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Comparative validations of non-aqueous capillary electrophoresis and high-performance liquid chromatography methods for the simultaneous determination of histamine H2 receptor antagonists in human urine

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In memoriam of professor Juan José Berzas Nevado.

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ABSTRACT

This paper reports a previously optimised method based on non-aqueous capillary electrophoresis (NACE) using UV detection for the separation and simultaneous determination of cimetidine (CIM), ranitidine (RAN), roxatidine (ROX), nizatidine (NIZ) and famotidine (FAM) in human urine. Separation is performed at 25 °C and at a separation voltage of 15 kV. Methanol containing 10 mM ammonium acetate and 0.2% acetic acid was used as background electrolyte, and detection at 214 nm. These conditions allow the five analytes to be separated within 4 min. In addition in the present paper a HPLC method using diode-array as well as detector, was proposed as standard analytical method, which chromatography conditions were following: a mobile phase consisting of 80:20 20 mM phosphate buffer (pH 7.5)/acetonitrile, and using 1 mL min^{-1} as flow rate of the mobile phase. Detection limits were evaluated on the basis of baseline noise and were establishing between 8 and 15 μg L^{-1} for NACE and between 16 and 162 μg L^{-1} for HPLC. The methods showed good precision with overall intra- and inter-day variations of 0.5–2.0% and 0.7–3.8%, respectively. Finally the proposed methods were successfully applied to the screening determination of the analytes in human urine, with recoveries between 97 and 105%, being able the use as pharmacokinetic data in clinical urine samples.

1. Introduction

Histamine H2 receptor antagonists constitute a drug class used to block the action of histamine on parietal cells in the stomach in order to reduce their acid production. Chromatographic methods are widely used to determine histamine H2 receptor antagonists, both individually and in combination. Most such methods use high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) with UV or mass spectrometry detection. Some have been specifically developed for determining antiulcers in pharmaceutical dosage forms, or their metabolites and impurities. Analyses with these methods are usually performed on biological fluids including urine, plasma and serum.

HPLC with UV detection has been used for the determination of ranitidine [1–5] or famotidine [6–10] in plasma, ranitidine [11,12] and famotidine [13] in serum, and ranitidine [14,15] and famotidine (together with its impurities) [16] in pharmaceutical preparations. Ranitidine and cimetidine have been determined jointly in aqueous samples [17] and plasma [18]. Other methods use MS detection to determine ranitidine and its metabolites [19] or famotidine in plasma [20]. Iqbal et al. developed a HPLC method for the determination of cimetidine in human plasma and urine [21] with a limit of quantitation of 100 ng mL^{-1} and 10 μg mL^{-1}, respectively.

Capillary electrophoresis with UV detection has been used to determine various histamine H2 receptor antagonists including cimetidine in plasma [22], and its degradation products and impurities [23]; and ranitidine in pharmaceutical preparations [24]. This drug was also determined in urine by capillary electrophoresis with electrochemiluminescent detection [25]. Finally, cimetidine, famotidine, nizatidine and ranitidine in plasma, urine and pharmaceutical preparations have been the subjects of screening analyses [26–32] by CE or HPLC.

Roxatidine is the least analytically documented histamine H2 receptor antagonist; in fact, only four papers have seemingly been devoted to its determination. In 1988, Burrows et al. [33]

Abbreviations: CIM, cimetidine; FAM, famotidine; NACE, non-aqueous capillary electrophoresis; NIZ, nizatidine; RAN, ranitidine; ROX, roxatidine.

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determined this drug in plasma, urine and milk by gas chromatography. Argekar and Kunjir quantified it in pharmaceutical preparations by HPLC-UV [34]; and, recently, two liquid chromatography/mass spectrometry (LC/MS) methods [35,36] for its determination in human plasma were developed and validated.

Most of these methods are only capable of analysing some of five H_2 antagonists. However, there has been no report a single universal assay by capillary electrophoresis for determining all five H_2 antagonists in human urine. In a previous work recently published [37], we developed a NACE method enabling control analyses for these drugs in pharmaceuticals formulations. In the present paper one of the main aim was thus to carry out a universal complete validate procedure using a new NACE-UV analytical methodology for the screening determination of cimetidine, famotidine, ranitidine, nizatidine and roxatidine in human urine. Since a considerable amount of five H2 antagonists gets excreted unchanged in urine, about 40–60% of an oral dose, in a first step their metabolites were not considered.

Other of the main aims of this research is also to prove the suitability of the proposed method for a reliable quantification of analytes at clinical levels. In this sense, we have carried out an extensive validation study, and specially applying a new design for a whole and simultaneous robustness/ruggedness evaluation. So, an integral evaluation including robustness and ruggedness tests was also performed on urine matrix for the electrophoretic method previously proposed.

Finally the proposed methods were successfully applied to the screening determination of the analytes in human urine, being able their use as pharmacokinetic data in clinical urine samples.

2. Materials and methods

2.1. Reagents, solutions and samples

2.1.1. Reagents

RAN, FAM and NIZ were supplied by Sigma (Madrid, Spain), CIM was obtained from Tocris (Biogen Cientifica S.L., Madrid, Spain) and ROX from Zambón (Barcelona, Spain). Ammonium acetate, sodium dihydrogen phosphate, ethanol and methanol (HPLC grade) were purchased from Panreac (Madrid, Spain). Ethanol and methanol were both obtained from Panreac, whilst acetonitrile was supplied by Sigma. Milli-Q water was used throughout.

