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RESEARCH ARTICLE

Use of a monolithic column for the development and validation of a HPLC method for the determination of famotidine, cimetidine and nizatidine in biological fluids

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A simple and selective HPLC method, using a monolithic column, was developed for the simultaneous determination of the histamine H_2-receptor antagonists: famotidine, cimetidine and nizatidine, in the presence of sulfadimethoxine as internal standard. The separation was performed on a Chromolith Performance RP-18 column (100 mm x 4.6 mm i.d.) with an isocratic mobile phase consisting of 0.05 mol/L acetate buffer (adjusted to pH 6.5 with triethylamine)/methanol/acetonitrile (85:10:5, v/v/v). The wavelength was set at 230 nm. Linearity was obtained for concentrations between 0.2 to 50 μg/mL and limits of detection were in the range 0.07-0.17 μg/mL. Full validation with respect to linearity, selectivity, detection and quantification limits, accuracy, precision and robustness, the latter using the Youden's test, was carried out. The method was successfully applied to the determination of the drugs in human serum and urine following solid phase extraction. Average recoveries between 88.0 to 104.4% and 88.0 to 108.0% in serum and urine samples, respectively, were obtained.

Keywords: cimetidine, famotidine, nizatidine, HPLC, monolithic column, serum, urine.

Introduction

Histamine H_2-receptor antagonists (H_2RAs)- famotidine (FAM), cimetidine (CIM) and nizatidine (NIZ)- constitute a drug class used to block the action of histamine on parietal cells in the stomach in order to reduce their acid production, therefore they are widely used in the treatment of gastro-esophageal reflux disease and gastric and duodenal ulceration [1]. The determination of these drugs in pharmaceutical preparations for quality control and in biological fluids has remained of analytical interest in studying drug interactions, preventing drug toxicity, and optimizing drug therapy in patients [2]. A literature survey retrieved a large number of references reporting analytical methods for the determination of these compounds.

Mainly separation techniques such as Gas Chromatography (GC) [2], High Performance Thin Layer Chromatography (HPTLC) [3-10], Capillary Electrophoresis (CE) [11-17] and High Performance Liquid Chromatography (HPLC) [18-34] have been used, with the latter being the predominant technique for the determination of these compounds in a variety of matrices, utilizing different methods of sample pretreatment and detection modes. HPLC with ultra-violet (UV) detection, employing reversed phase particle packed columns, is most frequently used for the analysis of these drugs in pharmaceutical preparations and biological fluids. An overview of recently reported HPLC methods for the determination of FAM, CIM and NIZ (either individually or along with

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other compounds) is presented in **Table 1**.

Monolithic stationary phases have attracted considerable attention in liquid chromatography due to their unique properties and excellent performance, especially for separation of drugs in biological samples. Zarghi et al. [34] proposed an HPLC method for the determination of FAM in human plasma using a monolithic column. However, to the best of our knowledge, there has been no report of a single universal HPLC assay capable of analyzing all three histamine H_2RAs, with a monolithic column, in biological samples, such as human serum and urine. Analysis, especially from urine, is a very useful means of determining the bioavailability of these drugs in human subjects. Blood, serum or plasma, are the preferred specimen for toxicological analyses if the objective is to assess the acute impact of a drug on a person. The present study describes a simple HPLC-UV method for the simultaneous determination of FAM, CIM and

Table 1. Overview of the most recent HPLC methods for the determination of histamine H_2RAs.

