* four tablets) of famotidine in the morning after an overnight fast. Multiple venous blood samples were drawn into Venoject heparin-containing tubes over the following 24 h. Plasma famotidine concentration–time profiles were analysed for the area under the curve (AUC) by a non-compartmental method [7]. The peak plasma concentration (C_{max}) and time to reach the peak plasma concentration (t_{max}) were denoted from the individual plasma concentration–time profiles. The following pharmacokinetic parameters were calculated using standard procedures [7]: elimination half-life ($t_{1/2}$) and relative bioavailability (F_r).

RESULTS AND DISCUSSION

Assay development

At the start of this investigation, we attempted to use the published methods [4–6] to analyse famotidine in plasma. The SPE technique for plasma samples was carried out according to the procedures described by Vincek *et al.* [4] and Carlucci *et al.* [5]. Both published methods employed silica SPE columns to absorb the hydrophilic drug, famotidine, from the biological matrices. The results obtained in our laboratories (Table I) have shown that these methods could not be reproduced. Their procedures for SPE sample preparation gave low recoveries (<

TABLE I

SOLID-PHASE EXTRACTION (SPE) OF FAMOTIDINE

SPE column used[a]	Procedure[b]	Results found[c]
(a) Silica	– 1 ml methanol – 1 ml water – 1 ml plasma – 5 ml water – then eluted with 2 ml acetonitrile	– Recovery of famotidine was only 15% from standard sample in water – Blank plasma had an endogenous peak interfering with the drug.
(b) Silica	– As above but eluted with 2 ml of a mixture of trifluoroacetic acid–acetonitrile (1:99, v/v)	– Recovery of famotidine was increased to 80%. – However, there was an endogenous interfering peak from plasma coeluted with famotidine.
(c) Silica	– As (a) but eluted with 0.25 ml of a mixture of methanol–phosphate buffer, pH 3 (35:65, v/v)	– 96% of the drug was recovered from the standard sample in water but there was still an endogenous plasma peak coeluted with famotidine.
(d) C_{18}	– As in (a)	– Only 10% recovery, and the endogenous interfering peak also appeared.
(e) C_{18}	– As in (b)	– Similar to (d) but the recovery was increased to 85%.
(f) C_{18}	– As in (c)	– 95% recovery was achieved but there was an endogenous peak from plasma interfering with famotidine.

[a] SPE columns (3 ml) were purchased from Analytichem International (Harbor City, CA, USA).

[b] The experiments were performed in six replicates and followed the details of procedures described previously [4,5].

[c] Recovery of famotidine was performed using a standard famotidine (500 ng/ml) in water. The procedure to assess the absolute recovery was similar to the method which was described in the Experimental section (see *Recovery*). Drug-free plasma samples (*i.e.* blank plasma) were used to test the interference from plasma endogenous substances on famotidine peak in our chromatographic conditions described above.

15%), and it was subsequently discovered that famotidine was strongly retained by the SPE silica column. Reversed-phase C_{18} SPE columns (Bond-Elut) were also tested and gave similar results. The recovery of famotidine from the standard samples prepared in water was increased markedly by eluting the drug from the SPE column by either a mixture of trifluoroacetic acid–acetonitrile (1:99, v/v) or a mixture of methanol–phosphate buffer, pH 3 (35:65, v/v). The C_{18} SPE column appeared to be as good as the silica column. However, using the chromatographic conditions described in the Experimental section, these SPE procedures were not suitable for the determination of famotidine in plasma since there was an endogenous substance coeluting with the drug.

Failure of the above procedures to isolate famotidine from plasma led us to investigate alternative techniques, *i.e.* solvent extraction. Famotidine is a base with a pK_a of 6.7. The solubility of famotidine in many organic solvents including chloroform and methanol is low [4]. Diethyl ether and acetonitrile–ethyl acetate (3:2, v/v) and diethyl ether–chloroform–isopropanol (2:1:1, v/v) mixtures are the common solvents employed for the extraction of cimetidine and ranitidine from plasma [8,9]. However, famotidine was not extracted from plasma with these solvents. Likewise diethyl ether and a mixture of 20% isopropanol–diethyl ether were unable to extract famotidine from plasma samples. We then tried to duplicate the method employed by Inotsume *et al.* [6]. This method involves two solvent extractions, diethyl ether and ethyl acetate. Blank plasma samples (*i.e.* drug-free plasma) were analysed by their procedures and it was found that there was an endogenous plasma peak interfering with famotidine. In their method the plasma was alkalinized before extraction with diethyl ether in order to eliminate any interfering endogenous substances, followed by extraction of the drug with ethyl acetate. We therefore modified their methods by acidification of plasma samples with 100 μl of 4 *M* HCl before extraction with diethyl ether. This obviated the problem with the plasma endogenous peak interfering with famotidine.