2.1.2. Standard solutions

Standard solutions of the histamine H2 receptor antagonists (1000 mg L^{-1}) were prepared in water. By exception, FAM was dissolved in an ethanol–water mixture owing to its low solubility in water. Working-strength solutions were prepared on a daily basis by appropriate dilution of the stock solutions with methanol.

2.1.3. Urine samples

Fresh urine samples were obtained from different healthy volunteers, whereas clinical urine samples were provided from patients under histamine H2 receptor antagonist treatment. These fresh urine samples were directly submitted to solid-phase extraction after a preliminary centrifugation step (1960 × *g*, 15 min, 20 °C).

2.2. Instrumentation

2.2.1. Capillary electrophoresis

Tests were performed on a Beckman (Fullerton, CA, USA) P/ACE System MDQ capillary electrophoresis system equipped with a diode-array detector and controlled via Beckman capillary electrophoresis software. Separations were done in a 31 cm (21 cm from inlet to detector) × 75 (m i.d. fused silica capillary accommodated in a cartridge that was thermostated at 25 °C. The detection window was 800 μm × 100 μm.

2.2.2. High-performance liquid chromatography

A Thermo Finnigan™ Surveyor® Plus HPLC system with a diode-array detector was used for this purpose. The system was monitored via a computer equipped with ChromQuest 5.0 software, which was used for both measurements and data processing. Compounds were separated on a 4.6 mm i.d. × 150 mm Kromasil C_{18} column of 5 μm particle size, using 20:80 acetonitrile/20 mM phosphate buffer (pH 7.5) as mobile phase. Elution was done in the isocratic mode, using an injected volume of 20 μL and a flow rate of 1 mL min^{-1}.

2.2.3. Others equipments

The stability of the solutions was assessed on a Beckman DU-70 spectrophotometer furnished with 1.0 cm quartz cells and connected to an IBM-PS 2 Model 30 computer running Beckman Data Leader software.

A vortex shaker for tubes (OVAN, Barcelona, Spain) was used to mix and agitate samples, and a model S240 centrifuge (Selecta, Barcelona, Spain) for centrifugation.

Extraction and preconcentration were carried out with a laboratory-made device consisting of a water manifold (Supelco Visiprep™ Sep-Pack system, Madrid, Spain) coupled to a Millipore XF 54 23050 vacuum pump. Solid-phase extraction (SPE) was performed with Sep-Pack Plus C_{18} cartridges (500 mg, Waters, Milford, MA). Samples were evaporated by using a dry block heater (Pierce Reactive-Therm™ Heating Modules) which provided precise, uniform temperature-controlled heating and N_2 flow.

pH was measured with a Crison model 2001 pH meter with a combined glass electrode.

HPLC mobile phases both aqueous and organic were filtered through 0.45–1 xm filters (MF membrane filters) and through 0.5–1 xm filters (Fluoropore membrane filters), respectively. Both types of membrane filter were purchased from Millipore.

2.3. Methods and procedures

As it has been commented later, in the present paper, comparative validate procedures have been carried out using a NACE-UV and HPLC-UV for the simultaneous determination of cimetidine, famotidine, ranitidine, nizatidine and roxatidine in biological samples. For that, two analytical methods have been proposed, being very important their chemical features of each analysed compounds. So, Fig. 1 shows the chemical structures of each drug used for both analytical methods.

Use of diode-array detection enabled extraction of chromatograms and electropherograms at different wavelengths. In the optimisation process we monitored the five analytes at 214 nm whereas validation procedure we monitored each antiulcers at its wavelength of maximum absorbance.

2.3.1. NACE-UV method

The capillary was used for hydrodynamic injection of samples for 3 s at 0.5 psi (25.8 nl). Separations were performed at 25 °C, using an applied voltage of 15 kV for 4 min (88 kV min^{-1} ramp). The current intensity thus obtained was 13 (A. Samples were injected in triplicate and corrected peak areas (CPA) (area/migration time ratio) used for quantitation.

Methanol containing 10 mM ammonium acetate and 0.2% acetic acid, and prepared on a daily basis, was used as background electrolyte. Each electrolyte solution was used in only six runs in order to avoid electrolysis.