Method	Column	Compounds	Detection	Application	Ref.
HPLC (isocratic)	Phenomenex Luna SCX (250 mm × 4.6 mm i.d., 5 µm)	FAM, CIM, RAN, NIZ	UV (λ=230 nm)	Urine	[18]
HPLC (isocratic)	Nova-Pack C18 (150 mm × 3.9 mm i.d., 4 µm)	CIM	MS/MS	Plasma	[19]
HPLC (isocratic)	n.r.	FAM, CIM, RAN, CET	UV (λ=230 nm)	Formulations, serum	[20]
LC (isocratic)	Zorbax SB-CN (150 mm × 2.1 mm i.d., 5 µm)	FAM, CIM, RAN, LAF	MS/MS	Plasma	[21]
HPLC (isocratic)	Purospher Star C18 (250 mm × 4.6 mm i.d., 5 µm)	FAM, CIM, RAN, MET	UV (λ=229 nm)	Formulations, serum	[22]
UPLC (gradient)	Acquity UPLCTM HSS T3 (100 mm × 2.1 mm i.d., 1.8 µm)	NIZ, MP, PP	UV (λ=254 nm)	Formulations	[23]
HPLC (gradient)	Phenomenex Synergi Hydro-RP (150 mm × 4.6 mm i.d., 4 µm)	FAM	MS	Plasma, urine	[24]
HPLC (ioscratic)	Thermo Hypersil BDS-C8 (250 mm × 4.6 mm i.d., 5 µm)	NIZ	DAD (λ=320 nm)	Bulk and capsules dosage form	[25]
HPLC (isocratic)	Nova Pack C18 (150 mm × 3.9 mm i.d., 5 µm)	FAM, CIM, NIZ, RAN, ROX	UV (λ=214 nm)	Urine	[26]
HPLC (isocratic)	Purospher Star C18 (250 mm × 4.6 mm i.d., 5 µm)	FAM, CIM, RAN, CPT	UV (λ=225 nm)	Serum	[27]
HPLC (isocratic)	Inertsil C18 (150 mm × 4.6 mm i.d., 5 µm)	NIZ	FL (λex/λem=461/517 nm)	Plasma	[28]
HPLC (isocratic)	Phenomenex Gemini C18 (150 mm × 4.6 mm i.d., 5 µm)	CIM	MS/MS	Plasma	[29]
HPLC (isocratic)	LiChrosorb C18 (250 mm × 4.6 mm i.d., 5 µm)	FAM	UV (λ=274 nm)	Plasma	[30]
HPLC (isocratic)	Eclipse Plus C18 (100 mm × 4.6 mm i.d., 5 µm)	NIZ	MS/MS	Plasma, urine	[31]
HPLC (isocratic)	Symmetry C18 (150 mm × 4.6 mm i.d., 3.5 µm)	FAM, IBP	UV (λ=236 nm)	Plasma	[32]
HPLC (isocratic)	Waters Symmetry C18 (250 mm × 4.6 mm i.d., 5 µm)	FAM	UV (λ=498 nm)	Pharmaceuticals	[33]
HPLC (isocratic)	Chromolith Performance C18 (100 mm × 4.6 mm i.d.)	FAM, CIM, NIZ	UV (λ=230 nm)	Serum, urine	This work

RAN = ranitidine, CET = cetirizine, LAF= lafutidine, MET = metformin, MP = methylparaben, PP = propylparaben, ROX = roxatidine, CPT = captopril, IBP = ibuprofen, DAD = diode array detection, FL = fluorescence, n.r. = not reported.

NIZ, in human serum and urine, utilizing a monolithic column and a solid phase extraction (SPE) clean-up step. An extensive validation study was also carried out including a robustness evaluation according to the Youden approach. A sensitive method for the simultaneous determination of the three histamine H_2-receptor antagonists could be very meaningful and imperative. It is also very useful in a routine laboratory to save time and solvents. Namely, the developed method allows determination of each drug without the need for development of separate and distinct methods for each analyte.

Materials and Methods

FAM, CIM, NIZ and the internal standard, sulfadimethoxine sodium salt (SDMX), were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The acetate (0.05 mol/L)-triethylamine buffer), used in the mobile phase, was prepared daily in a 500-mL volumetric flask by mixing 25 mL sodium hydroxide and 27 mL acetic acid from their respective equimolar solutions (1mol/L) and diluting to volume with double – deionised water. The resulting pH of the acetate buffer was around 5.8. The pH-adjustment to 6.5 was made with triethylamine (TEA), which also served as a peak modifier. Sodium hydroxide (min. 99%), acetic acid (glacial 100% w/w) and triethylamine TEA were supplied by Merck (Darmstadt, Germany). Potassium dihydrogen phosphate and phosphoric acid were obtained from Merck and Panreac (Barcelona, Spain) respectively. Methanol, acetonitrile and acetone, obtained from ChemLab (Zedelgem, Belgium), were of HPLC grade. All other chemicals were of analytical-reagent grade. Double-deionised water was used throughout the work. Both water and buffer solution were filtered under vacuum through 0.22-μm cellulose membrane filters (Whatman Labotatory Division, Maidstone, England) prior to use. ABS Elut-Nexus (60 mg/3 mL) cartridges obtained from Agilent Technologies (USA) were used for the SPE of the samples.

Centrifuged whole blood samples, collected from five healthy volunteers, were provided by the Laboratory of Toxicology of the Medical School of the Aristotle University of Thessaloniki and the supernatant serums were mixed in order to obtain a pooled sample. Urine specimens were collected from five healthy volunteers and were similarly mixed in order to obtain a pooled sample. All samples were stored at -20 ºC until analysis.

Equipment

The HPLC system consisted of a Shimadzu LC-9A binary pump (Kyoto, Japan), an AS3000 autosampler (Thermo Scientific), an Elite™ vacuum degasser (Alltech, USA) and a Shimadzu SPD-10A UV/VIS detector (Kyoto, Japan). Data acquisition was carried out via the Clarity® software (DataApex, Czech Republic) running under Windows® XP. The analytical column was a Chromolith Performance RP-18 end-capped (100 mm x 4.6 mm i.d.) column, obtained from Merck (Darmstadt, Germany).

