JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2001; **36**: 736–741

JMS

Determination of famotidine in low-volume human plasma by normal-phase liquid chromatography/tandem mass spectrometry

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Received 12 January 2001; Revised 27 March 2001; Accepted 29 March 2001

A rapid, sensitive and robust assay procedure using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) for the determination of famotidine in human plasma and urine is described. Famotidine and the internal standard were isolated from plasma samples by cation-exchange solid-phase extraction with benzenesulfonic acid (SCX) cartridges. The urine assay used direct injection of a diluted urine sample. The chromatographic separation was accomplished by using a BDS Hypersil silica column with a mobile phase of acetonitrile–water containing trifluoroacetic acid. The MS/MS detection of the analytes was set in the positive ionization mode using electrospray ionization for sample introduction. The analyte and internal standard precursor–product ion combinations were monitored in the multiple-reaction monitoring mode. Assay calibration curves were linear in the concentration range 0.5–500 ng ml^{-1} and 0.05–50 μg ml^{-1} in plasma and urine, respectively. For the plasma assay, a 100 μl sample aliquot was subjected to extraction. To perform the urine assay, a 50 μl sample aliquot was used. The intra-day relative standard deviations at all concentration levels were <10%. The inter-day consistency was assessed by running quality control samples during each daily run. The limit of quantification was 0.5 ng ml^{-1} in plasma and 0.05 μg ml^{-1} in urine. The methods were utilized to support clinical pharmacokinetic studies in infants aged 0–12 months. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: famotidine; infants; pharmacokinetics; liquid chromatography/tandem mass spectrometry; biological fluid

INTRODUCTION

Famotidine, 3-[({2-[(aminoiminomethyl)amino]-4-thiazolyl}methyl)thio]-*N*′-(aminosulfonyl)propanimidamide is a potent, competitive and reversible inhibitor of histamine action at the H_2 receptor. Famotidine has been approved to treat peptic ulcer disease, hypersecretory syndromes and gastroesophageal reflux disease (GERD) in adults and children 1–16 years of age.[1–3] However, there has been little published information regarding the use of famotidine in infants 0–12 months of age.

A number of assay procedures for the determination of famotidine in human biological fluids have been published. Liquid–liquid extraction and solid-phase extraction using a variety of cartridges, such as silica,[4] C_{18}[5] and SCX,[6] were employed to isolate famotidine from human plasma. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was utilized in these methods to separate further and quantify the analytes. To date, the most sensitive method published[6] had a limit of quantification (LOQ) of 1 ng ml^{-1} using 1 ml of plasma sample. In 1998, James *et al*. reported an assay with an LOQ of 7.81 ng ml^{-1} in plasma and 1.56 μg ml^{-1} in urine to support a pharmacokinetic/pharmacodynamic study in infants.[7] As the volume of plasma sample from infants was limited, it required the development of sensitive assay methods to investigate the pharmacokinetics of famotidine in this infant population.

This paper describes rapid and sensitive analytical methods to determine famotidine concentrations in plasma and urine. The plasma assay utilized solid-phase extraction for sample preparation. The urine assay was based on a direct injection of the urine sample premixed in the HPLC mobile phase. LC/MS/MS was employed to separate further and detect the analytes in the positive ionization mode using electrospray ionization (ESI) and monitoring their precursor–product ion combination in the multiple-reaction monitoring (MRM) mode. The methods were developed to support pediatric pharmacokinetic studies following administration of clinical doses of famotidine.

EXPERIMENTAL

Chemicals and reagents

Famotidine and the internal standard (IS), [4-({[2-(5-amino-4*H*-1,2,4,6-thiatriazin-3-yl)ethyl]thio}methyl)-2-thiazolyl] guanidine *S*,*S*-(thiatriazine)dioxide (see Fig. 1) were synthesized and purified at Merck.