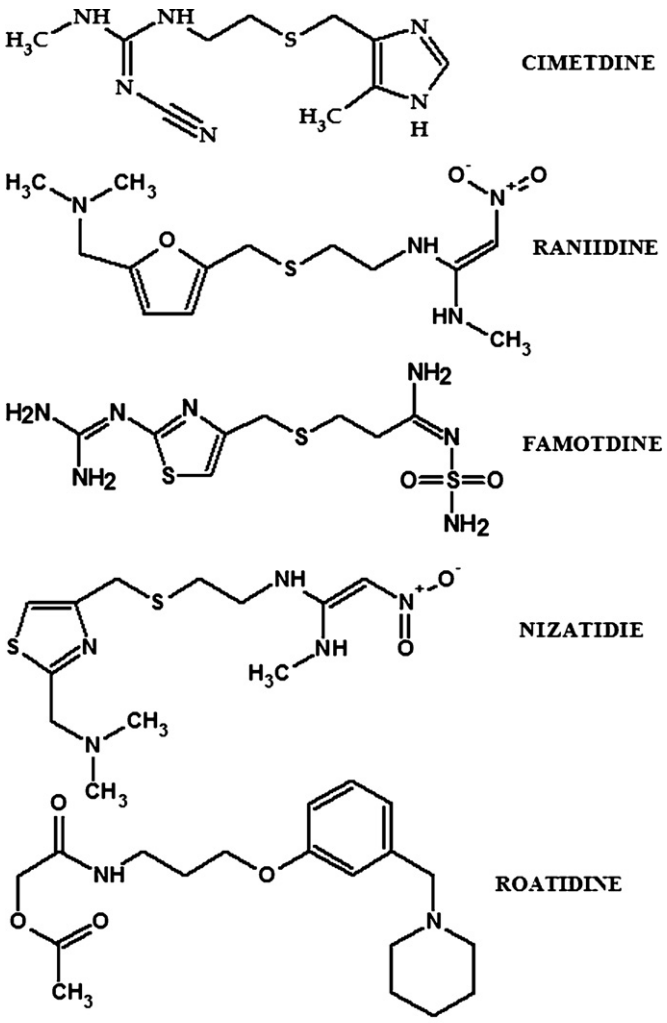

Fig. 1. Structures of the histamine H2 receptor antagonists (CIM, FAM, NIZ, RAN and ROX).

2.3.2. HPLC-UV method

Compounds were separated on at room temperature on a 3.9 mm i.d. × 150 mm, 5 μm particle, Nova Pack C_{18} reversed-phase column from Waters Millipore (Milford, MA, USA). A mobile phase consisting of 80:20 (v/v) of phosphate buffer, 20 mM pH 7.5 and acetonitrile as organic solvent, was used in preliminary tests. Isocratic elution was performed at a flow rate of 1.0 mL min^{-1}. The volume injected (samples and standards) was 20 μL. A wavelength of 214 nm was used since provided the best S/N ratio and a resolution better than 1.4 for the simultaneous separation of selected analytes.

2.3.3. Solid-phase extraction procedure for urine samples

Extraction of the histamine H2 receptor antagonists present in the urine samples was performed by solid-phase extraction (SPE) with reversed-phase C_{18} 500 mg cartridges (Sep-Pak Plus, Waters, Milford, MA, USA). Every step of this SPE procedure (nature and volume of organic and aqueous solvents in washing stages, sample volume, elution volume, final volume of extracts, etc.) was evaluated in an effort to achieve complete extraction. Owing to the high level of complexity of the urine matrix, where the analytes under investigation were present at low levels this SPE process was also necessary as a preconcentration and cleaning stage. It was also decided to submit the urine samples to a preliminary centrifugation step (1506 × *g*, 10 min, 25 °C).

Prior to loading with the sample, the sorbent was conditioned with 5 mL of methanol and 5 mL of 10 mM phosphate buffer (pH 7). Then, the urine sample (5 mL) was passed slowly through it. After loading, the sorbent was washed with 2 mL of 100 mM phosphate buffer (pH 7), 2.5 mL of 20:80 methanol/water and 2 mL of water. Finally, the target analytes were eluted with 2 mL of methanol.

3. Results and discussion

The stability of standards solutions of cimetidine (CIM), ranitidine (RAN), roxatidine (ROX), nizatidine (NIZ) and famotidine (FAM) was assessed from spectrophotometric measurements. All standard solutions were stored at room temperature. Working solutions were prepared on a daily basis by diluting the stock solutions with methanol.

The difference between absorption spectra of the stocks solutions were found to be unchanged of freshly prepared solutions and those aged for 60 days after this period. The solution can therefore be used within this period without the results being affected. So we can assure stock standard solutions used (1000 mg L^{-1}) were found to remain stable for at least 2 months.

3.1. NACE method

3.1.1. Separation conditions

Based on previous results [37] with a new, validated CE-UV method for the simultaneous detection and quantitation of the five antiulcers in pharmaceutical formulations, in this work we used it to determine the drugs in human urine. The optimum separation conditions were established by examining the influence of the electrolyte composition, proportion of acetic acid, concentration of ammonium acetate, applied voltage, capillary temperature and injection time; the optimum conditions thus identified are shown in Table 1. Fig. 2 shows an electropherogram for the five antiulcers as obtained under such conditions.

Prior to first use, the capillary was conditioned by rinsing with 0.1 M NaOH for 20 min, water for 10 min and separation electrolyte for 10 min. Also, prior to each analysis, the capillary was flushed with running electrolyte for 1 min in order to restore and re-equilibrate its wall surface. Once ready, methanol containing 10 mM ammonium acetate and 0.2% acetic acid, and prepared on a daily basis, was used as background electrolyte. Each electrolyte solution was used in only six runs in order to avoid electrolysis.

The current intensity thus obtained was 13 (A. Samples were injected in triplicate and corrected peak areas (CPA) (area/migration time ratio) used for quantitation.

Diode-array detection was used to obtain chromatograms at different wavelengths in CE and HPLC. A wavelength of 214 nm was used to simultaneously detect the five analytes. In other way, in order to improve some validations parameters ad limits of detection, the analytes were quantified at the highest absorbance

Table 1
Optimum capillary electrophoresis separation conditions.

NACE separation conditions[a]	
Electrolyte	10 mM ammonium acetate and 0.2% acetic acid in methanol
Voltage	15 kV, voltage ramp 88 kV min^{-1} in 0.17 min
Silica fused capillary	31 cm total length (21 cm effective length) × 75 μm i.d.
Sample injection	Hydrodynamic (0.5 psi), 3 s
Temperature	20 °C
Detection wavelength	214 nm

[a] Ref. [37].

Table 2
Precision in peak areas for samples analysed on different days.

