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Improved selectivity in detection of polar basic drugs by liquid chromatography–electrospray ionization mass spectrometry Illustration using an assay method for the determination of famotidine in human plasma

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Abstract

It is well to assume that bioanalytical chromatographic methods for the determination of polar basic drugs are developed and optimised according to a standardised procedure which involves two alternatives: (a) modifications in the sample preparation procedures, and (b) changes in the stationary phase of the chromatographic system. In this paper, a simple and rapid chromatographic procedure using a specific analytical detection method (ESI tandem mass spectrophotometric detection) in combination with a fast and efficient sample work-up procedure, protein precipitation, is presented. A demonstration of the entire chromatographic procedure is given for an HPLC method for the determination of famotidine in human plasma, a basic polar drug with poor solubility in organic solvents. In order to optimize the mass detection of famotidine, several parameters such as ionization mode, fragmentor voltage, m/z ratios of ions monitored, type of organic modifier and eluent additive, were investigated. Each analysis required 5 min. The calibration curve of famotidine in the range 1–200 ng/ml was linear with a correlation coefficient of 0.9992 ($n=6$), and a detection limit a signal-to-noise ratio of 3 was ~0.2 ng/ml. The within- and between-day variations in the famotidine analysis were 5.2 ($n=6$) and 6.7% ($n=18$), respectively. The applicability of this method was also demonstrated for the analysis of plasma samples in a Phase-I human pharmacokinetic study.

Keywords: Polar basic drugs; Famotidine

1. Introduction

In this study we try to evaluate if the combination of a simple extraction procedure with a specific analytical method (liquid chromatography with mass spectrometry as detection system) represents a good

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alternative to specific and complicated sample extraction procedures employed for the determination of polar drugs at low concentrations. In order to evaluate the viability of this combination famotidine, a polar molecule, has been elected as drug model.

Famotidine (Fig. 1) is a potent histamine H_2-receptor antagonist which differs structurally from its predecessors cimetidine and ranitidine in having a thiazole rather than an imidazole or furan nucleus. In humans, famotidine defectively inhibits basal and stimulated gastric acid secretion and has no apparent clinically significant activity at histamine H_2-receptors outside the gastrointestinal tract [1]. The recommended famotidine dose to achieve those gastrointestinal effects is very low (40 mg daily), and these doses produced peak plasma concentrations of ~75–100 ng/ml. As these doses produce very low therapeutic concentrations in plasma, sensitive methods are required in order to determine famotidine concentrations in samples from clinical studies and particularly in bioequivalence single-dose phase I studies.

Only a few high-performance liquid chromatographic (HPLC) methods have been reported for the determination of famotidine in biological fluids. Three methods [2–4] employed liquid–liquid extraction procedures of plasma samples for elimination of endogenous plasma interference and preconcentration followed by a reversed-phase HPLC analysis. The other HPLC methods involved solid-phase extraction procedures with [5] and without automated column switching [6–10]. However, these methods were not suitable under all conditions [7]. The first method requires labourious and time-consuming clean-up of the sample, and, nevertheless, in most cases endogenous peaks co-eluted with famotidine. The second method shows high variations in famotidine recovery, since the characteristic physicochemical properties of this drug. Famotidine is a base with a pK_a of 6.7 and low solubility in many organic solvents as chloroform, methanol and ether [6]. However, despite the different solid-phase extraction columns employed by many authors, the elution of famotidine from solid-phase extraction columns is low and highly variable. In fact, detection limits below 5 ng/ml are achieved with great difficulties.

2. Experimental

2.1. Chemicals, reagents and solutions

Famotidine and ranitidine (I.S.) (Fig. 1) were

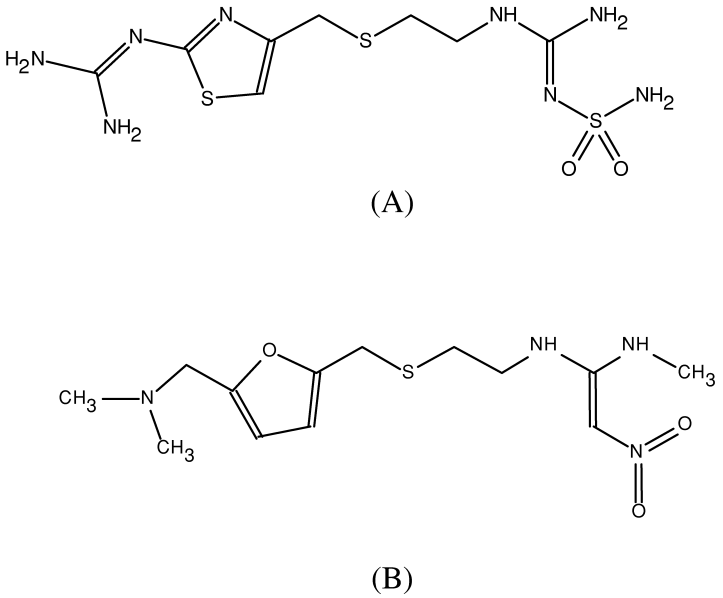

Fig. 1. Chemical structures of famotidine (A) and ranitidine (B).

provided by CINFA (Olaz, Spain). Formic acid 99% was purchased from Aldrich (Barcelona, Spain). Methanol (HPLC grade) and acetic acid were obtained from Merck (Darmstadt, Germany). Ammonium acetate RPE-ACS was obtained from Carlo Erba (Milan, Italy).