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DOI: 10.1002/jms.176

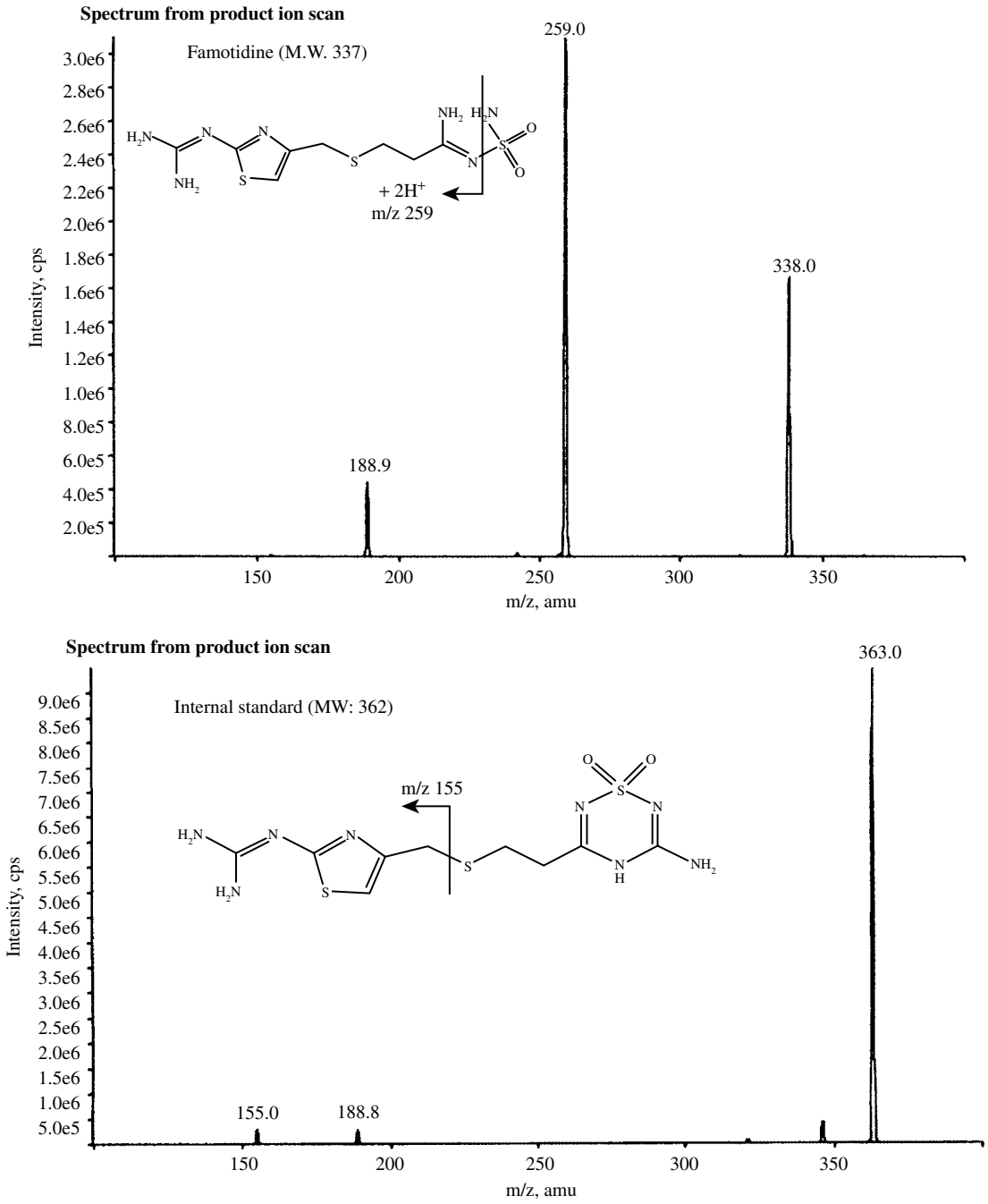

Figure 1. Product scan spectra of famotidine and the internal standard.

HPLC-grade acetonitrile and methanol were supplied by EM Science (Gibbstown, NJ, USA). Ammonia solution, formic acid (90%) and trifluoroacetic acid (TFA) were purchased from Fisher (Pittsburgh, PA, USA). Deionized water was HPLC filtered (>18 MΩ cm^{-1} resistivity) using a Milli-Q ultra-pure water system (Millipore, Bedford, MA, USA). All other reagents were of analytical-reagent grade. Control human plasma was purchased from Biological Specialty (Lansdale, PA, USA).

Instrumentation

The HPLC system consisted of two Perkin-Elmer (Norwalk, CT, USA) Series 200 micro pumps and a Perkin-Elmer ISS 200 autosampler equipped with a 20 µl sample loop (Alltech, Deerfield, IL, USA). The detection system was a Perkin-Elmer SCIEX API 3000 triple-quadrupole mass spectrometer interfaced with the HPLC system via a Turbo Ionspray source (Perkin-Elmer).