Chromatographic behaviour

The C_{18} bonded stationary phase is the most popular used in reversed-phase HPLC. ODS Hypersil, which is a typical modern-capped octadecyl stationary phase material, was thus selected as the stationary phase packing for the analytical column. The microbore column (100 mm × 2 mm I.D.) was packed with this stationary phase material according to the manufacturer's recommendations (Shandon). The column efficiency was over 4000 plates per 10 cm. Even though a guard column was not used, the analytical column life-time was found to be approximately 400 injections with a carefully extensive washing with water and then methanol–water (50:50) after each run.

The concentration of organic modifier, acetonitrile, in the mobile phase, ranging from 5 to 20%, had a marked effect on the retention of famotidine. The retention time of famotidine was increased dramatically when the concentrations of acetonitrile in the mobile phase were lower than 10% (*i.e.* 5–10%). The pH and

ionic strength of the mobile phase also had a profound effect on the retention of this basic drug (pK_a 6.7) [4]. The retention time of famotidine was unreproducible when the mobile phase containing 10 m*M* Na_2HPO_4 buffer was used. An ionic strength of the mobile phase of at least 20 m*M* was found to be necessary to give a reproducible retention time for famotidine. If the ionic strength of the eluent was increased, the retention time of famotidine was decreased. The retention time of the drug was found to be increased as the pH of the mobile phase was varied from 3 to 6. All attempts to resolve famotidine from endogenous plasma components by manipulation of mobile phase variables proved futile.

Because of the failure of reversed-phase chromatography to resolve famotidine from endogenous plasma components, ion-pair HPLC was investigated. In this study retention of famotidine was achieved by the addition of the anionic hydrophobic pairing ion SDS to the mobile phase at pH 3. The organic modifier concentration in the mobile phase used was 12% and ionic strength was kept constant with 20 m*M* Na_2HPO_4. The retention time or capacity factor (k') of famotidine was varied as a function of the mobile phases SDS concentation in the range 0–150 m*M*, and it goes through the predicted maxima at an SDS concentration of 50 m*M*. The chromatographic behaviour of famotidine as a function of SDS concentration was similar to that observed with other basic drugs and can be adequately explained by the ion-exchange desolvation [10,11]. The retention time of the internal standard, clopamide, was not significantly affected by the variation in mobile phase SDS concentration. Consequently, a mobile phase of acetonitrile–water (12:88, v/v) containing 50 m*M* SDS and 20 m*M* Na_2HPO_4 adjusted to pH 3 was chosen as it provides good resolution between famotidine and the internal standard.

Fig. 1 shows chromatograms of blank plasma, plasma spiked with 5 ng/ml famotidine and a typical subject's plasma chromatogram 4 h post-dose. Under these chromatographic conditions, no endogenous sources of interference were observed and the resolution between famotidine and the internal standard (clopamide) was satisfactory. Blank plasma samples from more than 40 subjects were analysed and no plasma endogenous peaks coeluting with famotidine and the internal standard were detected. The elution sequence and retention time were: clopamide (the internal standard) 7.5 min and famotidine 12.5 min.

Recovery

The mean (± S.D.) recovery for famotidine (n = 5) from plasma samples was 86.7 ± 7.3% at 25 ng/ml and 79.9 ± 4.8% at 200 ng/ml. The recovery for the internal standard, clopamide, was 75.1 ± 5.8% (n = 5).