A glass vacuum solvent-filtration apparatus, obtained from Altech Associates Inc. (Deerfield, IL, USA) was employed for the filtration of the aqueous portion of the mobile phase. An Orion EA940 pH meter was used to adjust the pH of the mobile phase. Degassing of solvents was carried out by helium sparging before use. Dissolution of compounds was aided by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany). UV-spectra were obtained with a Jasco V530 spectrophotometer. A Glass-col Terre Haute In 47802 small vortexer and a Hermle Z230 centrifuge (Gosheim, Germany) were used for the treatment of biological fluids.

SPE was performed on a Supelco (Bellefonte, PA, USA) VisiprepTM vacuum manifold system, having a 12-position capacity. Evaporation of solvents was carried out, at ambient temperatures, under a stream of nitrogen, by means of a model 18825 Reacti-Vap device from Thermofischer Scientific (Rackford, USA).

Chromatographic conditions

The analysis was performed under isocratic conditions, with a mobile phase consisting of acetate (0.05 mol/L)-TEA buffer)/methanol/acetonitrile (85:10:5, v/v/v). The pH of the acetate buffer was adjusted to 6.5 with TEA, which also served as a peak modifier. The flow rate was 1 mL/min, and the injected volume 20 μL. Under these conditions, the compounds were eluted in less than 15 min and detected at 230 nm.

Procedures

Preparation of standard solutions

Primary stock standard solutions (1000 μg/mL) of each compound and of the internal standard were prepared in methanol and stored in the dark at -20 ºC, for 1 month. Secondary stock solutions (100 and 50 μg/mL) were prepared by serially diluting the corresponding 1000 μg/mL primary stock solutions in 10-mL volumetric flasks with mobile phase and stored protected from light at 4 ºC, for 1 week. The multi-component working solutions were prepared just prior to use. Eight multi-component standard mixtures, containing all three compounds, at concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 μg/mL with respect to each compound, were prepared by transferring the appropriate volumes from the

individual secondary stock solutions of each compound to 10-mL volumetric flasks and bringing to volume with mobile phase, after the addition of SDMX (1 mL of a 100 μg/mL solution), so as to obtain a final internal standard concentration of 10 μg/mL. Calibration curves were constructed by plotting ratios of analyte to internal standard peak areas against concentrations of the analytes.

Sample treatment and solid phase extraction

In order to take into account the influence of the matrix on the measured signal, the matrix-matched calibration was adopted by fortifying pooled drug-free samples from healthy volunteers.

Fortified serum samples were prepared by spiking six 100 μL aliquots of the pooled human drug-free serum with 100 μL of aqueous standard mixtures of FAM, CIM and NIZ (without the internal standard) at concentrations ranging between 0.5 and 20 μg/mL in Eppendorf tubes. A volume of 100 μL of acetonitrile was then added. The samples were vortex-mixed and centrifuged at 3500 rpm (548 *x g*) for 15 min. The supernatants were transferred into clean Eppendorf tubes and the organic solvents were evaporated under a stream of nitrogen, at room temperature. The remaining aqueous phases of the supernatants were transferred to ABS Elut-Nexus SPE cartridges, which had been preactivated and preconditioned with one cartridge volume of methanol followed by one volume of water. The cartridges were subsequently washed with one cartridge volume of water and dried applying mild vacuum. The compounds were eluted with 2.5 mL of methanol and the eluates were evaporated to dryness, at room temperature, under a stream of nitrogen. The residues of the samples were reconstituted with 100 μL of a 10 μg/mL SDMX solution in mobile phase and vortex-mixed before injection into HPLC. A blank sample was also prepared, by adding a 100 μL volume of water (instead of a standard mixture), to a 100 μL aliquot of serum, following for the rest, the same SPE procedure, as that applied to fortified samples. The residue of the blank sample was reconstituted with 100 μL of mobile phase (no internal standard added).

Fortified urine samples were prepared by spiking six 100 μL aliquots of the undiluted human drug-free urine with 100 μL volumes of aqueous standard mixtures of the compounds (without the internal standard), at concentrations ranging between 0.5 and 20 μg/mL in Eppendorf tubes. For the preparation of the blank sample, a 100 μL aliquot of the undiluted human urine was placed in an Eppendorf tube with 100 μL of water. The samples were vortex-mixed and transferred to the SPE cartridges. The SPE procedure was identical to that applied to serum samples, except for the washing step which was conducted with one cartridge volume of a 5% volume fraction of methanol/water rather than water. Reconstitution of the residues was performed with a 100 μL volume of a 10 μg/mL SDMX solution in mobile phase. The residue of the blank sample was reconstituted with 100 μL of mobile phase (no internal standard added).