LC/MS/MS conditions

The chromatographic system consisted of a BDS Hypersil silica column (3 µm particle size, 50 × 3 mm i.d.) (Keystone Scientific, Bellefonte, PA, USA) with a mobile phase of acetonitrile–water (60 : 40, v/v) containing 0.1% TFA (the pH of the aqueous portion was adjusted to 2.75 with ammonia solution). The flow-rate was set at 0.2 ml min^{-1}. The MS/MS detection of the analytes was set in the positive ionization mode with a dwell time of 1000 ms for both famotidine

and the IS with the Turbo Ionspray interface maintained at 450 °C. Quantitation was performed by using MRM and monitoring their precursor–product ion combination of m/z 338 (MH^+) → 259 for famotidine and m/z 363 (MH^+) → 155 for the internal standard. The product ion scan spectra of famotidine and the IS are shown in Fig. 1. The collision potential ($Q_0 - RO_2$) was optimized at 15.5 V. The molecules were fragmented by collisionally activated dissociation (CAD) with N_2.

Preparation of working standard solutions and calibration curves

Standard solutions of famotidine were prepared in methanol. Famotidine working standard solutions chosen for the calibration curve were at eight concentration levels: 0.5, 1, 2, 5, 20, 100, 200 and 500 ng ml^{-1} for the plasma assay and 0.05, 0.1, 0.2, 0.5, 2, 10, 20 and 50 μg ml^{-1} for the urine assay. These solutions were stable at 4 °C and prepared weekly. A standard solution of the internal standard was prepared in methanol at 125 and 500 ng ml^{-1} for the plasma and urine assay, respectively. The solutions were stored at 4 °C until required. No degradation was observed under these conditions for a period of 4 months.

A daily plasma calibration curve was prepared by aliquoting 100 μl of control plasma into glass culture tubes, followed by the addition of 100 μl of each of the famotidine working standard solutions and 50 μl internal standard solution. This yielded calibration curve concentrations of famotidine in the range 0.5–500 ng ml^{-1}. A daily urine calibration curve was prepared by mixing 50 μl of control urine, 50 μl of each of the famotidine working standard solutions and 500 μl of internal standard solution into glass culture tubes. This yielded calibration curve concentrations of famotidine in the range 0.05–50 μg ml^{-1}. These standard samples were subjected to further sample preparation as described in the next section. The calibration curve was constructed by plotting the peak area ratio of famotidine to the internal standard (ordinate) versus famotidine concentration (abscissa). Peak area ratios were calculated using Perkin-Elmer SCIEX MacQuan software residing on an Apple Macintosh system. Famotidine concentrations in clinical plasma and urine samples were calculated from the equation $y = mx + b$ as determined by the $1/x$ and $1/x^2$ weighted linear regression analysis of the calibration curves, respectively.

Sample preparation

Plasma

A vacuum manifold (J. T. Baker) was used in the sample preparation procedure. A single solid-phase extraction with an SCX solid-phase cartridge was used to isolate the drug and IS from a 100 μl plasma sample buffered with 0.4% formic acid solution. The cartridge was conditioned by sequential elution of methanol, water and 0.4% formic acid solution. The buffered plasma sample was transferred to the cartridge under vacuum at 4 psi. Further clean-up was accomplished by additional elution of water and methanol. The analytes were eluted with 1% ammonia in methanol solution with centrifugation at 1000 rpm. The eluate was evaporated to dryness under N_2 for 20 min at 40 °C and the residue was reconstituted in 100 μl of acetonitrile–water (60 : 40, v/v) containing 0.1% TFA solution, then 30 μl were injected on to the LC/MS/MS system.

Urine

A 50 μl urine sample was aliquoted into 4.5 ml of acetonitrile–water (60 : 40, v/v) and mixed with 500 μl of IS standard solution; 50 μl of methanol–water were added to complete the mixture. A 5 μl volume of the sample mixture was injected on to the LC/MS/MS system.

Recovery

The absolute recovery from the plasma extraction procedure was determined at 1, 10 and 100 ng ml^{-1} for famotidine and at 25 ng ml^{-1} for the internal standard. The recovery was obtained by comparing the absolute peak area of the analyte from plasma extracts before and after the extraction procedure.