Linearity and precision

The standard curve for famotidine in plasma was linear over the concentration range 5–500 ng/ml with the square of the correlation coefficient (r^2) greater than 0.99. The typical linear relationship for the calibration curve can be expressed by

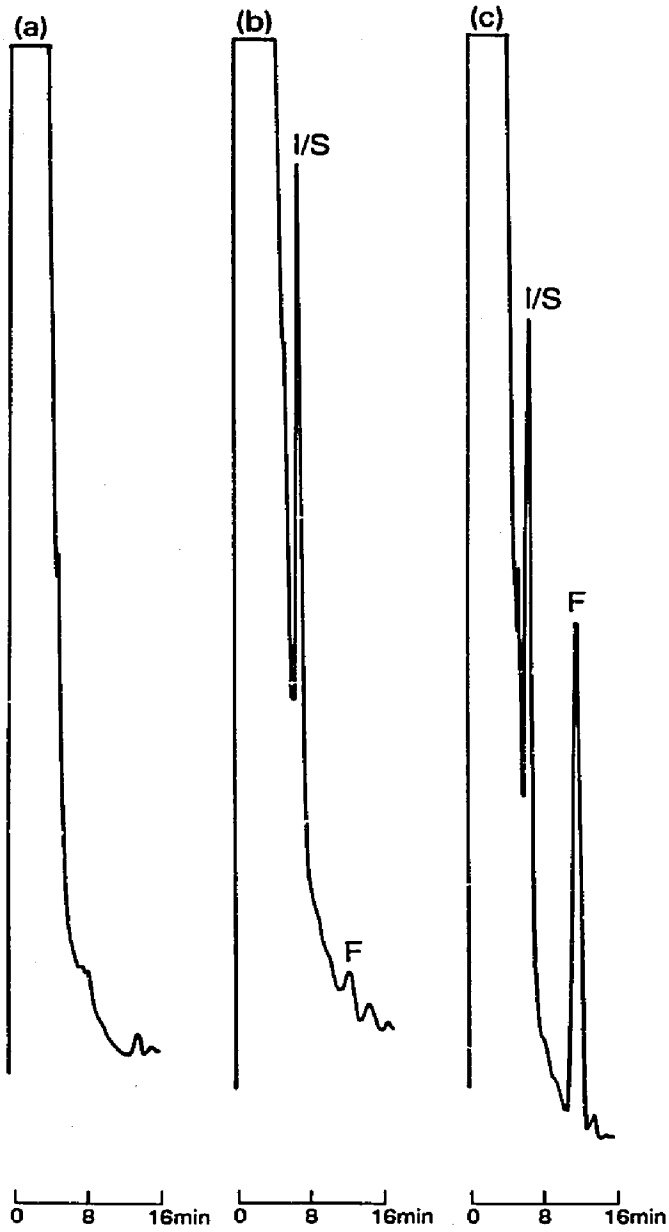

Fig. 1. Typical chromatograms of extracts of human plasma. (a) Blank plasma; (b) plasma spiked with 5 ng/ml famotidine; (c) plasma with 157 ng/ml famotidine from a healthy subject 4 h after an oral dose of 80 mg famotidine. Peaks: I/S = internal standard (clopamide); F = famotidine.

the equation: $y = 0.0047x$, where y is the peak-height ratio (drug/internal standard) and x is the plasma famotidine concentration. The intercepts (a) in all calibration curves were found to be statistically insignificant ($p > 0.1$) and were thus not included for the calculations. The day-to-day coefficient of variation (C.V.) of the slope of the calibration curves of famotidine was 7.3% ($n = 5$).

Standard plasma samples spiked with 5, 50 and 200 ng/ml famotidine were

TABLE II

PRECISION AND ACCURACY OF THE ASSAY FOR FAMOTIDINE: WITHIN-DAY REPRODUCIBILITY

Spiked concentration (ng/ml)	n	Observed concentration (mean ± S.D.) (ng/ml)	C.V (%)	Accuracy[a] (%)
5	6	5.6 ± 0.32	5.7	112
50	6	49.8 ± 4.1	8.2	99.6
200	5	204 ± 6	2.9	102

[a] Accuracy (%) = $\frac{\text{observed concentration}}{\text{nominal concentration}} \times 100$.

analysed by our method. The results of precision and accuracy of the assay (within-day reproducibility) are given in Table II. Within-day C.V. at all three concentrations studied were less than 9%, illustrating the precision of the method for routine purposes. To examine the reproducibility of the procedure further, between-day variation was assessed by re-analysing 36 samples obtained from the volunteers who participated in the clinical study. The results showed that the concentration values were reproducible and the results of any repeated determination fell within 15% of its original value.