	Roxatidine		Ranitidine		Famotidine		Cimetidine		Nizatidine	
	NACE[a]	HPLC[b]	NACE[a]	HPLC[b]	NACE[a]	HPLC[b]	NACE[a]	HPLC[b]	NACE[a]	HPLC[b]
Day 1	0.86	9.48	4.26	1.99	1.13	0.55	1.01	1.85	1.15	1.79
Day 2	1.31	5.98	3.65	3.85	1.24	1.17	1.06	0.69	1.16	1.06

[a] Corrected peak area (% RSD).
[b] Peak area (% RSD).

Table 3
Linearity in the signal–concentration relation for the analytes as determined by NACE and HPLC.

Analyte	Method	Equation	R^2
Famotidine	NACE	$y = (18978.44 \pm 307.06)x + (2.43 \pm 2357.18)$	0.9990
	HPLC	$y = (195796.40 \pm 4179.59)x + (37041.00 \pm 55290.79)$	0.9990
Cimetidine	NACE	$y = (30646.57 \pm 1689.71)x + (-7787.30 \pm 12970.73)$	0.9940
	HPLC	$y = (321137.07 \pm 56726.85)x + (525710.50 \pm 750425.69)$	0.9810
Nizatidine	NACE	$y = (10076.87 \pm 511.85)x + (-2432.83 \pm 263.30)$	0.9950
	HPLC	$y = (206789.03 \pm 1072.64)x + (124226.00 \pm 14189.72)$	0.9982
Ranitidine	NACE	$y = (10566.06 \pm 537.97)x + (-2927.46 \pm 463.56)$	0.9951
	HPLC	$y = (169903.93 \pm 27920.99)x + (144230.50 \pm 369360.04)$	0.9934
Roxatidine	NACE	$y = (14402.97 \pm 775.58)x + (-2992.20 \pm 5953.55)$	0.9946
	HPLC	$y = (8903.64 \pm 1836.55)x + (-10138.5 \pm 24295.34)$	0.9882

wavelength for each (200 nm for ROX, 231 nm for RAN, 280 nm for FAM, 219 nm for CIM and 325 nm for NIZ).

3.1.2. Validation procedure

The specificity or purity of the peaks for the histamine H2 receptor antagonists was assessed from several injections of urine samples previously subjected to SPE and spiked with the target analytes. Absorbance peaks at different wavelengths were used to compare the spectra for the analytes. The results revealed the absence of interferences due to matrix effects.

The reproducibility of corrected peak areas (CPA) was evaluated over a period of 2 days by performing five successive injections of urine samples spiked with each analyte at a 10 mg L^{-1} concentration and subjected to SPE prior to CE. The results, calculated as percent RSD values, are shown in Table 2. A comparison via Snedecor's test revealed the absence of significant differences at the 5% confidence level.

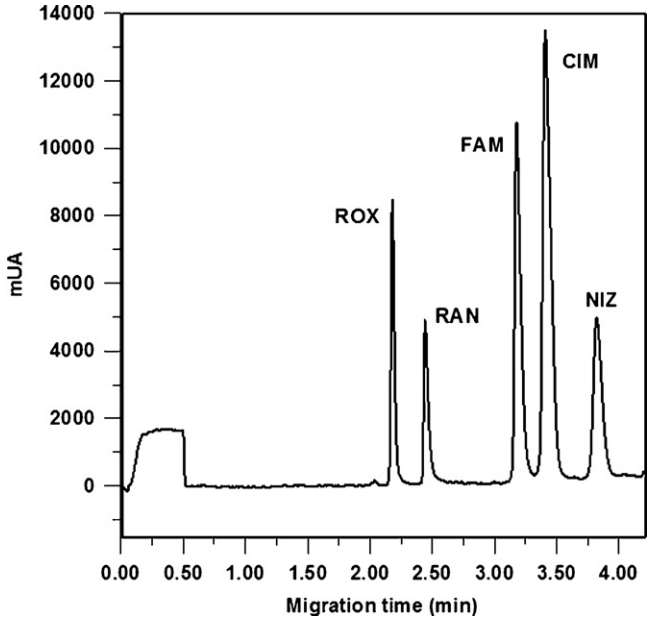

Fig. 2. Electropherogram for a urine sample spiked with a 5 mg L^{-1} concentration of each histamine H2 receptor antagonist and subjected to the SPE treatment under the optimum NACE separation conditions.

Linearity was assessed by using spiked urine samples subjected to the SPE treatment. The results (CPA) were obtained by triplicate injection of nine concentrations levels (0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10 and 20 mg L^{-1}) for each analyte and revealed good linearity between concentrations and relative peak areas for all analytes, with correlation coefficients higher than 0.99. Table 3 lists the regression equations obtained and their coefficients.

The accuracy of the proposed method was validated by applying it to various human urine samples spiked with ROX, RAN, FAM, CIM and NIZ. Their recoveries, which were calculated against spiked blank urine extracts used as external standards, are shown in Table 4.

The limits of detection (LOD) and quantitation (LOQ) for each analyte were calculated as 3 and 10 times, respectively, their S/N ratios. In addition in order to boost sensitivity up and improve LODs and LOQs a bubble cell capillary was used (see Table 5).

3.1.3. Integral evaluation of robustness and ruggedness

The United States Pharmacopeia (UPS) defines ruggedness as "the degree of reproducibility of test results obtained by the analysis of reproducibility of the same samples under a variety of normal test conditions such as different days, several reagent lots, different

Table 4
Recoveries from human urine samples spiked with the analytes at variable concentrations using NACE analytical method.

Analyte	Added (ppm)	Recovery (%)
Roxatidine	5	101
	12.5	105
Ranitidine	5	99
	12.5	101
Famotidine	5	100
	12.5	101
Cimetidine	5	97
	12.5	102
Nizatidine	5	97
	12.5	104

Table 5
Limits of detection (LOD) and quantitation (LOQ) for the NACE and HPLC methods.