Urine recovery was obtained through the comparison of absolute peak area with and without added control urine over the calibration curve range.

Clinical study

The assay methods were applied to support clinical pharmacokinetic studies. Prior to the analysis of clinical samples, quality control (QC) samples were prepared at concentration levels of 1, 40 and 400 ng ml^{-1} in plasma and 0.1, 4 and 40 μg ml^{-1} in urine. The QC samples were stored at −70 °C. Two sets of these QC samples were then assayed daily along with the clinical samples.

RESULTS AND DISCUSSION

LC/MS/MS conditions

A polar compound such as famotidine is difficult to assay using typical reversed-phase chromatography because of the early elution of polar compounds under such conditions. Because of the extremely high polarity of famotidine, the analyte elutes with the solvent front under various reversed-phase conditions. Prior assay[6] by reversed-phase HPLC involved two-dimensional chromatography with the use of an ion-pair reagent to improve analyte retention and assay sensitivity. Normal-phase chromatography has long been successfully utilized to assay polar compounds, ranging from small drug molecules such as clomipramine[8] and chloroquine[9] to large molecules such as cholesterol,[10] in biological fluids. In order to retain the analyte better to achieve the desired separation and to increase the sample throughput, normal-phase chromatography coupled with MS/MS detection was considered. Famotidine and the IS were well retained on the BDS Hypersil silica stationary phase. The capacity factor of famotidine was >3.5. The chromatographic resolution of famotidine and the IS on the silica stationary phase was easily optimized using modifiers compatible with the MS/MS interface. Two acids, formic acid and TFA, were tested during method development. TFA had a better effect on peak shape and column capacity factor (k'). As shown in Fig. 2, k' decreased as the TFA concentration increased. A concentration of 0.1% TFA was adopted considering the reasonable k' and peak shape. The

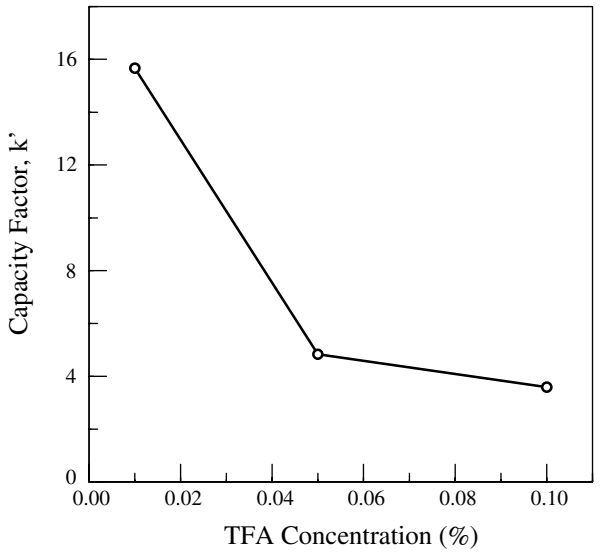

Figure 2. Effect of trifluoroacetic acid concentration on column capacity factor, k'.

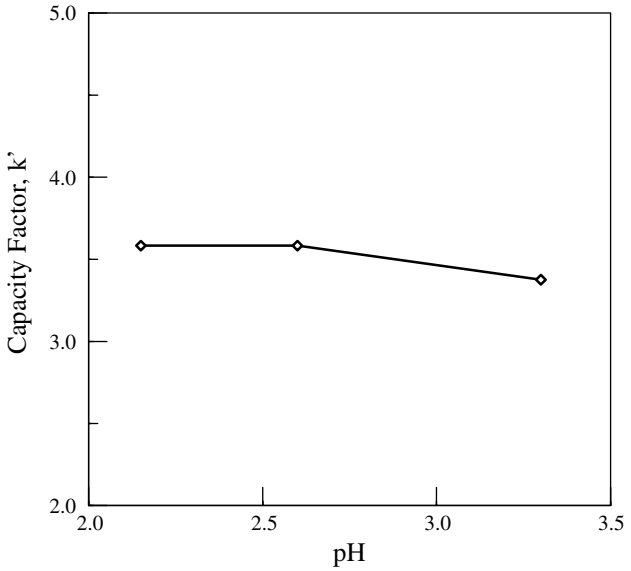

Figure 3. Effect of pH on column capacity factor, k'.

apparent pH of the mobile phase did not have a significant effect on k' (Fig. 3). An aqueous pH of 2.75 was selected to help maintain the stability of the silica stationary phase. The chromatographic conditions selected were compatible with the sample processing procedures, both precluding the use of non-volatile reagents. The retention time of famotidine and the IS remained reproducible among columns with different lots of silica stationary phase. The silica column was stable for at least 300 injections.