2.2. Standard solutions and samples

Stocks solutions of famotidine and ranitidine, with a concentration of 1 mg/ml, were prepared separately by dissolving 10 mg of each drug in methanol. Eight standard solutions of famotidine (0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1 and 2 μg/ml) were made by further dilution of the stock solution with appropriate volumes of methanol. The standard solution of ranitidine (0.5 μg/ml) was similarly prepared. Standard and stock solutions of famotidine and ranitidine were stored at 4°C.

2.3. Sample preparation

Plasma samples (1 ml) were spiked with the I.S. (25 μl of 0.5 μg/ml of ranitidine). Blank plasma samples were also spiked with the famotidine solutions mentioned above to reach the concentration range of 1–200 ng/ml. After addition of 2 ml methanol, the samples were centrifuged at 2000 *g* for 20 min at −2°C. A 10-μl aliquot of the supernatant was injected onto the HPLC column.

2.4. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Model 1100 series LC coupled with an atmospheric pressure (AP)-electrospray ionisation (ESI) mass spectrometer (Hewlett-Packard, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett-Packard computer using the CHEMSTATION G2171 AA programme.

Separation was carried out at 50°C on a reversed-phase, 250×4 mm base stable column packed with 5 μm C_{18} silica reversed-phase particles (Tracerkromasil 100). This column was obtained from Teknokroma (Barcelona, Germany). Mobile phases were: (A) methanol–1% formic acid (24:76, v/v); and (B) methanol–50 m*M* ammonium acetate with 1% acetic acid (24:76, v/v). Separation was achieved by isocratic solvent elution at a flow-rate of 1 ml/min.

ESI-MS conditions were as follows:

Ionization mode	ESI, positive Selected ion monitoring (SIM)
Ions (m/z)	
Famotidine	338.0
Ranitidine	315.0
Interface variables	
Temperature	350°C
Drying gas	N_2 (12.5 l/min)
Nebulizer gas	N_2 (55 p.s.i.; 1 p.s.i.=6894.76 Pa)
Capillary voltage	4000 V
Fragmentor voltage	55 V

Optimization of the interface variables, such as gas flows and voltages was done manually during direct infusion of 10 μg/ml separate solutions of the target analytes dissolved in methanol. Mass spectra are presented in Figs. 2 and 3 for famotidine and ranitidine, respectively.

2.5. Validation

The method was validated by analysis of human plasma quality control samples prepared at four concentrations spanning the calibration range. Three samples of each quality control pool and calibration samples were analysed on 6 different days. On day 1 the number of samples of quality control was six. Precision and accuracy was determined. Precision of a method is expressed as the percentage of the relative standard deviation (RSD) of replicate measurements. Accuracy was measured according to the following equation

$$\text{Percentage difference from theoretical value} = \left[\frac{X - C_T}{C_T}\right] \cdot 100$$

where X is the determined concentration of a quality control and C_T is the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

The selectivity of the assay was determined by the

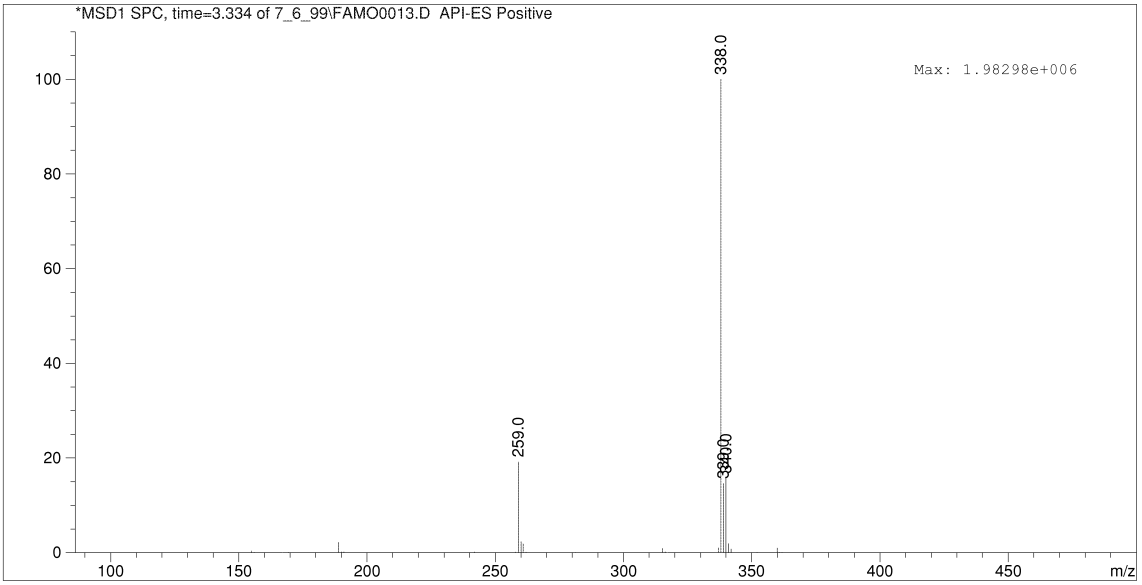

Fig. 2. ESI$^+$ spectra obtained by LC–MS of 10 μg/ml famotidine.

individual analysis of blank samples. The retention times of endogenous compounds in the matrix were compared with those of famotidine and ranitidine.

LOD was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration which can be determined with an accuracy and precision $<$20%. In this work LOD of the assay method was determined by analysis of the peak baseline noise in ten blank samples.