Stability

The stability of the analytes in serum and urine during the sample storage period was assessed. Stability was evaluated under different conditions that occurred during sample analysis. The short-term stability was evaluated by keeping spiked samples at room temperature for 24 h. The long-term stability was assessed by spiked samples kept at low temperature (-20 ºC) for 10 days. The post-preparative stability was measured by placing spiked samples under the autosampler conditions for 24 h. The freeze- and thaw stability was tested by analyzing spiked samples undergoing three freeze (-20 ºC)–thaw (room temperature) cycles on consecutive days. When completely thawed, the samples were refrozen for 24 hours under the same conditions. The freeze–thaw cycle was repeated two more times, then analysed on the third cycle. The stock solutions were also evaluated for stability at room temperature for 24h and at 4 ºC for 10 days. All samples were spiked at two concentration levels (low and high) and were run in triplicate.

Results and discussion

Optimization of chromatographic conditions

Keeping the flow rate at 1 mL/min, and detection at 230nm, a multi-component mixture of the analytes was run with different isocratic elution systems, in order to achieve optimum separation. Testing different volume fractions of potassium dihydrogen phosphate/phosphoric acid (0.02 mol/L, pH<5) and methanol, it was observed that at acidic pH, the retention of FAM, CIM and NIZ decreased and the compounds co-eluted. Increasing the pH (by means of either TEA or NaOH), different mixtures of potassium dihydrogen phosphate/phosphoric acid (0.02 mol/L)/methanol or acetate buffer (0.05 mol/L)/methanol were also tested. Improved separation of the drugs was observed at pH-values higher than 6, although the separation of the CIM-NIZ pair was still inadequate.

Addition of acetonitrile to the mobile phase improved the peak shape and symmetry, as well as the separation of the CIM-NIZ pair, which in all other cases was in-

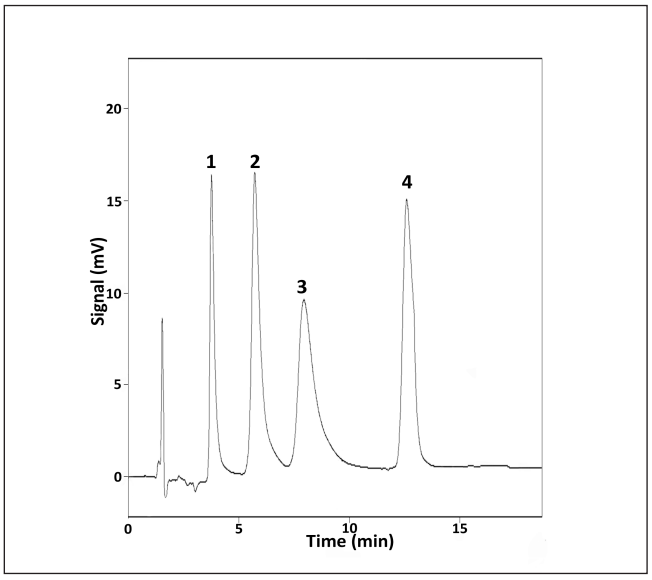

Figure 1. Representative chromatogram of a standard multi-component mixture (5 μg/mL) of the H_2RAs with internal standard; peaks: 1, FAM; 2, CIM; 3, NIZ; 4, internal standard (SDMX) (10 μg/mL). Chromatographic conditions as described under Experimental.

adequate. Optimum separation was finally obtained with an isocratic mobile phase consisting of acetate (0.05 mol/L)-TEA buffer (pH 6.5)/methanol/acetonitrile (85:10:5, v/v/v). The pH of the acetate buffer was adjusted to 6.5 with TEA. The use of higher pH values can also provide improved separation, but may also cause partial dissolution of the silica stationary phase. Under the selected HPLC conditions, well resolved peaks of all three H_2RAs, and the internal standard SDMX, eluted in less than 15 minutes, as shown in the chromatogram of **Figure 1**. SDMX was selected among several other compounds, which were tested for their suitability as internal standards.

SPE optimization and extraction efficiency

Methanol and acetonitrile were tested for their elution capability in a 10 μg/mL standard multi-component mixture of the analytes, and the results were compared with those of a standard non-extracted mixture of identical concentration. In each case a 2.5 mL volume of the solvents was used. Conditioning was carried out with one cartridge volume of methanol followed by one cartridge volume of water, while washing was with one cartridge volume of water. The recoveries were calculated and methanol proved to be the best choice.