Extraction recovery

For the plasma assay, cartridges with a strong cation-exchange functional group, such as SCX, provided sufficient separation and relatively high recovery. The ionic interactions between famotidine and benzenesulfonic acid allowed the use of various solvents with different polarities to wash out endogenous material.

Following the solid-phase extraction and evaporation, the final reconstitute should be weaker than the HPLC mobile phase. For normal-phase chromatography, increasing organic concentration makes a weaker solvent. Hence the reconstituted solution adopted consisted of 80% acetonitrile and 20% of water containing 0.1% TFA.

The recovery of the plasma assay averaged 62% for famotidine over the calibration curve range and 63% for the IS. The urine assay showed a recovery of 102% for famotidine and 108% for the IS.

Assay specificity

Figure 4 shows typical chromatograms of control plasma (A) and a plasma extract containing 0.5 ng ml^{-1} famotidine and 125 ng ml^{-1} IS (B). Representative urine extract chromatograms for control urine (A) and a urine extract containing 0.050 μg ml^{-1} famotidine and 5 μg ml^{-1} IS (B) are presented in Fig. 5. None of the predose or control plasma

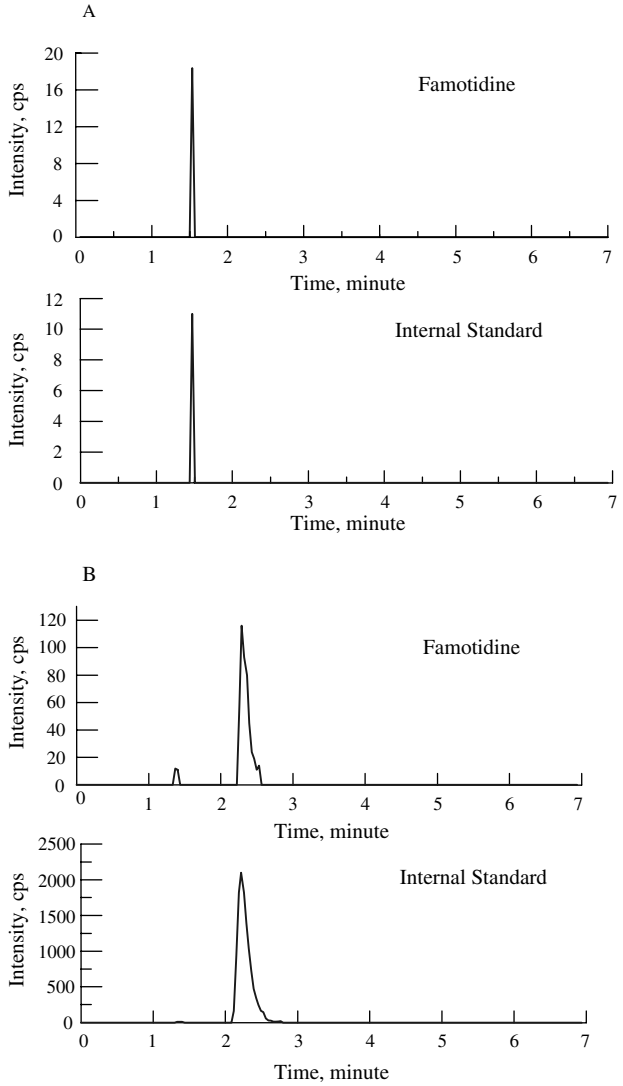

Figure 4. Representative chromatograms of plasma extracts by multiple reaction monitoring at m/z 338 → 259 for famotidine and m/z 363 → 155 for the internal standard (IS). (A) Control human plasma; (B) plasma spiked with 0.5 ng ml^{-1} of famotidine and 125 ng ml^{-1} of IS.