The stability of famotidine in both frozen plasma (−70°C) over 3 months, and in processed samples

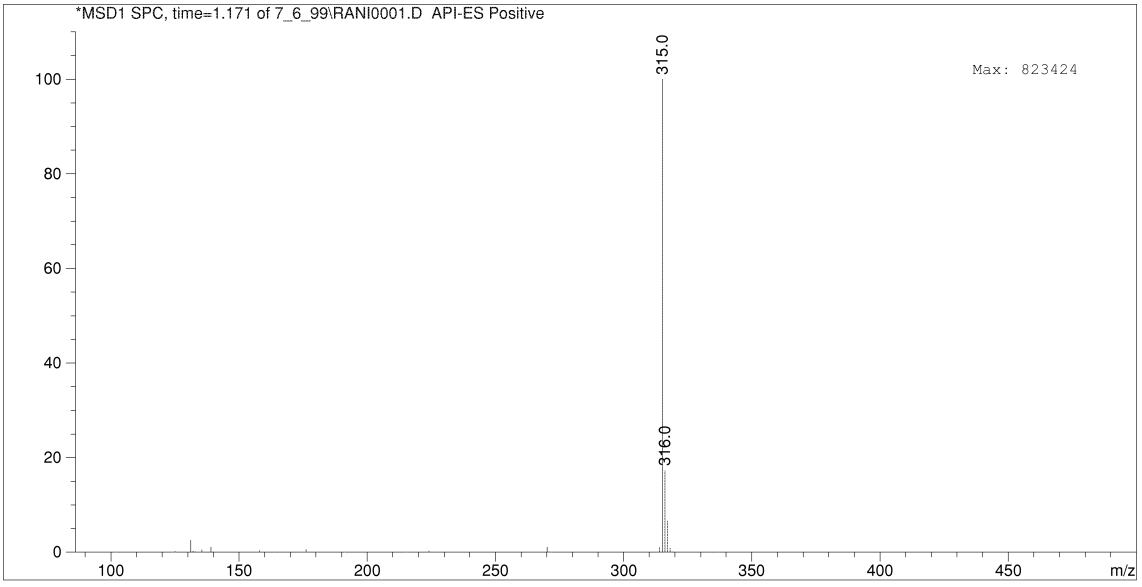

Fig. 3. ESI$^+$ spectra obtained by LC–MS of 10 μg/ml ranitidine.

left at room temperature (20±3°C) over 24 h, was also studied.

2.6. Quantitation

Each calibration curve consisted of 8 calibration points (1, 2, 5, 10, 0.2, 25, 50, 100 and 200 ng/ml). Calibration curves were determined by least square linear regression analysis (weighting $1/\chi^2$). Peak area ratio of famotidine and ranitidine versus the corresponding famotidine concentration was plotted. The linearity of the method was confirmed by comparing the slopes, the intercepts of calibration curves with zero and the correlation coefficients with 1. Moreover, a Student's *t*-test was used to compare the back-calculated concentrations with each calibration curve versus the nominal ones.

2.7. Application of the method

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. Thirty healthy volunteers received a single oral dose of famotidine (40 mg). Venous blood samples were withdrawn in heparinized tubes and plasma fractions were separated immediately prior to dosing and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16 and 24 h after drug administration. Plasma samples were stored frozen (−20°C until analysis).

3. Results and discussion

HPLC–MS has grown into one of the most powerful analytical techniques currently available. Among its advantages, HPLC–MS technology has provided a high level of sensibility and selectivity previously not available, especially for weak ultraviolet absorbing compounds. Therefore, with on-line HPLC–MS it is possible to analyze non-volatile, high-polar compounds in biological samples, which cannot be dealt with effectively by conventional GC and HPLC. One of these compounds is famotidine. At the start of this investigation, we attempted to use the published methods [2–10] to analyze famotidine in plasma with ultraviolet detection. However, the results obtained in our laboratories showed that these methods could not be reproduced. Liquid–liquid extraction procedures with ethyl acetate of plasma samples previously purified with diethyl ether, or *t*-butyl methyl ether gave highly variable recoveries (50–80%). The solid-phase extraction procedures for sample preparation also gave low recoveries (<50%) in most cases, irrespectively of the cartridge type (silica or C_{18} SPE) used. In addition, these extracts contained much endogenous interferences, which could not be resolved from famotidine using a variety of normal- or reversed-phase columns. A combination of liquid–solid-phase extraction was also evaluated, and yielded similar results. In this paper we have demonstrated that HPLC–ESI-tandem MS detection can facilitate the development of quick, automated, and highly reproducible analytical methods for the determination of low concentrations of polar drugs in biological samples.

The methodology described in this paper was developed to provide a simple and fast famotidine determination in human plasma for a routine assay in large sample series, i.e. hundreds of samples analysed in sequential series, for pharmacokinetic purposes.

3.1. Mass spectrometry conditions

The response obtained in the mass spectrometer depends on the compound, i.e. how easily it is ionized, the amount of organic solvent and the nature and concentration of buffer used in the mobile phase. Famotidine is a weak base with a pK_a of 6.7. The alkaline characteristics of this molecule are due to the presence of amine functional groups in the structural chain. These primary amines are fully ionized when pH<4, yielding positive ions. Therefore, positive electrospray ionization is the best option to obtain a high sensitivity.