Analyte	LOD ($\mu g\,L^{-1}$)			LOQ ($\mu g\,L^{-1}$)		
	NACE		HPLC	NACE		HPLC
	Normal conditions	Bubble cell capillaries	Normal conditions	Normal conditions	Bubble cell capillaries	Normal conditions
Roxatidine	26	9	162	87	28	540
Ranitidine	41	13	22	138	45	75
Famotidine	45	15	23	150	49	76
Cimitidine	23	8	16	77	26	52
Nizatidine	48	15	18	160	52	60

Table 6
Experimental design for the integral evaluation of robustness and ruggedness with the Plackett–Burman model.

Number of experiments	Selected factors (external and internal)										
	A	B	C	D	E	F	G	H	I	J	K
1	+	+	–	+	+	+	–	–	–	+	–
2	–	+	+	–	+	+	+	–	–	–	+
3	+	–	+	+	–	+	+	+	–	–	–
4	–	+	–	+	+	–	+	+	+	–	–
5	–	–	+	–	+	+	–	+	+	+	–
6	–	–	–	+	–	+	+	–	+	+	+
7	+	–	–	–	+	–	+	+	–	+	+
8	+	+	–	–	–	+	–	+	+	–	+
9	+	+	+	–	–	–	+	–	+	+	–
10	–	+	+	+	–	–	–	+	–	+	+
11	+	–	+	+	+	–	–	–	+	–	+
12	–	–	–	–	–	–	–	–	–	–	–

instruments, various laboratories, different elapsed assay times. . .", where all of these factors are external to the written analytical method.

The robustness of a method is defined by both the USP and ICH Tripartite guidelines (International Conference on Harmonization) as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters" and "provides an indication of its reliability during normal use" [38]. Ruggedness can therefore be regarded as a measure of the absence of external influences on the test results, and robustness as one of the lack of internal influence on such results. In this work, we assessed the potential influence of both internal and external factors of the method on performance. To this end, we used the Plackett–Burman factorial model, which is based on balanced incomplete blocks. For statistical reasons concerning effects on interpretation, no designs involving fewer than 8 experiments were used and designs involving more than 24 were deemed impractical [39]. To date, this model has been successfully used to assess robustness only.

Table 6 shows the figures of merit of a novel Plackett–Burman design allowing the effects on both robustness and ruggedness to be assessed; the design, which involves 11 factors and 12 experiments ($N = 12$), is described in detail elsewhere [40]. The choice of variables (factors) and the levels at which they are tested is very important for a reliable robustness/ruggedness test. In fact, the variables should be significant in practice and their levels reflect their typical variations. The external (ruggedness) and internal (robustness) factors (A–K) used in our model are shown in Table 7 together with the (+) and (−) levels for each factor; these two signs denote whether the value in question was greater or lower than the optimum value for the procedure.

The effects of varying the levels of the most critical electrophoretic responses of the method were investigated in terms of CPA, peak heights and migration times for the analytes.

The ranked effects of each factor for a selected electrophoretic response were calculated simply by adding its (−) and (+) assay test results, the total being divided into one-half the number of runs (i.e., by 6 in a 12-run design). *M* values are constant for any given design and are in fact the means of the order statistics (reference) for a sample size of 11. The ranked effects of the 12 factors (on the *X*-axis) were plotted against the *M* values (on the *Y*-axis) for each critical electrophoretic response. The result should be a near-straight line. If a value lies outside the line, then the method can be deemed not rugged or not robust (as classified by its corresponding factor). However, if all the plots form (nearly) straight line, the analytical method can be deemed rugged and robust over the conditions tested in the run design.

Table 7
Factors used to evaluate robustness and ruggedness in the NACE method.

Factor	External/internal	Optimum	Level (−)	Level (+)	*M*
A: Ammonium acetate concentration (mM)	Internal	10	8	12	−1.59
B: % acetic acid	Internal	0.20	0.16	0.24	−1.06
C: Voltage (kV)	Internal	15	13	17	−0.73
D: Temperature (°C)	Internal	25	23	27	−0.46
E: Injection time (s)	Internal	3	2.8	3.2	−0.22
F: Urine volume (mL)	Internal	5	4.8	5.2	0.00
G: Elution volume (mL)	Internal	2	1.9	2.1	0.22
H: Different days	External	–	I	II	0.46
I: Various capillaries	External	–	I	II	0.73
J: Different urine samples	External	–	I	II	1.06
K: Different SPE cartridge lots	External	–	I	II	1.59

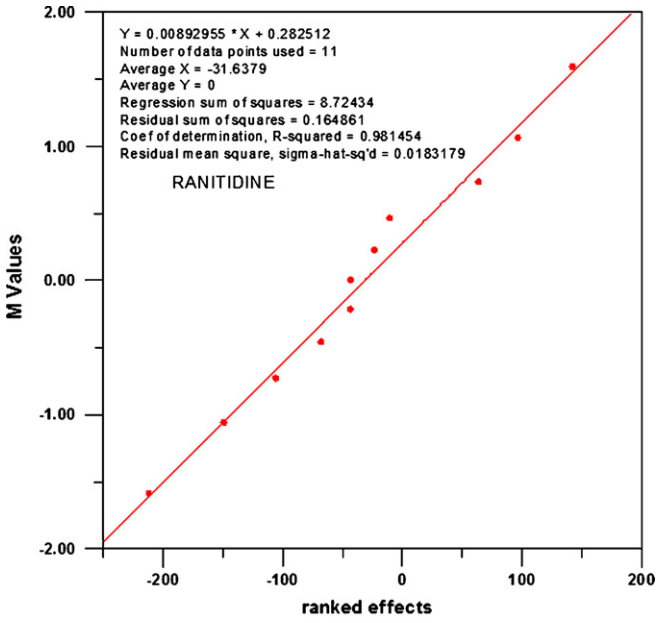

Fig. 3. Ranked effects of the 12 selected factors against the *M* values for the ranitidine response (corrected peak areas).