To assess the accuracy of the assay, quality control samples were analysed each day of analysis along with the plasma samples from the subjects. The results of this assessment are shown in Table III. The concentration values of each quality control sample had an accuracy between 89 and 116%.

TABLE III

QUALITY CONTROL (QC) DATA

QC concentration[a] (ng/ml)	n	Observed concentration (mean ± S.D.) (ng/ml)	Accuracy[b] (%)	
			Mean	Range
16	8	16.6 ± 1.6	104	89–116
80	15	80.4 ± 6.3	101	91–112
160	11	155 ± 9	97	89–108
300	13	298 ± 12	99	108–104

[a] Three quality control samples were selected at each day of analysis and assayed with the subject plasma samples.

[b] Accuracy is defined in Table II.

TABLE IV

STABILITY OF FAMOTIDINE IN PLASMA SAMPLES AFTER STORAGE AT −70°C FOR THREE MONTHS

Sample[a]	*n*	Famotidine concentration[b] (ng/ml)	
		Fresh	Stored
S.W. (2 h)	6	372 ± 14	380 ± 15
J.W. (2 h)	5	169 ± 9	164 ± 13
J.W. (10 h)	5	24 ± 2	22 ± 2

[a] Plasma samples were obtained from two healthy volunteers, at the times specified in the parentheses, after an oral dose of 80 mg famotidine (Pepcidine).

[b] Results given are mean ± S.D. There was no significant difference between the fresh and the stored samples ($p > 0.3$).

Limit of detection

Six 5 ng/ml plasma famotidine standards gave observed values of 5.2, 5.6, 5.7, 6.1, 5.3 and 5.6 ng/ml (mean and S.D., 5.58 ± 0.32 ng/ml). The C.V. of the assay at this concentration was 5.7% (with an accuracy of 112%), which is much lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). A typical chromatogram of a 5 ng/ml plasma famotidine standard is presented in Fig. 1b. Thus the MQC or the detection limit for this assay was assigned at 5 ng/ml.

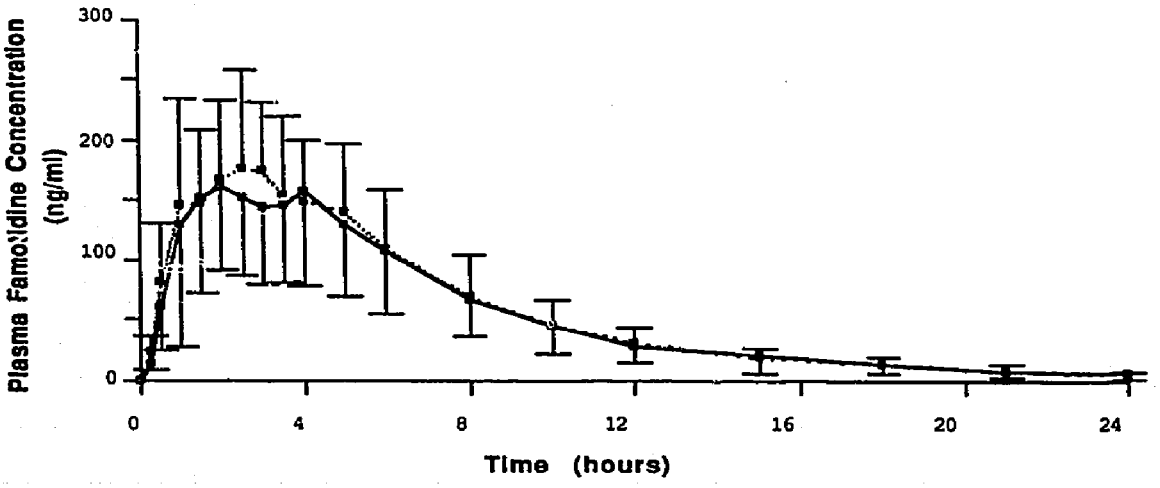

Fig. 2. Mean plasma concentrations of famotidine *versus* time profiles in eighteen healthy subjects after an oral administration of either an 80-mg famotidine formulation (Douglas Pharmaceuticals, ■.....■) or an 80-mg Pepcidine tablet (Merck Sharp & Dohme, ■———■). The vertical bars represent one standard deviation from the mean.