Different washing solvents were tested in order to establish the most efficient matrix clean-up in urine and serum samples, in terms of recovery. In order to find the best washing solvent, for urine samples, a 5% volume fraction of methanol/water, acetonitrile/water and acetone /water, as well as plain water, were tested for their ability to remove matrix interferences, and achieve an efficient matrix clean-up during the washing step, with no analyte loss. In each case, one cartridge volume was used. The procedure was applied to both a 10 μg/mL standard multi-component mixture of the analytes and a pooled urine sample, spiked with a standard mixture of identical concentration. Conditioning was carried out with one cartridge volume of methanol followed by one cartridge volume of water, while elution was with methanol. The recoveries were calculated to evaluate the extraction efficiency from urine samples, relative to standard solutions, and on this basis, to select the best washing solvent, which in this case was a 5% volume fraction of methanol /water. The above procedure was repeated in serum samples, spiked with a 10 μg/mL standard multi-component mixture of the analytes. Plain water provided the highest relative recoveries for all compounds and was therefore chosen as washing solvent. The optimum SPE results are summarized in **Table S1** in the Supplementary Material, where the extraction efficiency, is expressed as absolute and relative recovery of the extracted analytes from standard and spiked samples, respectively. Higher relative recoveries of the analytes from spiked samples than from the processed standard solution, might be attributed to matrix-sorbent interactions, i.e. endogens from matrix facilitate the elution of the drugs by occupying polar groups of the sorbent surface, thus reducing the possibility of polar interactions between isolate-sorbent. Lower relative recoveries in spiked samples than in the standard might be due to isolate-matrix interactions. Variations in absolute recoveries of the drugs may be due to polar interactions between isolate-sorbent.

Method validation

The validation of the method was carried out through evaluation of selectivity, linearity, sensitivity, precision, accuracy and robustness.

Selectivity

The specificity of the peaks for the histamine H_2-receptor antagonists was assessed from several injections of urine and/or serum blank samples previously subjected to SPE. The results revealed the absence of interferences due to matrix effects, i.e. no peaks from endogenous compounds were observed at the retention time of each analyte and internal standard in any of the blank extracts evaluated.

Table 2. Linearity data in standard solutions and spiked pooled samples.

H_2RA	Regression equation $y = (a \pm S_a) + (b \pm S_b)x$	Correlation coefficient	Linear range (μg/mL)	LOD (μg/mL)
FAM	$y^w = (-0.0088 \pm 0.0031) + (0.0799 \pm 0.0002)x^w$	0.9999^w	$0.2\text{-}50^w$	0.07^w
	$y^u = (-0.0056 \pm 0.0041) + (0.0544 \pm 0.0004)x^u$	0.9997^u	$0.5\text{-}20^u$	0.17^u
	$y^s = (-0.0171 \pm 0.0013) + (0.0456 \pm 0.0001)x^s$	0.9999^s	$0.5\text{-}20^s$	0.17^s
CIM	$y^w = (-0.0193 \pm 0.0057) + (0.1499 \pm 0.0003)x^w$	0.9999^w	$0.2\text{-}50^w$	0.07^w
	$y^u = (-0.0157 \pm 0.0059) + (0.1061 \pm 0.0006)x^u$	0.9999^u	$0.5\text{-}20^u$	0.17^u
	$y^s = (-0.0233 \pm 0.0078) + (0.1011 \pm 0.0008)x^s$	0.9997^s	$0.5\text{-}20^s$	0.17^s
NIZ	$y^w = (-0.0108 \pm 0.0018) + (0.1487 \pm 0.0001)x^w$	0.9999^w	$0.5\text{-}50^w$	0.17^w
	$y^u = (-0.0044 \pm 0.0022) + (0.1057 \pm 0.0002)x^u$	0.9999^u	$0.5\text{-}20^u$	0.17^u
	$y^s = (-0.0296 \pm 0.011) + (0.0904 \pm 0.0012)x^s$	0.9993^s	$0.5\text{-}20^s$	0.17^s

y = peak area ratio of H_2RA to internal standard (n = 3); x = concentration in μg/mL; a = intercept; b = slope; S_a, S_b = standard error of intercept and slope, respectively; ^{w} in standard solutions; ^{u} in urine; ^{s} in serum.

Linearity and sensitivity

Linearity was evaluated by aqueous and matrix-matched calibration. Calibration curves in standard solutions, as well as in serum and urine samples (matrix-matched calibration), were constructed by plotting the analyte to internal standard peak-area ratios (mean values of three replicate measurements of standards or samples) as a function of the analyte concentrations. Linear least squares regression was used to calculate the slopes, intercepts and correlation coefficients. Linearity in aqueous solutions was obeyed in the range 0.2-50 μg/mL (**Table 2**) with limits of detection (LOD) of 0.07 μg/mL, for FAM and CIM and 0.17 μg/mL for NIZ, calculated on the basis of a peak-to-peak signal-to noise ratio (S/N) 3:1. The limits of quantification (LOQ) were the lowest concentrations determined, with acceptable precision, with a signal-to-noise ratio (S/N) of 10:1 and were found to be 0.2 μg/mL for FAM and CIM and 0.5 μg/mL for NIZ. The linearity of the method in the biological matrices was evaluated in spiked pooled samples from healthy individuals (n=5). The samples were treated according to the procedure described in Sample treatment and solid

Table 3. Within-day and between-day precision of assay for the determination of FAM, CIM and NIZ in standard solutions.