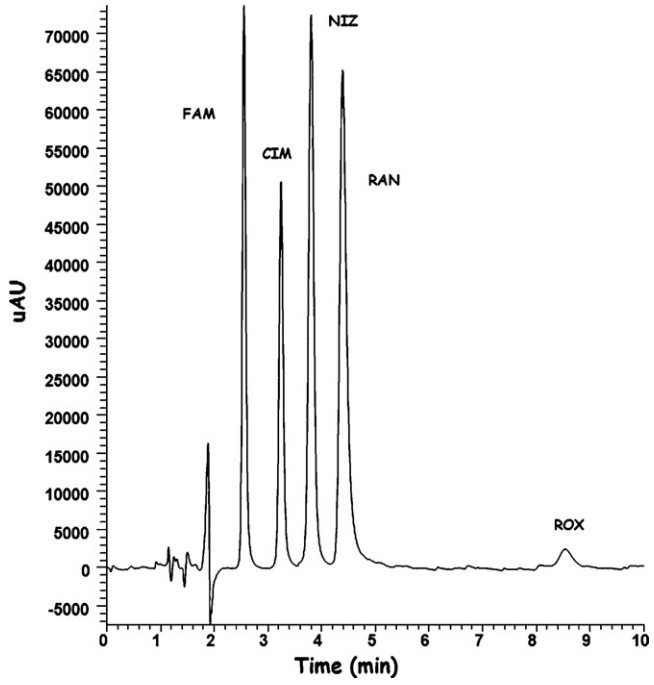

Fig. 4. Chromatogram for a urine sample spiked with a 5 mg L^{-1} concentration of each histamine H2 receptor antagonist and subjected to the SPE treatment under the optimum HPLC separation conditions.

The robustness/ruggedness pair was assessed from triplicate injections of spiked urine samples containing a 5 mg L^{-1} concentration of each analyte. Based on the results of the test, the proposed SPE–electrophoretic method is both robust and rugged for the critical electrophoretic responses assessed under all variations tested.

By way of example, Fig. 3 shows the plot for the ranked effects of the 12 selected factors against the *M* values for ranitidine. As can be seen, all points lay on a straight line; therefore, the proposed method is robust and rugged with regard to the electrophoretic response. A similar evaluation was done for the rest of electrophoretic responses.

3.2. HPLC standard method

The HPLC method used for validation was developed in accordance with previous work in this direction. Because some of the analytes studied here were not addressed in such work, a new method for the determination of the five antiulcers in urine samples had to be optimised and validated.

3.2.1. Separation conditions

A mobile phase consisting of 80:20 20 mM phosphate buffer (pH 7.5)/acetonitrile was used in preliminary tests. Samples were injected in triplicate and peak areas used for quantitation.

The influence of the pH of the mobile phase on separation was studied by using various 20 mM phosphate solutions containing 20% acetonitrile and adjusted to pH 6.5–7.5. Retention times were found to increase with increasing pH under these conditions, so pH 7.5 was selected as it ensured efficient separation of the analytes.

The influence of the buffer concentration in the mobile phase was examined over the range 10–50 mM and 20 mM selected on the grounds of resolution.

The effect of the organic solvent in the mobile phase was investigated by using 20 mM phosphate buffer containing variable proportions of acetonitrile from 20 to 40%. Retention times were found to decrease and overlap between peaks to increase as the acetonitrile content was raised. A proportion of 20% was thus chosen as optimal based on peak resolution.

The influence of the flow rate also was studied and 1 mL min^{-1} selected inasmuch as it provided good resolution and short analysis times.

Under the above-described optimum separation conditions the analytes were eluted in the following sequence: famotidine, cimetidine, nizatidine, ranitidine and roxatidine (Fig. 4).

3.2.2. Validation procedure

After the optimum separation conditions were established, appropriate validation tests for precision, linearity and limits of detection and quantitation were conducted. To this end, a standard solution containing a 10 mg L^{-1} concentration of each histamine H2 receptor antagonist was injected 5 times. The repeatability thus obtained was quite acceptable. Between-day precision (reproducibility) was evaluated by analysing several standards on two consecutive days; the results are shown in Table 2. As in capillary electrophoresis, a Snedecor test revealed the absence of significant differences in peaks areas at the 5% confidence level.

The analytical signal (area) versus concentration relationship was found to be quite linear for the five analytes. Such a relationship, however, was lost at concentrations above 20 mg L^{-1}. Table 3 shows the regression equations and coefficients of determination for the five analytes.

Accuracy was assessed by determining the five antiulcers in various standards and recoveries calculated by reference to the capillary electrophoresis results. The recoveries thus obtained were 99%, 92%, 93%, 105% and 106% for ROX, RAN, FAM, CIM and NIZ, respectively.

The limits of detection and quantitation, calculated on the basis of a detector signal-to-noise ratio of 3:1 and 10:1, respectively, were obtained from the average value for several chromatograms. The results are shown in Table 5.