The level of sensitivity in HPLC–MS is related to the percentage of organic content in the mobile phase solvent. As the mobile phase organic concentration increases (relative to water) the signal-to-noise ratio of polar compounds increases. In this work methanol was selected as organic modifier since in a positive mode, methanol generally gave stronger signals than acetonitrile (10–20% higher with methanol [11]).

Electrolyte additives are included into mobile phase to facilitate the ionization of basic compounds. In positive electrospray ionization mode volatile acids are usually used as electrolyte additives. To study the effect of the nature and concentration of volatile acids in the analytical response of famotidine, two test eluents were prepared, one with formic acid and other with ammonium acetate and acetic acid (added to pH adjust below 4). The concentration range studied was 1–250 m*M*. The results obtained showed that in a positive mode, the peak intensities observed with ammonium acetate were 80% lower than those achieved with formic acid at the same concentration. In general, the increase of the concentration of the buffer additives would lead to a decreased analyte response in both positive and negative electrospray ionization [12]. The intensity of this effect is different with each type of buffer employed. With ammonium acetate, the intensity of the response decreased 60% as the concentration changed from 1 to 100 m*M* (Fig. 4). However, with formic acid the situation was somewhat different, since the response shows a slight increase when the concentration of the formic acid increased (Fig. 4).

3.2. Sample preparation

The design of a sample preparation method is heavily dependent on the context in which it is intended to be used. Since we intended to analyse series containing 150–200 samples from clinical trials, speed and simplicity are critical issues. In this way, protein precipitation can provide the speed and simplicity necessary to make this kind of sample series possible. However, the outcome extracts contained many endogenous interferences that must be

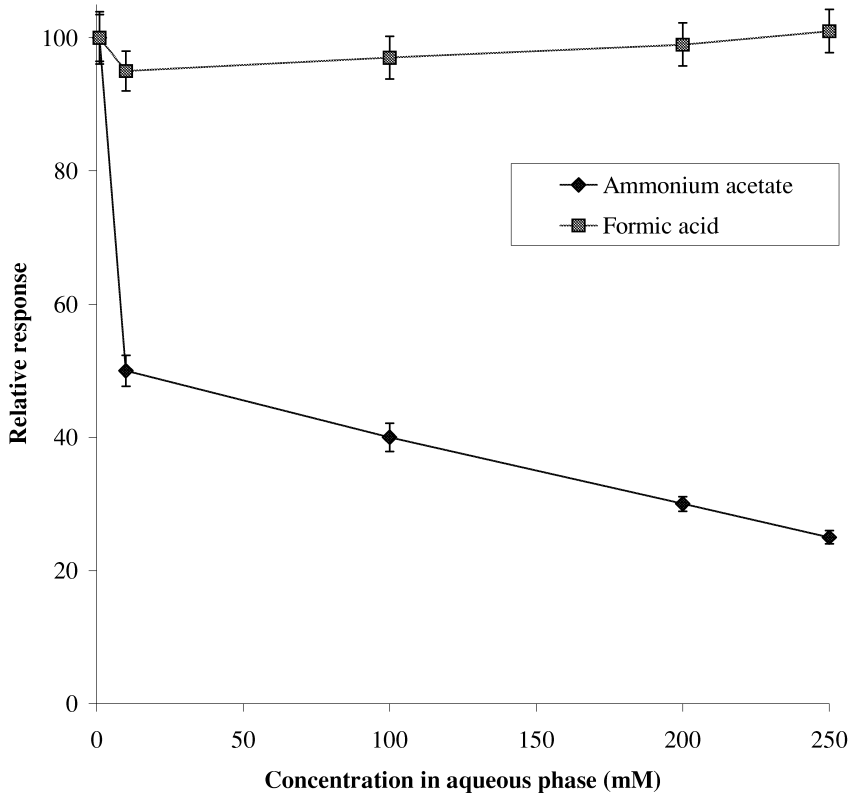

Fig. 4. Comparison of the relative response shown by famotidine with varying concentrations of formic acid, or ammonium acetate in a methanol–water based eluent.

avoided. In this work, we have exclusively used the potential of HPLC–MS to achieve this goal.

In preliminary studies, famotidine gave a very simple ESI mass spectrum under standard operation conditions (see Experimental). A strong signal corresponding to m/z 338 $[M+H]^+$ was observed in the mass spectrum. Other minor peak appears at m/z 259 $[(M-SO_2NH_2)+H]^+$ (Fig. 2). In order to optimize the extraction procedure of famotidine several parameters, such as type and volume of solution precipitant, centrifugation speed and type of electrolyte additive were investigated by monitoring of the ionic current of m/z 258 and 338 in the SIM mode. The best results were obtained using methanol as a precipitating agent and formic acid as a mobile phase additive. In contrast to ammonium acetate–acetic acid, formic acid decreases the signal intensity of protein extract. This can be explained by two approaches: (a) it has been shown that better ESI sensitivity is attainable with acetic acid when proteins were analyzed by HPLC–MS [13], and (b) a competition of extract ions and hydronium ions in the conversion process from solution to gas phase ions was observed at formic acid concentrations higher than 25 m*M* [14].

Moreover, it is interesting to note that the baseline mass spectrum showed ions with lower m/z ratio (below m/z 275) when formic acid was used, in contrast with the observed when ammonium acetate, or acetic acid were utilised as additive (see Fig. 5). These results can be explained by its high capacity to increase the ionisation of the sample, independently of fragmentor voltage or other MS parameters.

