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HPLC METHOD FOR QUANTIFICATION OF NIZATIDINE AND ITS N-DESMETHYLMETABOLITE IN HUMAN PLASMA

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A simple and fast HPLC method for quantification of nizatidine and N-desmethylnizatidine in human plasma has been developed and validated. After protein precipitation from plasma sample with perchloric acid, clear supernatant was injected at 42 ºC into a C18 column. The elution was made in 4 minutes at 1 ml min^{-1} in gradient mode, with an UV detection at 314 nm, using a mixture of aqueous potassium dihydrogenophosphate 40 mM pH 2.3, methanol and acetonitrile. The linearity domains were established between 80 to 3200 ng ml^{-1} for nizatidine and between 12 and 484 ng ml^{-1} for N-desmethylnizatidine, respectively. Accuracy (bias%) and precision (CV%) were less than 5.3% for intra-day assay and 8.1%, for inter-day assay, and less than 11.4% and 17.2%, respectively, at the limits of quantification. The recovery ranged between 93.9 and 104.2%. The analytes were proved to be stable in plasma over a period of 6 months below -20 ºC.

INTRODUCTION

Nizatidine, N-[2-[[[2-[(dimethylamino)methyl]-4-thiazolyl]methyl]thio]ethyl]-N'-methyl-2-nitro-1,1-ethenediamine, is a competitive reversible H_2-receptor antagonist approved in adults for the treatment of acid-related gastrointestinal disorders, including duodenal and gastric ulcers, erosive and ulcerative esophagitis, and gastroesophageal reflux disease.[1,2]

The literature of the analytical chemistry of nizatidine in human plasma is extensive, with methods based on HPLC with ultraviolet[3-5], MS[6-8] or electrochemical detection.[9,10] The sample preparation approach was mainly liquid-liquid extraction[11,13], but solid-phase extraction was also used.[6,7,12]

In a single-dose pharmacokinetic/ bioequivalence (PK/BE) study of a medicinal product, the extrapolated area of the curve which describes plasma concentration of the drug and metabolites versus time after administration must not be greater than 20% of total calculated area. To achieve this requirement a quantifiable concentration must be obtained after five half-life times and the sensitivity of the applied analytical method plays an important role. In the mean time, a bioequivalence study involves analysis of hundred of samples and the development of a suitable chromatographic method must take into account not only a sensitive procedure, but also a fast one.

Taking into account these facts, the primary aim of the study was to develop a fast HPLC method with enough sensitivity to quantify simultaneously nizatidine and N-desmethylnizatidine in human plasma by applying protein precipitation after oral administration of 300 mg nizatidine. None of the studied articles applied protein precipitation, a simple and low cost plasma sample preparation. The developed and validated method was successfully applied for bioequivalence investigation of two medicinal products containing nizatidine by monitoring the signal of both parent drug and its N-desmethylmetabolite.

RESULTS AND DISCUSSION

The endogenous compounds or the used anticoagulant did not interfere at the retention

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times of nizatidine and N-desmethylnizatidine (figure 1). The short gradient with high concentration of acetonitrile was introduced just before analytes elution only for within-run column recondition and to have a run-time as short as possible; in fact the elution of the analytes is on the isocratic condition.