3.3. Applications

Because histamine H2 receptor antagonists are excreted mainly in urine, about 40–60% of an oral dose is excreted as unchanged

Table 8
Application of the NACE and HPLC methods to real urine samples.

Active principle	Pharmaceutical formulation	Urine collection time after administration (h)	NACE (mg L^{-1})	HPLC (mg L^{-1})
Roxatadine (roxatidine acetate hydrochloride)	Zarcos, 150 mg (tablets)	2	92.28	91.50
Ranitidine (ranitidine hydrochloride)	Ranitidina Ratiopharm 150 mg (tablets)	2	42.95	36.15
Famotidine	Famotidina Ratiopharm 20 mg (tablets)	2	8.11	7.51
Cimetidine	Tamaget, 200 mg (tablets)	2.5	247.02	263.00
Nizatidine	Distaxid, 150 mg (capsules)	2.5	132.13	142.5

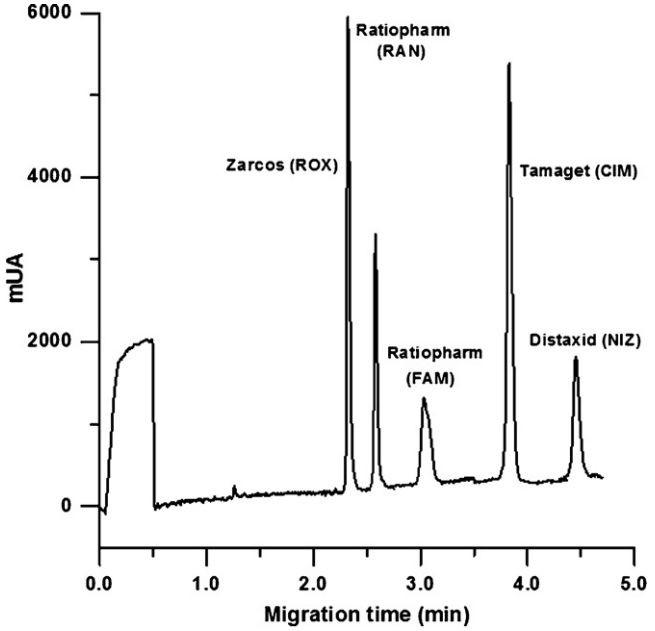

Fig. 5. Electropherogram for a mixture of human urine samples from five patients under treatment with roxatidine, ranitidine, famotidine, cimetidine and nizatidine, respectively.

drug, we assessed the ability of the proposed method to determine the analytes in this biological fluid. To this end, we analysed several urine samples from patients under medical treatment. The samples were subjected to the straightforward, expeditious extraction–preconcentration procedure described in Section 2.3, prior to CE separation.

Using a photodiode detector allowed us to confirm the identity of the peaks, not only from migration times, but also by comparing the UV–vis spectra for samples with those for a standard. Peak purity was also assessed in all tests.

So, the utility of our off-line SPE electrophoretic and chromatographic procedures were demonstrated by means of the qualitative and quantitative analysis of ROX, RAN, FAM, CIM and NIZ in several urine samples.

All samples were analysed in triplicate. The results were confirmed by using the HPLC method described in Section 3.2. There was no evidence of systematic errors affecting the determination of the antiulcers in urine with the proposed method. The analyte concentrations found are shown in Table 8.

Fig. 5 shows the electropherogram for a mixture of five human urine samples obtained from several patients under treatment with roxatidine, ranitidine, famotidine, cimetidine and nizatidine, respectively. The unknown peaks in it may be due to metabolites or interferents present in the urine samples.

4. Conclusions

In this work, we used two complementary techniques based on different physical principles (HPLC and CE) in order to develop two methods for the simultaneous detection and quantitation of ROX, RAN, FAM, CIM and NIZ in human urine using UV detection. We report for the first time a universal and full NACE validated method for determining five H_2 antagonists in human urine. It provides lower LOD than those reported by Ashiru et al. HPLC method. Furthermore, the use of an orthogonal HPLC method allowed compare and validate the results achieved by NACE. The reliability of both methods were certified by means of an exhaustive validation on urine extracts in terms of precision, selectivity, linearity, LODs and LOQs. Also, CE uses solvents and reagents sparingly, is more expeditious and provides higher peak efficiencies.

The main contribution of the work lies in the simultaneous robustness/ruggedness evaluation proposed, where the overall influence of external and internal (chemical and instrumental) factors on method parameters was simultaneously considered.

Having proved the reliability of our off-line SPE electrophoretic and chromatographic methods, it was successfully applied to the qualitative and quantitative analysis of the analytes in actual clinical urine samples. The utility of our method for obtaining relevant data concerning the pharmacokinetic screening of active principle was demonstrated. As a consequence, we can conclude that the proposed methods are suitable and more sensitive for the analysis of five H_2 antagonists in urine.