3.3. Liquid chromatography

Changing methods from ultraviolet to ESI-MS detection is rarely straightforward because conventional eluents usually contain buffers and other additives to mediate the separation and improve chromatographic performance. Typical mobile phase additives such as phosphoric acid and phosphate salts are not suitable for ESI-MS since they are deposited in the ion source, resulting in decreased ion transmission and eventual blockage of bores which conduct the ions to the mass analyzer [15,16].

On the other hand, the retention and chromatographic performance of drugs is strongly modified by the mobile phase additives used in ESI-MS. Among the three different additives tested, acetic acid and formic acid resulted in most pronounced band broadening with peak widths at half height 70–100% larger than those obtained with ammonium acetate. Although the peak width in ESI-MS detection also depends on the data acquisition rate [17], the first approach to optimize the chromatographic performance involves the variation of the percentage of organic modifier in the mobile phase. If organic modifier manipulations cannot provide the retention and selectivity needed, the remaining route is the change of stationary phase. This last procedure is tedious since it has to test more than 30 HPLC columns in most cases [18]. However, the optimization of chromatographic performance under the aforementioned procedure did not need to be done since famotidine has both different mother ion and product ion that blank plasmatic extracts.

The next step was the selection of an appropriate I.S. Contrary to what happens in UV chromatography, the I.S. can be eluted at the same retention time as the analyte, provided that the mother ion, or one of product ions of this molecule have different m/z values to the ion selected for quantification of analyte in the SIM mode. In this work, ranitidine was selected as I.S. since it gave a very simple ESI mass spectrum under the operation conditions with a signal to m/z 315 $[M]^+$, and other minor peak to m/z 316 $[M+H]^+$, (Fig. 3).

Figs. 6 and 7 shows the ion chromatograms of human plasma obtained from a pharmacokinetic bioequivalence study. Retention times of the two compounds were approximately as follows: ranitidine 1.86 min; famotidine 1.87 min.

3.4. Assay performance

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, LOQ, stability and applicability in pharmacokinetic studies.

The assays exhibited linearity between the response (y) and the corresponding concentration of famotidine (x), over the 1–200 ng/ml range in the samples. Results are presented in Table 1. For each point of calibration standards, the concentrations were back-calculated from the equation of the regres-

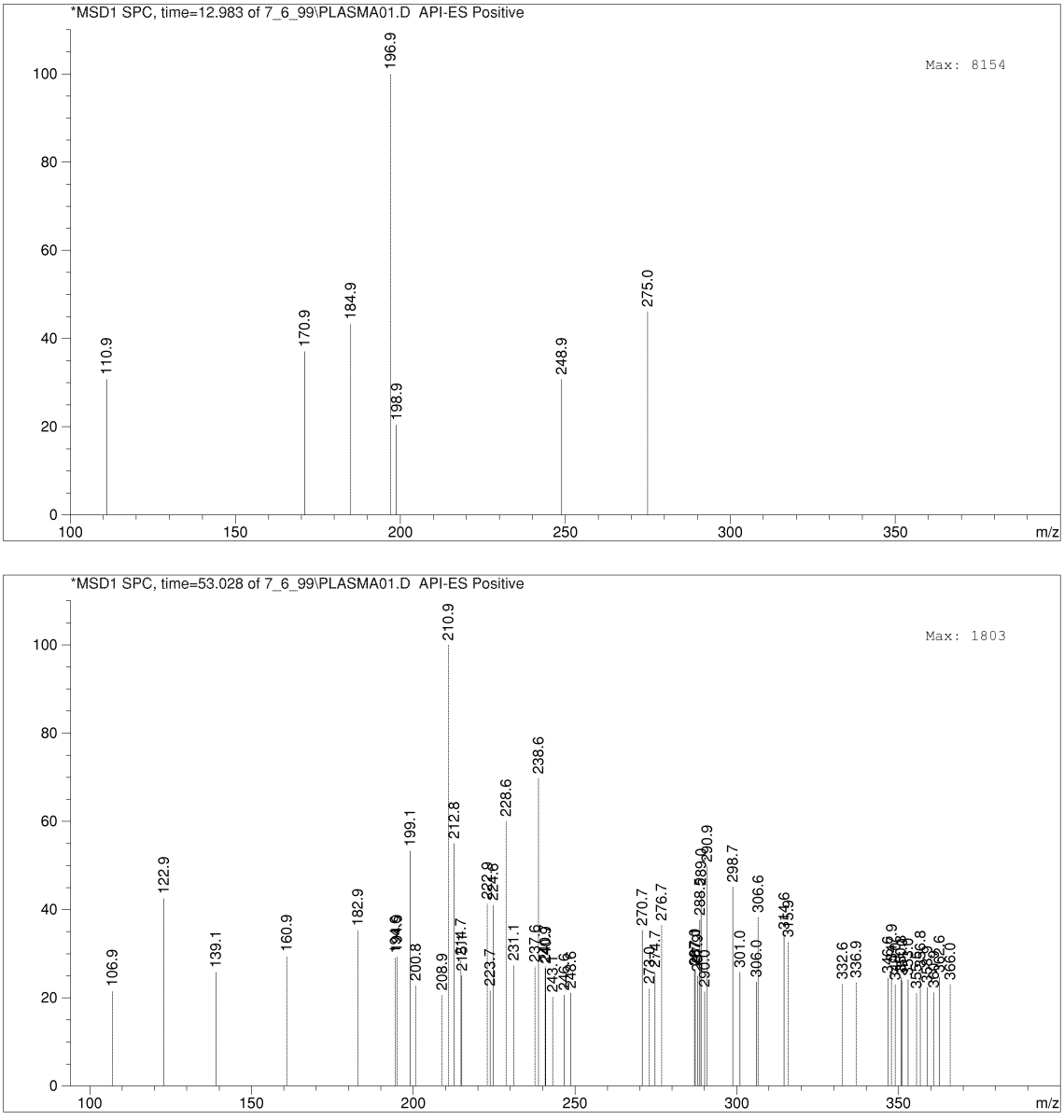

Fig. 5. ESI^+ spectra obtained by LC–MS of blank human plasma. Traces, top with formic acid and bottom, with ammonium acetate–acetic acid as electrolyte additives of the mobile phase.