H_2RA	Injected concentration μg/mL	Measured concentration ± SD[a] μg/mL		RSD (%)	
		WD	BD	WD	BD
FAM	0.5	0.53 ± 0.003	0.52 ± 0.001	0.57	0.19
	5.0	5.09 ± 0.008	5.08 ± 0.003	0.16	0.06
	50.0	50.02 ± 0.007	50.01 ± 0.007	0.01	0.01
CIM	0.5	0.51 ± 0.002	0.51 ± 0.003	0.39	0.59
	5.0	5.02 ± 0.025	5.03 ± 0.001	0.50	0.02
	50.0	50.04 ± 0.004	50.04 ± 0.004	0.01	0.01
NIZ	0.5	0.51 ± 0.002	0.51 ± 0.001	0.39	0.20
	5.0	5.02 ± 0.07	5.01 ± 0.001	1.39	0.02
	50.0	49.99 ± 0.006	49.99 ± 0.003	0.01	0.01

SD = standard deviation, RSD = relative standard deviation.[a] Mean of values calculated from the regression line equation for eight determinations within a day (WD) and three determinations per day over five days (BD).

Table 4. Within-day precision and accuracy of assay for the determination of FAM, CIM and NIZ in serum and urine samples.

H_2RA	Added concentration to serum and urine (μg/mL)	Mean measured concentration ± SD[a] (μg/mL)		RSD (%)		Recovery (%)	
		Serum	Urine	Serum	Urine	Serum	Urine
FAM	0.5	0.52 ± 0.001	0.48 ± 0.006	0.19	1.25	104.0	96.0
	1.0	1.03 ± 0.011	1.08 ± 0.001	1.07	0.09	103.0	108.0
	2.0	1.94 ± 0.009	2.10 ± 0.008	0.46	0.38	97.0	105.0
	5.0	5.05 ± 0.003	4.77 ± 0.004	0.06	0.08	101.0	95.4
	10.0	9.34 ± 0.006	10.05 ± 0.010	0.06	0.10	93.4	100.5
	20.0	20.02 ± 0.003	20.02 ± 0.005	0.01	0.02	100.1	100.1
CIM	0.5	0.48 ± 0.015	0.48 ± 0.040	3.13	8.33	96.0	96.0
	1.0	0.88 ± 0.001	1.07 ± 0.017	0.11	1.59	88.0	107.0
	2.0	1.93 ± 0.005	1.89 ± 0.001	0.26	0.05	96.5	94.5
	5.0	5.22 ± 0.003	5.12 ± 0.002	0.06	0.04	107.4	102.4
	10.0	10.07 ± 0.003	9.92 ± 0.001	0.03	0.01	100.7	99.2
	20.0	19.93 ± 0.006	20.02 ± 0.007	0.03	0.03	99.7	100.1
NIZ	0.5	0.50 ± 0.001	0.44 ± 0.001	0.20	0.23	100.0	88.0
	1.0	0.96 ± 0.001	1.04 ± 0.003	0.10	0.29	96.0	104.0
	2.0	2.04 ± 0.002	2.02 ± 0.001	0.10	0.05	102.0	101.0
	5.0	4.77 ± 0.005	5.01 ± 0.002	0.10	0.04	95.4	100.2
	10.0	10.35 ± 0.006	9.98 ± 0.002	0.06	0.02	103.5	99.8
	20.0	19.88 ± 0.007	20.00 ± 0.009	0.04	0.05	99.4	100.0

SD = standard deviation, RSD = relative standard deviation[a] Means of values calculated from the regression line equation for three determinations within a day (WD).

phase extraction and were spiked with elevated amounts of the H_2RAs (n=6). The linearity, expressed as the correlation coefficient of the calibration slopes from both aqueous and matrix-matched calibration, was higher than 0.999 for all compounds.

Precision and accuracy

The precision of the method in standard solutions was evaluated in terms of injection repeatability (within-day precision) and intermediate precision (between-day precision), both expressed as a relative standard deviation (RSD), and it was assessed at a low, medium and high concentration level of standard solutions. Repeatability was determined by performing eight repeated injections of each of the three multi-component H_2RAs standards (0.5, 5.0 and 50.0 μg/mL) on the same day, with the same instrument and the same operator, while intermediate precision was calculated on the basis of the results from triplicate measurements of each of the three standard H_2RAs mixtures (0.5, 5.0 and 50.0 μg/mL) conducted during routine operation of the system over five consecutive days. The data collected are illustrated in **Table 3**. The within- and between-day RSDs ranged from 0.01 to 1.39% and from 0.01 to 0.59% respectively, for all the substances.