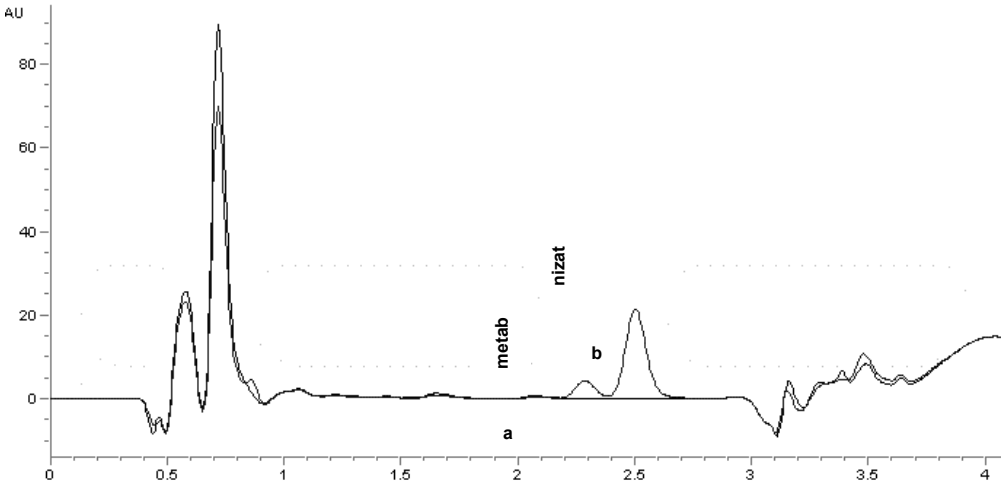

Fig. 1 – Chromatogram of a. blank plasma and b. plasma sample of a healthy volunteer at 1.5 hours after oral administration of 300 mg nizatidine (retention times: 2.5 minutes nizat – nizatidine; 2.3 minutes metab – N-desmethylnizatidine).

The calibration curves showed linear response over the range of concentration used in the assay procedure. The mean calibration curves were: for nizatidine in the concentration range 80 - 3200 ng/ml, Area = 0.1117 c (±0.0002) – 2.088(±0.240), N = 7 calibration points, n = 3 days, weigth factor 1/c, coefficient of correlation greater than 0.999, with the residuals distribution within ±16%; for N-desmethylnizatidine in the concentration range 12 - 484 ng/ml Area = 0.1106 c (±0.0002) – 0.3854(±0.0334), N = 7 calibration points, n = 3 days, weigth factor 1/c, coefficient of correlation greater than 0.999, with the residuals distribution within ±18%.

The inter- and intra-day precision and accuracy results are showed in Table 1 for nizatidine and in Table 2 for desmethylmetabolite and were less than 5.3 % for intra-day assay and 8.1 %, for inter-day assay. The lower limits of quantification were established at 80 ng/ml nizatidine and 12 ng/ml N-desmethylnizatidine, with intra- and inter-day accuracy and precision less than 15.7% and 17.2%, respectively, and were sensitive enough to monitor concentrations of analytes over 12 hours after oral administration of 300 mg nizatidine.

The recovery was consistent and ranged between 93.9 and 102.5% for nizatidine and between 93.5 and 104.2%, for N-desmethylnizatidine (Tables 1 and 2), with a standard deviation which allowed the quantitative determination of the analytes without using internal standard.

The stability study demonstrated that nizatidine and N-desmethylnizatidine could be stored below -20 ºC at least six months. The back calculated concentrations ranged between -0.7 to 3.8 % for nizatidine and between 4.6 to 8.9%, for metabolite (Table 3), respectively. The values were between ±15%, the acceptable limits for bioequivalence methods accuracy.

The validated method was applied for bioequivalence investigation of two medicinal products containing nizatidine. The concentration profiles of nizatidine and its desmethylmetabolite after oral administration of 300 mg nizatidine are given in Figure 2. In six assay runs 576 unknown plasma samples were analyzed, each analytical run consisting of seven calibration standards, six quality control samples (QC) and 96 unknown samples of four volunteers in the study. The calibration curve slopes had a normal variation domain (slope ± 2s) of 0.1119 ± 0.0028 for nizatidine and 0.1109 ± 0.0012 for metabolite

(n = 6). The results of the quality control QC samples analysis provided the basis of accepting or rejecting the runs during clinical samples analysis. At least four out of six QC samples were within ±15% of their respective nominal value in each analytical run and no tendency of concentration variation was observed (Figure 3). All the runs were validated.