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References

[1] C.F. Perez, H.J. Olguin, J.F. Perez, I.L. Asseff, C.A. Garcia, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 795 (1) (2003) 141.
[2] A. Castro, A. Arancibia, P. Romero, M.N. Gai, Pharmazie 58 (10) (2003) 696.
[3] A. Ahmadiani, H. Amino, J. Chromatogr. B: Biomed. Appl. 751 (2) (2001) 291.
[4] V. David, M. Ionescu, F. Tache, A. Medvedovici, Chem. Anal. (Warsaw) 46 (6) (2001) 865.
[5] M.A. Campanero, A. Lopez Ocariz, E. García Quetglas, B. Sadaba, A. de la Maza, Chromatographia 47 (7–8) (1998) 391.
[6] E. Anzenbacherova, K. Filipova, M. Nobilis, P. Anzenbacher, J. Sep. Sci. 26 (8) (2003) 722.
[7] W.X. Peng, H.D. Li, B.K. Zhang, Yaowu Fenxi Zazhi 22 (1) (2002) 34.
[8] T.C. Dowling, R.F. Frye, J. Chromatogr. B: Biomed. Appl. 732 (1) (1999) 239.
[9] A. Zarghi, H. Komeilizadeh, M. Amini, L. Kimiagar, Pharm. Pharmacol. Commun. 4 (2) (1998) 77.
[10] L. Zhong, K.C. Yeh, J. Pharm. Biomed. Anal. 16 (6) (1998) 1051.
[11] M. Krishna Kumar, G. Jayasagar, K. Chandrasekhar, T. Ashok, Y. Madhusudan Rao, Pharmazie 58 (4) (2003) 284.
[12] H.L. Li, S.Y. Gao, D. Jin, G.Z. Ying, Yaowu Fenxi Zazhi 21 (6) (2001) 434.
[13] Y.R. Tahboub, M.F. Zaater, N.M. Najib, Quim. Anal. (Barcelona) 17 (13) (1998) 117.
[14] M.J. Nozal, J.L. Bernal, L. Toribio, M.T. Martin, F.J. Diez, J. Chromatogr. A 919 (1) (2001) 87.
[15] W.V. Caulfield, J.T. Stewart, Chromatographia 54 (9–10) (2001) 561.
[16] N. Helali, F. Darghouth, L. Monser, Chromatographia 60 (7–8) (2004) 455.
[17] D.E. Latch, B.L. Stender, J.L. Packer, W.A. Arnold, K. McNeill, Environ. Sci. Technol. 37 (15) (2003) 3342.
[18] D. Zendelovska, T. Stafilov, J. Pharm. Biomed. Anal. 33 (2) (2003) 165.
[19] E.W. Chung, E.N.M. Ho, D.K.K. Leung, F.P.W. Tang, K.C.H. Yiu, T.S.M. Wan, Chromatographia 59 (Suppl. 1) (2004) S29.
[20] Y. Zhang, X.Y. Chen, H.Y. Yang, D.F. Zhong, Shenyang Yaoke Daxue Xuebao 19 (5) (2002) 340.

[21] T. Iqbal, C.S. Karyekar, M. Kinjo, G.C. Ngan, T.C. Dowling, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 799 (2) (2004) 337.
[22] J.W. Luo, H.W. Chen, Q.H. He, Chromatographia 53 (5–6) (2001) 295.
[23] D.R. Ellis, M.E. Palmer, L.W. Tetler, C. Eckers, J. Chromatogr. A 808 (1–2) (1998) 269.
[24] M.A. Nelly, K.D. Altria, C. Grace, B.J. Clark, J. Chromatogr. A 798 (1–2) (1998) 297.
[25] Y. Gao, Y.L. Tian, X.H. Sun, X.B. Yin, Q. Xiang, G. Ma, E.K. Wang, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 832 (2) (2006) 236.
[26] T. Perez Ruiz, C. Martinez Lozano, V. Tomas, E. Bravo, R. Galera, J. Pharm. Biomed. Anal. 30 (4) (2002) 1055.
[27] S.M. Wu, Y.H. Ho, H.L. Wu, S.H. Chen, H.S. Ko, Electrophoresis 22 (13) (2001) 2717.
[28] S.M. Wu, Y.H. Ho, H.L. Wu, S.H. Chen, H.S. Ko, Electrophoresis 22 (13) (2001) 2758.
[29] Y.S. Chang, Y.R. Ku, K.C. Wen, L.K. Ho, J. Liq. Chromatogr. Relat. Technol. 23 (13) (2000) 2009.
[30] K.M. Kalani, M. Aziz, M.A. Hegazt, L.A. Fattah, J. AOAC Int. 85 (5) (2002) 1015.
[31] C. Ho, H.M. Haung, S.Y. Hsu, B.L. Chang, Drug Dev. Ind. Pharm. 25 (23) (1999) 379.
[32] D.A.I. Ashiru, R. Patel, A.W. Basit, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 860 (2) (2007) 235.
[33] J.L. Burrows, K.W. Jolley, D.J. Sullivan, J. Chromatogr. Biomed. Appl. 76 (1988) 199 (J. Chromatogr. 432).
[34] A.P. Argekar, S.S. Kunjir, Indian Drugs 32 (7) (1995) 333.
[35] B.S. Shin, J.W. Choi, J.P. Balthasar, D.K. Hong, J.J. Kim, S.D. Yoo, Rapid Commun. Mass Spectrom. 21 (3) (2007) 329.
[36] J.H. Ryu, S.J. Choi, H.W. Lee, S.K. Choi, K.T. Lee, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 876 (1) (2008) 143.
[37] J.J. Berzas Nevado, G. Castañeda Peñalvo, R.M. Rodríguez Dorado, Anal. Sci. 27 (4) (2011) 427.
[38] ICH Guidance for Industry: Q2B Text on Validation of Analytical Procedures, US Department of Health and Human Services, Foods and Drugs Administration, CDER, Rockville, USA, 1996.
[39] R.L. Plakett, J.P. Burman, Biometrika 33 (1946) 305.
[40] Handbook of Pharmaceutical, Generic Development, Sect. 13.41, Chapter 13, http://www.locumusa.com/pdf/general/article01.pdf