For the assessment of the within-day precision and accuracy of the assay in serum and urine samples, the within-day RSD and the recovery of the analytes, from spiked samples respectively, was calculated by means of the matrix-matched calibration. The accuracy of the method is reflected upon the recovery of FAM, CIM and NIZ from serum- and urine drug free samples, spiked at six concentration levels of the H_2RAs. Recovery was calculated as the percentage of the mean measured versus the added concentrations of the analytes. The experimental findings are summarized in **Table 4** and are based on

triplicate measurements. The recoveries were acceptable in all cases, ranging between 88.0 and 104.4% in serum and between 88.0 and 108.0% in urine samples.

Stability

The stability of FAM, CIM and NIZ in human serum and urine samples under different conditions was evaluated. The results are shown in **Tables S2 and S3** in the Supplementary Material and were expressed as mean percentage of the analyte concentration determined at certain time point relative to that at time zero (freshly prepared solutions). No significant degradation of the analytes occurred after short-term storage for 24 h at room temperature, long-term storage for 10 days at -20 ºC, three freeze-and thaw cycles, or post-preparative storage for 24h. The stock solutions were stable for 24h at room temperature and for 10 days at 4 ºC.

Robustness evaluation

The robustness evaluation of the chromatographic method was performed using the method proposed by Youden and Steiner [35]. The basic idea is instead of studying one alteration at a time, several variations are introduced at once. This was performed by adopting the experimental design described in Decision 2002/657/EC and tested by the introduction of seven small but deliberate changes in the analytical parameters and by the consequent assessment of their influence on the method results [36]. Seven analytical parameters were selected, on the basis of their influence on the chromatographic behavior of the method, and small variations (higher and lower) were introduced in the standard (nominal) conditions of the method. The analytical parameters at the higher values than nominal are represented by capital letters A to G, and the conditions with the lower values than nominal are denoted by the corresponding lowercase letters a to g. Eight runs, which use a combination of the chosen factors (A-G) were performed, in order to determine the influence of each parameter in the final result. The results of the determinations are shown by letters from s to z.

The seven parameters and its respective variations were combined in eight assays or chromatographic runs, performed in a random order. **Table 5** presents the seven analytical parameters employed, the introduced variations and the factorial combination of the parameters for the Youden's test. In each combination, two injections of a multicomponent standard solution (10 μg/mL) were made. The influence of the seven analytical parameters regarding retention time (tR) and resolution (Rs) was evaluated. By means of the Youden's test it is possible to establish the parameters which have a higher impact on the final result of the analyses and perform a more thorough control in the eventual variations of these parameters that may occur during a routine analysis.

To determine the influence of variations of each parameter in the final result, the difference D_i was calculated comparing the mean of the four values corresponding to the capital letters (higher values than nominal) to the mean of the four values corresponding to the lowercase letters (lower values than nominal). For example, to evaluate the effect of the pH-value of the buffer solution in the final result of the analyses, the following equation

Table 5. Analytical parameters with their variations and factorial combination for robustness evaluation by Youden's test.

Analytical parameter	Nominal condition	Higher level (capital letters)	Lower level (lowercase letters)	Factorial combination							
				1	2	3	4	5	6	7	8
A/a: Buffer solution pH	6.5	6.7	6.3	A	A	A	A	a	a	a	a
B/b: Methanol concentration (%) in mobile phase	10.0	12	8	B	B	b	b	B	B	b	b
C/c: Methanol supplier	ChemLab	ChemLab	Lichrosolv Merck	C	c	C	c	C	c	C	c
D/d: Acetonitrile supplier	Sigma Aldrich	Sigma Aldrich	ChemLab	D	D	d	d	d	d	D	D
E/e: Detection wavelength (nm)	230	232	228	E	e	E	e	e	E	e	E
F/f: Flow-rate (mL/min)	1.0	1.1	0.9	F	f	f	F	F	f	f	F
G/g: Concentration of CH_3COOH, NaOH (mol/L) in the buffer solution	1.0	1.05	0.95	G	g	g	G	g	G	G	G
Observed results				*s*	*t*	*u*	*v*	*w*	*x*	*y*	*z*

was employed:

$$D_{A/a} = \frac{s+t+u+v}{4} - \frac{w+x+y+z}{4}$$

Thus, the influence of all seven analytical parameters regarding the retention time (tR) and resolution (Rs) were evaluated.