Table 1

Intra- and inter-day precision and accuracy (n = 3) for nizatidine

$c_{nominal}$, ng ml^{-1}	Intra-day				Inter-day		
	Mean c_{found}, ng ml^{-1} (±SD)	CV%	Bias, %	Recovery % (±SD)	Mean c_{found}, ng ml^{-1} (±SD)	CV%	Bias, %
80.06	88.6(3.7)	4.2	10.7	102.5(5.5)	92.6(2.1)	2.3	15.7
160.1	166.6(8.9)	5.3	4.1	93.9(5.6)	172.8(3.6)	2.1	7.9
1601	1559(38.5)	2.5	-2.6	100.3(2.5)	1564(33.2)	2.1	-2.3
2402	2363(42)	1.8	-1.6	100.8(1.8)	2365(47.8)	2.0	-1.5

Table 2

Intra- and inter-day precision and accuracy (n = 3) for N-desmethylnizatidine

$c_{nominal}$, ng ml^{-1}	Intra-day				Inter-day		
	Mean c_{found}, ng ml^{-1} (±SD)	CV%	Bias, %	Recovery % (±SD)	Mean c_{found}, ng ml^{-1} (±SD)	CV%	Bias, %
12.12	13.5(0.7)	5.2	11.4	104.2(7.5)	14.2(0.4)	2.8	17.2
24.24	25.5(0.4)	1.6	5.2	93.5(1.9)	26.2(0.7)	2.7	8.1
242.4	237.2(6.3)	2.7	-2.2	104.2(2.8)	238.3(4.0)	1.7	-1.7
363.6	361.1(6.8)	1.9	-0.7	104.1(2.0)	358.0(7.1)	2.0	-1.5

Table 3

Results of the stability study below -20 ºC (n = 3)

Tested sample / Time, months		0	2	4	6
347.0 ng ml^{-1} nizatidine	CV%	1.0	3.2	3.5	1.0
	Bias%	-0.7	3.8	3.3	0.5
67.2 ng ml^{-1} N-desmethylnizatidine	CV%	2.0	1.2	4.2	1.6
	Bias%	5.6	8.9	8.2	4.6

In conclusion, the proposed method proved to be accurate and precise and, in less than four minutes, both analyte and its N-desmethylmetabolite are well separated from endogenous compounds. Without using internal standard and applying a single and simple extraction step, a specific and efficient analysis of a large number of plasma samples could be performed. The successfully transfer of the method between two laboratories proved the reproducibility of the method.