sion curves, and RSD values were computed. The obtained values are also reflected in Table 1. For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from 0. Moreover, a linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and intercept equal to 0 (Student's *t*-test). The extraction recoveries of famotidine and ranitidine in plasma were 99.80 ± 1.28 and $99.23 \pm 2.38\%$, respectively.

Accuracy values were within acceptable limits (Table 2). The results for within- and between-day precision for our sample are presented in Table 3 and the values ranged between 2.69 and 5.72%, 5.63 and

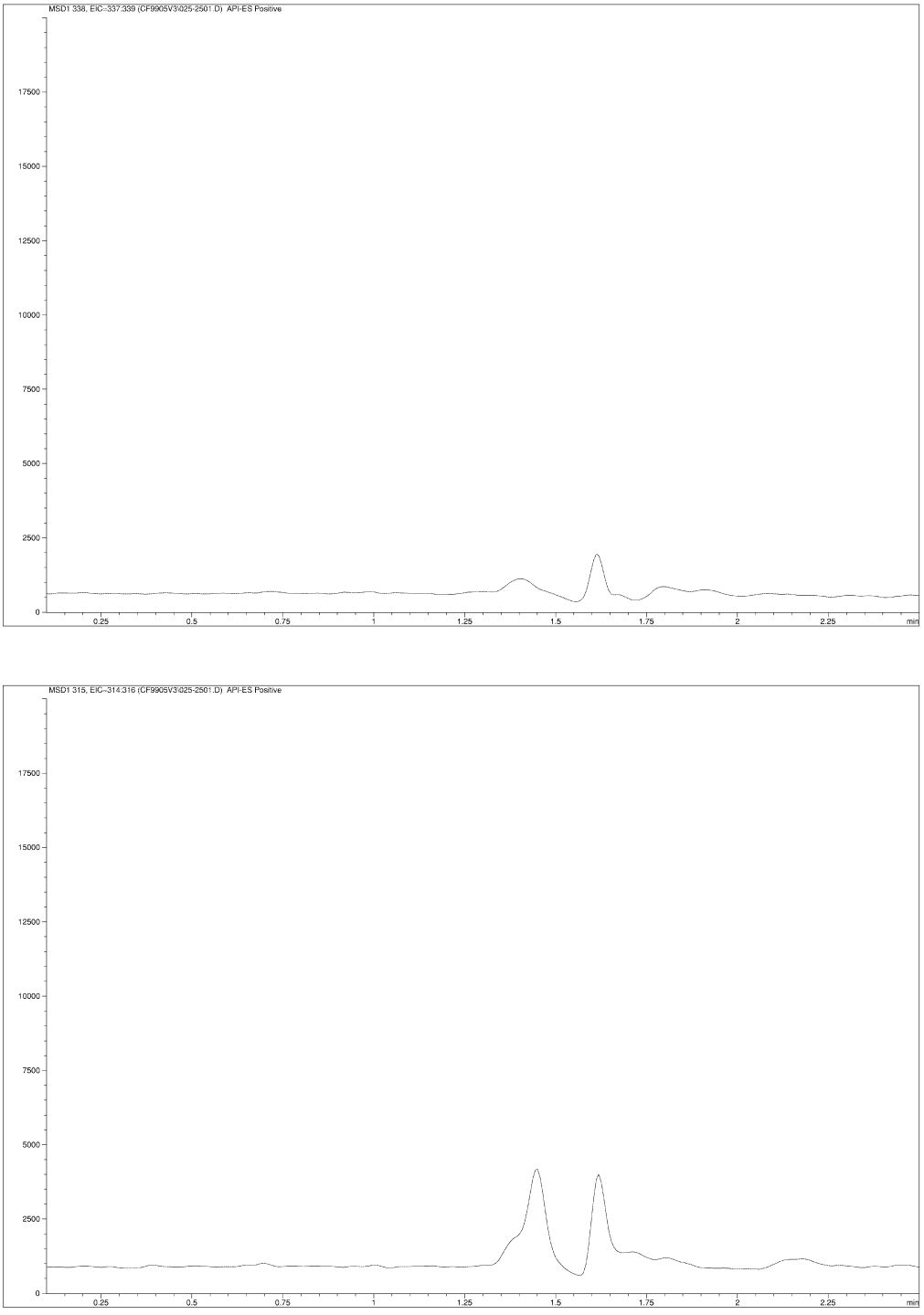

Fig. 6. Chromatograms of blank human plasma. Top, famotidine; bottom, ranitidine.