The results obtained from the eight robustness experiments are reported in **Table S4** in the Supplementary Material, indicating that the parameter that affects primarily the resolution (Rs) is the pH of the buffer solution. This constitutes a logical conclusion, since the separation of these compounds is particularly susceptible to the mobile phase pH-changes, as shown by preliminary method development experiments. On the other hand, the parameters that seem to affect the retention time (tR) of the compounds are the percentage (%) of methanol in the mobile phase and to a lesser extent the apparent pH of the mobile phase and the flow-rate. The results for the other Some parameters variation show values that present such as methanol and acetonitrile suppliers, detection wavelength and concentration of buffer components presented low influence in the evaluated factors of the chromatographic method.

Method application

The quantification of FAM, CIM and NIZ in biological samples was performed by using the matrix-matched calibration in order to compensate for matrix effects, after the SPE step. The chromatograms of blank and spiked human serum- and urine samples are shown in **Figures 2 and 3**, respectively. The absence of interfering peaks in the samples confirms the efficiency of the applied SPE clean-up procedure which offers excellent selectivity to the method.

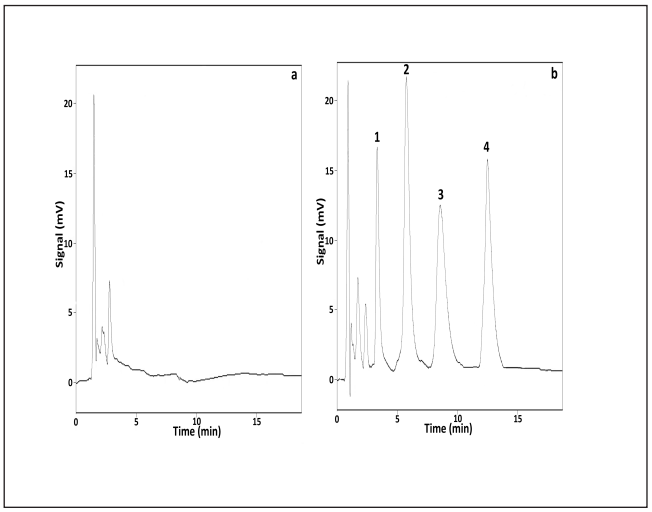

Figure 2. Chromatogram of a drug-free pooled serum sample; (a) blank and (b) spiked with a 10 μg/mL standard multi-component H_2RA mixture. Conditions and peak identification as in Figure 1.

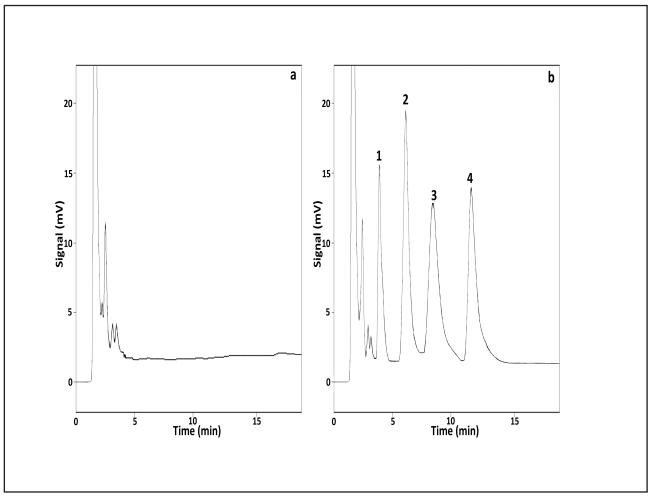

Figure 3. Chromatogram of a drug-free pooled urine sample; (a) blank and (b) spiked with a 10 μg/mL standard multi-component H_2RA mixture. Conditions and peak identification as in Figure 1.

Conclusions

The proposed method includes an useful and low cost analytical protocol for the determination of famotidine, cimetidine and nizatidine in the presence of an internal standard, sulfadimethoxine, in human serum and urine. The method is characterized by the following novel features: i) It is the first reported simultaneous determination of the three histamine H_2-receptor antagonists in both biological fluids employing a monolithic column. ii) It is sufficiently simple: it includes isocratic elution and a simple SPE clean-up step which offers high selectivity to the method. iii) It is fully validated with respect to selectivity, linearity, precision, accuracy and chromatographic method robustness, the latter using the Youden's test, by means of which it was possible to establish the parameters which present higher influence in the final result of the analyses, so as to perform a more rigorous control in the eventual variations of these parameters that may occur during routine operation. iv) The method exhibits very good linearity, precision and accuracy, as well as sufficient sensitivity, which can be controlled and further increased through preconcentration in the SPE procedure, so to meet the levels of the drugs in the specimens tested, after administration to patients. v) Without any

special requirement for the instruments and a high cost, the developed method can be used for results of high analytical quality, using conventional instruments in common laboratories for pharmacokinetic studies conducted in humans. Also this method offers the potential to be applied for analysis of real serum and /or urine samples after administration of famotidine or cimetidine or nizatidine, using the same mobile and stationary phase.

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