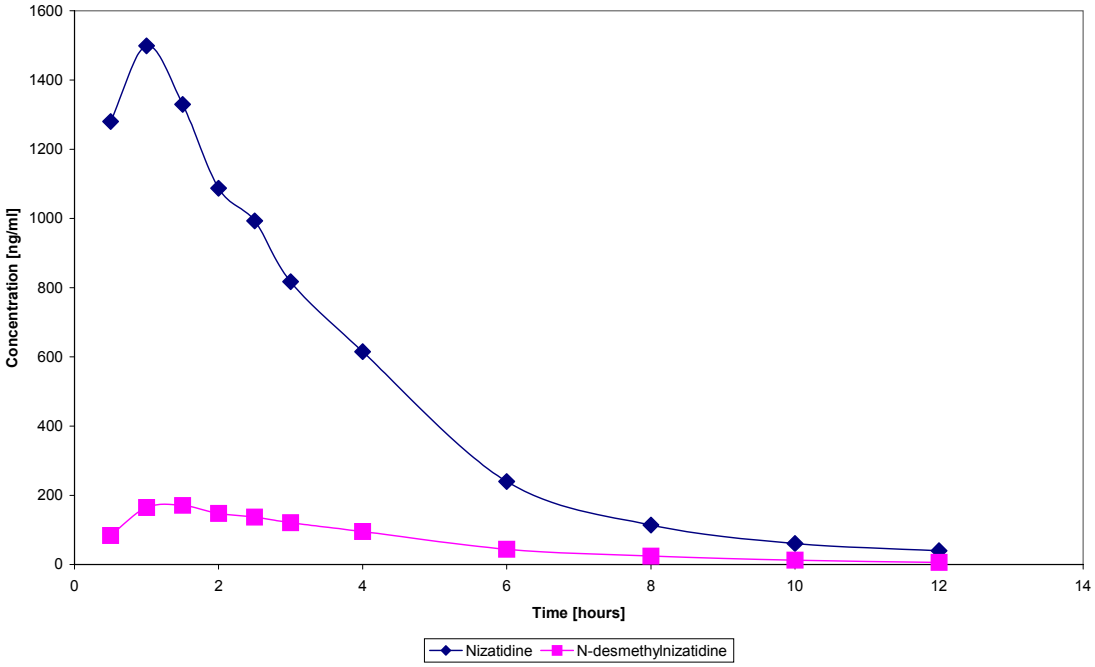

Fig. 2 – Concentration profiles of nizatidine and N-desmethylnizatidine in plasma of a healthy volunteer after receiving 300 mg nizatidine.

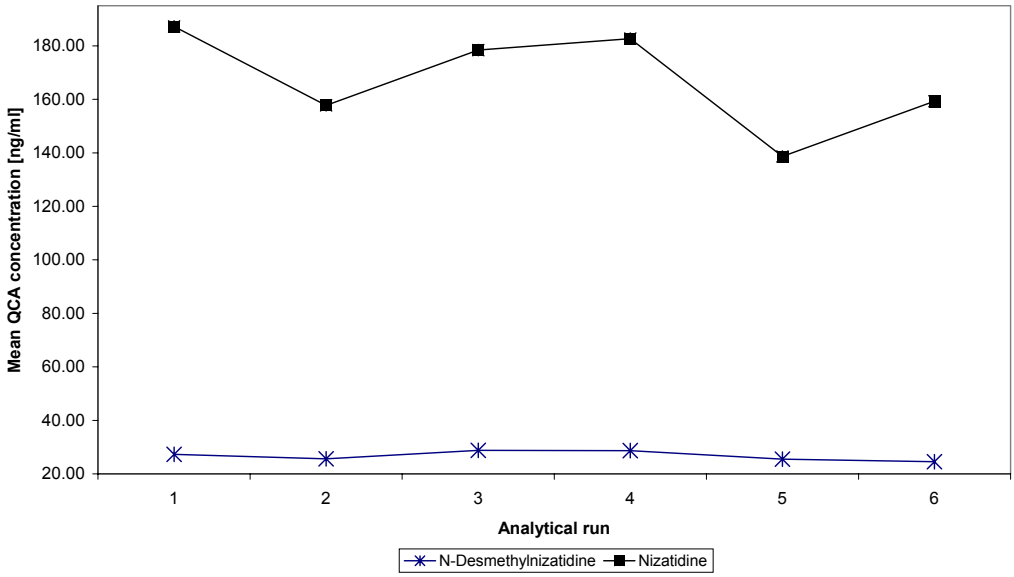

Fig. 3 – Mean QCA values of the analytical runs during clinical samples analysis.

EXPERIMENTAL

Materials

Nizatidine and N-desmethylnizatidine were working standards provided by Utraco Holland B.V. (Netherland). Acetonitrile, methanol, potassium dihydrogenophosphate and perchloric acid 85% were Merck products (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The human blank plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Preparation of plasma calibrator and control samples

Stock solutions of 1000 µg ml^{-1} nizatidine and desmethylnizatidine, respectively, were prepared by dissolving an appropriate quantity of standard substance (weighted on a

Mettler Toledo AB 54-S balance from Mettler Toledo, Greifenseee, Switzerland) in 10 ml methanol. Working solutions were then obtained by diluting specific volumes of stock solutions with water and used to spike 0.5 ml of plasma blank, providing finally seven plasma standards with the concentrations ranged between 80 and 3200 ng ml^{-1} nizatidine and 12-484 ng ml^{-1} N-desmethylnizatidine. Accuracy and precision were verified at four levels of concentration, including lower limits of quantification: 80, 160, 1600 and 2400 ng ml^{-1} for nizatidine, and 12, 24, 242 and 363 ng ml^{-1} for metabolite. Duplicated quality control samples, namely QCA, QCB and QCC, of 160, 800 and 2400 ng ml^{-1} nizatidine and 24, 121 and 363 ng ml^{-1} N-desmethylnizatidine, were used during clinical samples analysis, in order to ensure that the method continues to perform adequately.