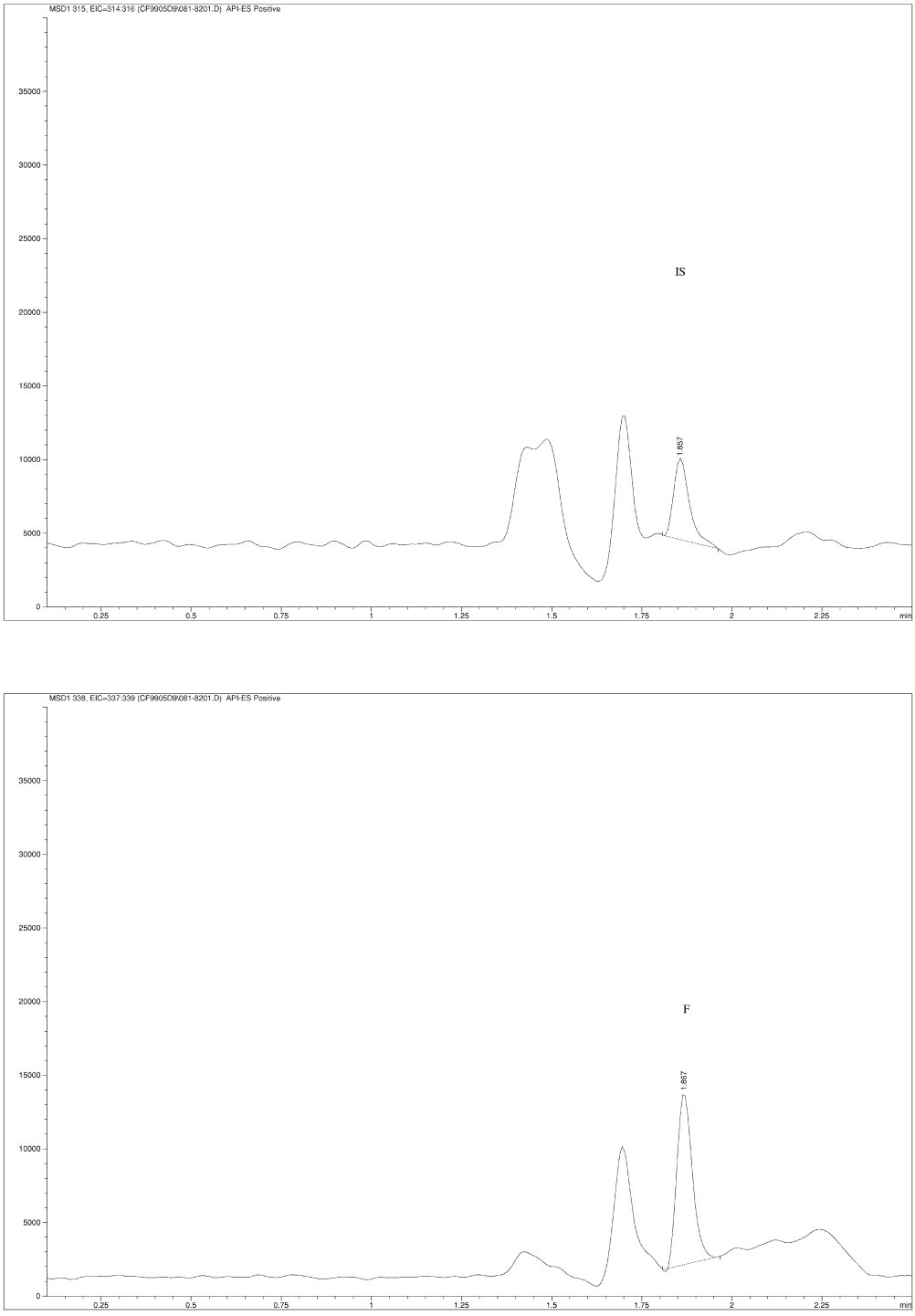

Fig. 7. Chromatograms resulting from the analysis human plasma samples obtained at 3 h (39.3 ng/ml famotidine) from a subject who received a single oral dose of Tamin®, (40 mg). Top, famotidine; bottom, ranitidine. I.S., ranitidine; F, famotidine.

Table 1
Standard calibration curves of famotidine in human plasma

	r	*a*	*b*	Concentration (ng/ml)							
				1	2.5	5	10	20	50	100	200
n	6	6	6	12	12	12	12	12	12	12	12
Mean	0.9991	0.0394	−0.0001	1.00	2.50	5.00	10.05	19.7	48.3	100.6	207.1
SD	0.0001	0.0013		0.03	0.08	0.16	0.26	0.84	0.93	4.08	2.66
RSD (%)	0.015	3.28		2.58	3.30	3.22	2.54	4.28	1.93	4.05	1.28
Accuracy (%)				−0.08	0.10	−0.02	0.53	−1.42	−3.44	0.62	3.56

r, Correlation coefficient; *a*, slope; *b* intercept.

Table 2
Accuracy of the method, expressed as relative error (%), for determining famotidine concentrations

Concentration added (ng/ml)	Concentration found (mean±SD) (ng/ml)	Accuracy (%)
1	1.09±0.06	8.97
5	5.23±0.23	4.64
20	18.9±0.70	5.07
100	102.0±2.74	2.03

7.83%, respectively. The obtained values for the precision were acceptable, although less variability could be expected if slow flow-rates (under 0.4 ml/min) were used.