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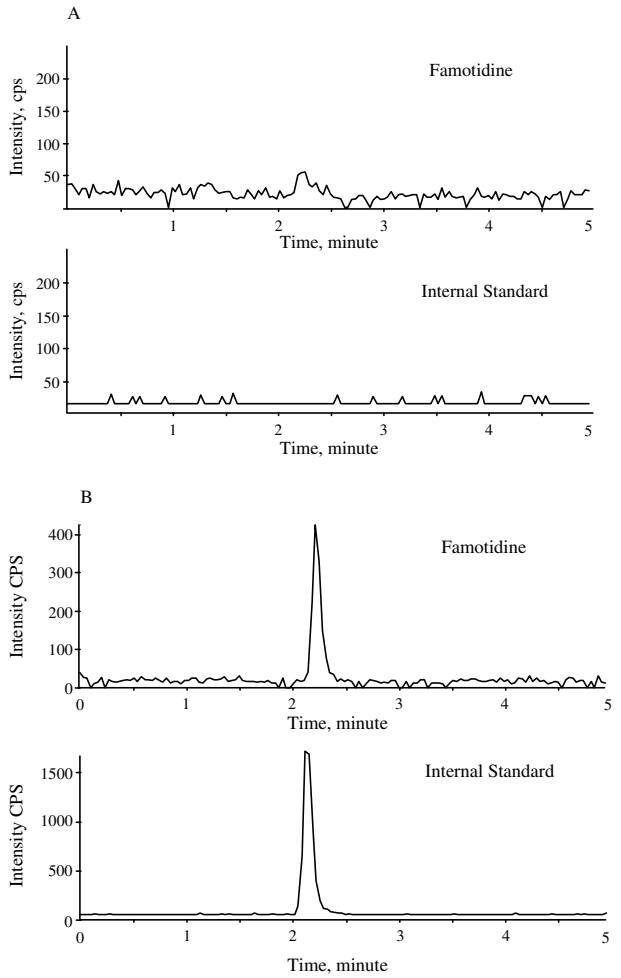

Figure 5. Representative chromatograms of urine extracts by multiple reaction monitoring at m/z 338 → 259 for famotidine and m/z 363 → 155 for the internal standard (IS). (A) Control human urine; (B) urine spiked with 0.050 μg ml^{-1} of famotidine and 5 μg ml^{-1} of IS.

and urine samples analyzed contained a significant interference in the retention time range of interest.

In the clinic, certain subjects received ranitidine concomitantly with famotidine. Experiments were conducted to determine any effect that the presence of ranitidine might have on the assay performance. The results indicate that the two analyte peaks were baseline separated under the current chromatographic conditions. The assay selectivity was further enhanced by the mass channel for ranitidine, which was different from that for famotidine.

Linearity, precision and accuracy

The weighted least-squares regression Calibration curve was linear over the concentration ranges 0.5–500 ng ml^{-1} and 0.05–50 μg ml^{-1} for plasma and urine assay, respectively. Replicate standards ($n = 5$) were analyzed to assess the within-day variability of the assay. The precision, expressed as relative standard deviation (RSD), and accuracy, expressed as percentage deviation from the theoretical value, are listed in Table 1 for the plasma assay and Table 2 for the urine assay.

Table 1. Intra-day precision and accuracy data for the determination of famotidine in plasma as assessed by the replicate ($n = 5$) analysis of standards

Nominal concentration (μg ml^{-1})	Mean concentration found[a] (μg ml^{-1})	Precision, RSD (%)	Accuracy[b] (%)
0.5	0.44	5.3	88.6
1	0.99	3.8	99.3
2	2.11	3.6	105.5
5	4.97	1.4	99.5
20	21.40	4.5	107.0
100	102.02	5.3	102.0
200	195.96	3.9	98.0
500	500.61	2.9	100.1

[a] Mean concentrations calculated from the weighted ($1/x$) linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as 100× (mean calculated concentration)/(nominal concentration).

Table 2. Intra-day precision and accuracy data for the determination of famotidine as assessed by the replicate ($n = 5$) analysis of standards spiked in human urine

Nominal concentration (ng ml^{-1})	Mean concentration found[a] (ng ml^{-1})	Precision, RSD (%)	Accuracy[b] (%)
0.05	0.050	2.5	99.7
0.1	0.097	4.0	96.9
0.2	0.21	3.7	106.6
0.5	0.51	4.8	101.4
2	2.1	2.7	105.5
10	10.1	2.0	101
20	19.5	5.8	97.7
50	46.0	7.0	92

[a] Mean concentrations calculated from the weighted ($1/x^2$) linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as 100× (mean calculated concentration)/(nominal concentration).