Stability in plasma

Plasma samples stored at −70°C for up to three months showed no signs of decomposition and practically the same concentration values were obtained (Table IV). This suggests that famotidine is stable under these storage conditions for at least three months.

Possible interference from other drugs

Possible interference by other drugs was evaluated. Tested drugs which did not interfere with the assay included aspirin, salicylic acid, paracetamol, cimetidine, ranitidine, diazepam, atenolol, lorazepam, labetalol, metoprolol, propranolol, chlorothiazide, cyclopenthiazide, frusemide, amiloride, indapamide, phenytoin and theophylline.

Pharmacokinetic application

The applicability of the present method was demonstrated by the determination of famotidine in plasma samples from eighteen subjects receiving oral doses of famotidine in a bioavailability study (Fig. 2). The sensitivity of the assay was such that famotidine concentrations in plasma could be quantitated for 24 h after a single oral dose of 80 mg of famotidine. Fig. 2 shows the mean plasma famotidine concentration–time profiles following a single oral dose of either 80 mg Pepcidine (a famotidine proprietary product) or 80 mg famotidine (a generic formulation of famotidine). The mean derived pharmacokinetic parameters and the relative bioavailability of the generic famotidine tablet (Douglas Pharmaceuticals) are summarized in Table V. The plasma samples at 24 h post-dose in most

TABLE V

DERIVED FAMOTIDINE PHARMACOKINETIC PARAMETERS AFTER A SINGLE 80-mg ORAL DOSE OF EITHER A GENERIC FAMOTIDINE TABLET (DOUGLAS PHARMACEUTICALS) OR A CURRENTLY MARKETED FAMOTIDINE TABLET, PEPCIDINE (MERCK SHARP & DOHME, MSD)

Results are given as mean ± S.D. (n = 18).

Pharmacokinetic parameter[a]	Famotidine tablet (Douglas Pharmaceuticals)	Pepcidine tablet (MSD)	ANOVA test
C_{max} (ng/ml)	219 ± 84	203 ± 90	$p > 0.3$
t_{max} (h)	2.6 ± 1.5	2.6 ± 1.3	$p > 0.2$
$t_{1/2}$ (h)	4.6 ± 1.4	4.3 ± 1.4	$p > 0.1$
$AUC_{0-\infty}$ (ng h/ml)	1391 ± 501	1327 ± 540	$p > 0.3$
F_r	1.05 ± 0.25	–	–

[a] C_{max} = peak plasma famotidine concentration; t_{max} = time to achieve the peak concentration; $t_{1/2}$ = elimination half-life; $AUC_{0-\infty}$ = area under the plasma concentration–time curve from 0 to infinity; F_r = relative bioavailability defined as $AUC_{Famotidine}/AUC_{Pepcidine}$.

of the subjects (fifteen out of eighteen) had plasma famotidine concentrations of greater than 5 ng/ml, *i.e.* higher than the detection limit. The C_{max}, t_{max}, AUC and $t_{1/2}$ obtained in this study were consistent with those of previous reports [1,3,6,12]. There were also no differences in these parameters between the two formulations ($p > 0.05$). The relative bioavailability of the new formulation investigated, *i.e.* famotidine tablet (Douglas Pharmaceuticals), was 105% of the proprietary product currently marketed, Pepcidine. Therefore, it can be concluded that 20-mg famotidine tablets (Douglas Pharmaceuticals) are bioequivalent to 20-mg Pepcidine tablets (Merck Sharp & Dohme).

CONCLUSION

In summary, the present HPLC method employing two solvent extractions before chromatographic separation enables the quantitation of famotidine in human plasma. This assay affords a sensitive and reproducible method for the determination of famotidine at concentrations observed in clinical study samples.

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