The LOD of famotidine was 0.2 ng/ml ($S/N=3$) and the estimated LOQ was calculated as low as 1 ng/ml ($S/N=10$) which is lower than that described by other authors (5 ng/ml [6,10]) in biological media. The last value was confirmed for our samples. The mean assay result was 1.0 ng/ml ($n=12$), with RSD$<$2.5%.

Famotidine is stable in plasma samples stored at −70°C for at least 3 months [6]. The stability of famotidine in processed samples left at room temperature (20±3°C) over 24 h was also studied from our laboratory quality control set up for the drug at concentrations of 5, 20 and 100 ng/ml, respectively. Famotidine and the I.S. were also stable under these conditions.

The applicability of this method has been demonstrated by the determination of famotidine in plasma samples from 30 subjects receiving oral doses of famotidine in a bioavailability study (Fig. 5). The sensitivity of the assay was such that famotidine concentrations in plasma could be quantitated over a period of 24 after a single oral dose of 40 mg of famotidine. Fig. 8 shows the mean plasma famotidine concentration–time profile following a single oral dose of 40 mg Tamin®.

Finally, it is interesting to note that under the chromatographic conditions described above we can avoid the slow and tedious extractive procedures usually used to increase the analytical sensitivity. Furthermore, for famotidine or other basic polar drugs, these extractions can be one of the most important causes of the lack of analytical reproducibility and the high variability observed in the pharmacokinetic parameters obtained in some bioavailability studies.

Table 3
Between and within-day variability of the HPLC method for determining famotidine concentrations

Concentration added (ng/ml)	Between-day variability ($n=6$)		Within-day variability ($n=18$)	
	Concentration found (Mean±SD) (ng/ml)	RSD (%)	Concentration found (Mean±SD) (ng/ml)	C.V. (%)
1	1.09±0.06	5.72	1.03±0.08	7.83
5	5.23±0.23	4.30	4.97±0.36	7.24
20	18.9±0.70	3.67	19.5±1.21	6.23
100	102.0±2.74	2.69	100.0±5.63	5.63

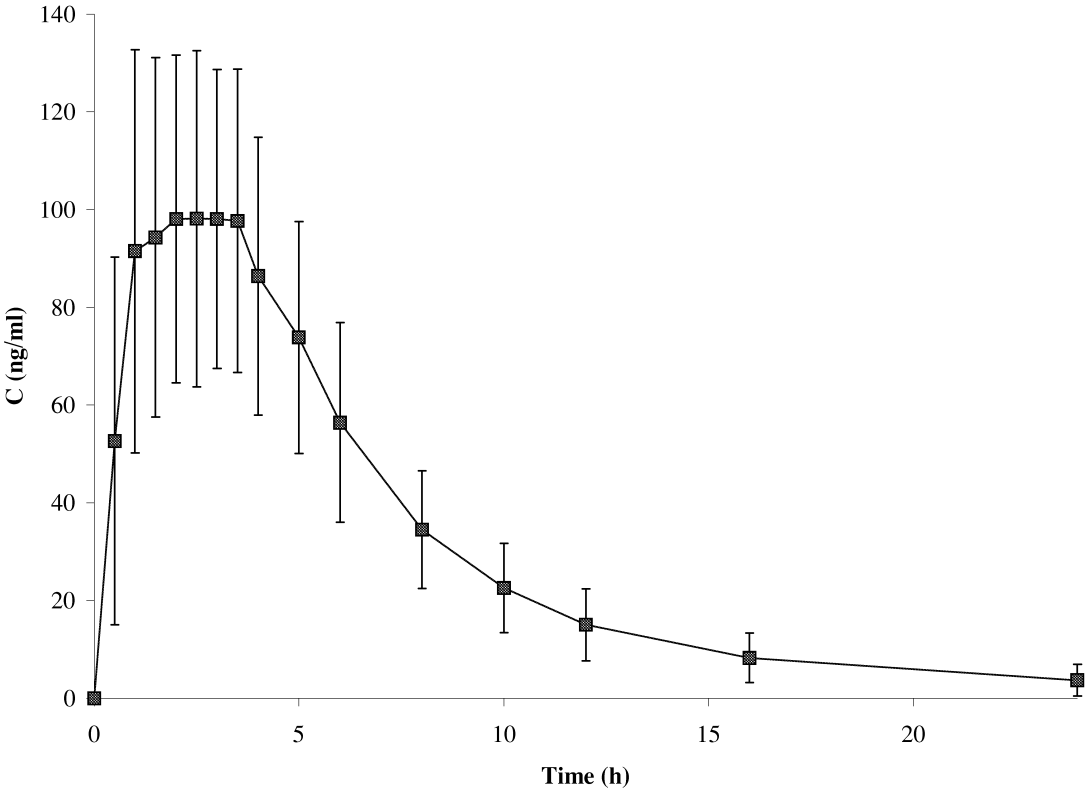

Fig. 8. Mean plasma famotidine concentrations versus time after oral administration of Tamin® to 30 healthy volunteers.

4. Conclusion

The experiments have demonstrated that HPLC–ESI-tandem mass spectrometric detection, together with the simplest extraction procedure, protein precipitation, is a good alternative to UV chromatographic methods traditionally employed for the determination of polar compounds in biological fluids. A simple chromatographic method has been developed for the rapid and precise determination of famotidine in plasmatic samples. The simplicity of the technique, the shorter analysis time and the high sensitivity makes this technique particularly attractive for pharmacokinetic studies. We believe that this method provides a useful tool in biomedical and clinical research, and the application of these tandem techniques can be easily increased for other basic polar compounds.

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