The limit of quantification was 0.5 ng ml^{-1} for the plasma assay and 0.05 μg ml^{-1} for the urine assay.

Quality control samples at low (1 ng ml^{-1} for plasma, 0.1 μg ml^{-1} for urine), medium (40 ng ml^{-1} for plasma, 4 μg ml^{-1} for urine) and high (400 ng ml^{-1} for plasma, 40 μg ml^{-1} for urine) concentrations were prepared prior to the start of the study and subjected to replicate ($n = 5$) within-day analysis. Two sets of QCs at each of the three concentration levels were analyzed daily over a period of 4 months to assess the inter-day precision. The results are shown in Table 3. Analysis of clinical samples was considered valid when two-thirds, but not from the same concentration, of the QCs had a <20% deviation from either the nominal or the day-zero concentration. The overall inter-day variability of the assay, as measured by the RSD, is

Table 3. Inter-day variability of the plasma and urine assay as assessed by the RSD of quality control samples at three concentration levels

Assay	Nominal concentration	Mean ($n = 24$) concentration found	RSD (%)
Plasma[a]	1	1.0	11.1
	40	39.1	8.4
	400	389.7	5.1
Urine[b]	0.1	0.11	11.9
	4	4.1	6.0
	40	35.7	9.7

[a] Plasma concentrations in ng ml^{-1}.
[b] Urine concentrations in μg ml^{-1}.

Table 4. Representative famotidine urinary recovery from subject A, a 35-day-old neonate weighing 3.1 kg, following intravenous administration of famotidine at 0.5 mg kg^{-1}

Sampling time (h)	Concentration (ng ml^{-1})	Collection volume (ml)	Urinary excretion (μg)
0–4	4814.3	80	385.2
4–8	21862.8	8	174.9
8–12	9499.5	28	266.0
12–24	1313.3	233	306.0
24–36	430.2	195	83.9

<12%. These data also indicate that famotidine is stable in plasma and urine stored at −70 °C for at least 4 months.

Table 4 exhibits the recovery of famotidine in urine for a representative infant following intravenous administration of famotidine at 0.5 mg kg^{-1}. The corresponding plasma profile and a profile from a representative subject following administration of an oral suspension of famotidine at 0.5 mg kg^{-1} are shown in Fig. 6.

CONCLUSION

Normal-phase chromatography interfaced with MS/MS and solid-phase extraction with SCX cartridges offers significant advantages in the determination of famotidine in low-volume human plasma. The urine assay requires very little sample preparation. The high-throughput methods are robust and sensitive. The limit of quantification in plasma is improved to 0.5 ng ml^{-1} using 100 μl of plasma sample, which represents a 20-fold increase in comparison with the published values. Both methods have been successfully utilized in a multi-center pediatric pharmacokinetic study.

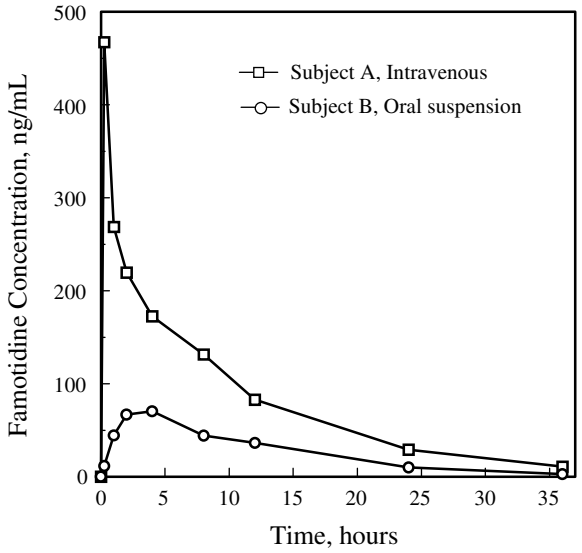

Figure 6. Representative famotidine plasma profiles from subject A, a 35-day-old neonate weighing 3.1 kg, following intravenous administration, of famotidine at 0.5 mg kg^{-1} and subject B, a 6-day-old neonate weighing 2.8 kg, following oral suspension administration of famotidine at 0.5 mg kg^{-1}.

Acknowledgement

The authors thank Dr Larissa Wenning of Merck Research Laboratories, Department of Drug Metabolism, for providing the patient and sampling information for these studies.

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