Sample preparation

At 0.5 ml plasma sample, 0.08 ml water (or total working solutions of analytes) were added and mixed with 0.12 ml perchloric acid 14% for five seconds via a vortex mixer (Genie 2, Scientific Industries Inc., USA). After 15 minutes of rest, a centrifugation for eight minutes at 5000 rpm was made, using a Sigma 204 centrifuge (Germany). The supernatant was transferred in an autosampler vial and 75 µl were injected into the HPLC system.

Chromatographic system and conditions

The HPLC system was an 1100 series model (Agilent Technologies) consisted of a binary pump, an in line degasser, an autosampler, a column thermostat and an UV detector. Data were collected and computed by ChemStation software (ver. A.09.03). The detector was operated at 314 nm. Chromatographic separation was performed in 4.1 minutes at 42 °C with a Zorbax SB-C18 100 x 3 mm, 3.5 µm (Agilent Technologies) column, protected by an on-line filter (2 µm). The elution was made in gradient mode at 1 ml min^{-1}, with a pre-run equilibrating time of 1 minute: 0-2 minutes 100% A (4% methanol and 96% KH_2PO_4 40 mM pH 2.3 with H_3PO_4 85%); between 2-2.01 minutes solvent A decreases from 100% to 35% and B (acetonitrile) increases from 0% to 65%; 2.01-2.4 minutes 35% A and 65% B; 2.4-2.41 minutes A increases from 35% to 100%; 2.41-4.1 minutes 100% A. The mobile phase was degassed, before elution, for 10 minutes in an Elma Transsonic 700/H (Singen, Germany) ultrasonic bath.

Method validation

As a first step of method validation[14,15], specificity was verified using six different plasma blanks obtained from healthy human volunteers who did not take before nizatidine and any other medication. The anticoagulant (Li-heparin) interference was also verified during this stage.

The linearity of the peak area against standard concentration was verified between 80-3200 ng ml^{-1} nizatidine and 12-484 ng ml^{-1} N-desmethylnizatidine in three different days, by applying least-squares linear regression. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion.

The limits of quantification were established as the lowest calibration standard with an accuracy and precision less than 20%.

The intra- and inter-day precision (expressed as coefficient of variation %, CV%) and accuracy (relative difference % between found and theoretical concentration, bias%) of the assay procedure were determined by the analysis in the same day of three samples at each of three levels of concentration in the considered concentration range and one sample of each in three different days, respectively. The recoveries at each of previously levels of concentration were measured by comparing the response of the treated plasma standards with the response of standards in water with the same concentration in nizatidine and N-desmethylnizatidine as the final extract from plasma standards.

Aliquots of plasma samples containing 347 ng ml^{-1} nizatidine and 67.2 ng ml^{-1} N-desmethylnizatidine were frozen for six months below -20 ºC and with four occasions during this period, each of three samples were analyzed.

Clinical application and in-study validation

The validated method was applied in a bioequivalence study of two dosage forms containing 300 mg nizatidine. The collecting times were 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12 hours after oral administration of 300 mg nizatidine. The accuracy and precision of the validated method was monitored to ensure that it continued to perform satisfactorily during analysis of volunteer samples. To achieve this objective, a number of QC samples prepared in duplicate at three levels of concentration were analyzed in each assay run and reported at the calibration curve of the run. The runs were validated if four out of six QC samples were within ±15% of their nominal concentration. Two but not all QC samples at the same concentration could be outside this range.

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