BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **21**: 40–47 (2007)
Published online 2 November 2006 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/bmc.715

ORIGINAL RESEARCH

High-throughput liquid-chromatography method with fluorescence detection for reciprocal determination of furosemide or norfloxacin in human plasma

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Received 15 May 2006; revised 12 June 2006; accepted 13 June 2006

ABSTRACT: A simple, high-throughput, highly selective and sensitive HPLC-FLD method for isolation and determination of furosemide and/or norfloxacin in human plasma samples following a simple organic solvent deproteinization step with acetonitrile as sample 'clean-up' procedure is reported. One of the two drug substances plays the internal standard role for the determination of the other. Separation of analyte and internal standard was achieved in less than 5.3 min (injection to injection) on a Chromolith Performance RP-18e column, using an aqueous component containing 0.015 mol/L sodium heptane-sulfonate and 0.2% triethylamine brought to pH = 2.5 with H_3PO_4. The composition of the mobile phase was: acetonitrile–methanol–aqueous component = 70:15:15 (v/v/v) and the flow-rate was set up to 3 mL/min. The chromatographic method applied to the determination of furosemide relies on fluorescent detection parameters of 235 nm for the excitation wavelength, and 402 nm for the emission wavelength. In case of norfloxacin, the excitation wavelength is set up to 268 nm and the emission wavelength is set up to 445 nm. The overall method leads to quantitation limits of about 27 ng/mL for furosemide, and 19.5 ng/mL for norfloxacin, using an injection volume of 250 µL. The method was applied to the bioequivalence study of two furosemide-containing formulations. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: furosemide; norfloxacin; fluorescence detection; liquid chromatography; human plasma

INTRODUCTION

Furosemide is an active diuretic, greatly increasing renal salt elimination and also effective in the case of reduced glomerular filtration. The diuretic effect of the drug starts 20–60 min from oral administration, lasting for about 4–6 h and reaching maximum intensity after 2–3 h; it reduces high arterial pressure and increases the effect of anti-hypertensive medication. The pharmacologic effects of furosemide are similar to those of ethacrynic acid. The exact mode of action of furosemide has not been clearly defined; in contrast to ethacrynic acid, it does not bind sulfhydryl groups of renal cellular proteins. Furosemide inhibits the reabsorption of electrolytes in the ascending limb of the loop of Henle. The drug also decreases reabsorption of sodium and chloride and increases potassium excretion in the distal renal tubule and exerts a direct effect on electrolyte transport at the proximal tubule (Uchida *et al*., 1991; McCurley *et al*., 2004; Buffin-Meyer *et al*., 2004).

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Abbreviations used: ACN, acetonitrile; FLT, fluorescence detector; IPA, ion-pairing agent; PMT, photomultiplier.

Literature data report several works on furosemide determination from biological matrices by HPLC. Most of them are based on the reversed-phase mechanism using octadecylsilicagel as stationary phase; other types of encountered silica modifications are octyl (Singh *et al*., 1989) and cyano (Abdel-Hamid, 2000). As mobile phases, mixtures of aqueous–polar organic solvent (usually acetonitrile) have been used, the aqueous component often containing phosphate (pH 2–7; Gomez *et al*., 2005; Okuda *et al*., 1996; Abou-Auda *et al*., 1998; Gaillard and Pepin, 1997; Campins-Falco *et al*., 1997; Sidhu and Charles, 1993; Farthing *et al*., 1992; Takamura *et al*., 1997) or acetate buffers (pH 5–7; Jankowski *et al*., 1997; Nava-Ocampo *et al*., 1999; Abdel-Hamid, 2000). Detection is generally achieved by absorption or fluorescence spectrometry. Mass spectrometry has also been used in APCI negative mode by monitoring the 329 mass channel (Abdel-Hamid, 2000). The detection limits obtained with these methods varied between 2 and 10 ng/mL.

The most common sample preparation procedure that was applied for furosemide isolation from different matrices was liquid–liquid extraction (Gomez *et al*., 2005; Jankowski *et al*., 1997; Abou-Auda *et al*., 1998; Gaillard and Pepin, 1997; Reeuwijk *et al*., 1992; Takamura *et al*., 1997; Abdel-Hamid, 2000). The extraction solvent most employed in this procedure following

sample acidification, deproteinization steps or internal standard addition is ethyl acetate (Gomez *et al*., 2005; Jankowski *et al*., 1997; Reeuwijk *et al*., 1992; Abdel-Hamid, 2000). Other mentioned solvents are diethyl ether (Abou-Auda *et al.*, 1998) and dichloromethane (Takamura *et al*., 1997). An acidic additive in various amounts and concentrations is often mixed with plasma samples prior to extraction to increase the furosemide extraction yield. After extraction, centrifugation allowed for phase separation and then the organic phase was evaporated to dryness under a nitrogen stream at 40°C.

Other sample preparation procedures encountered in the literature were deproteinization using acetonitrile (ACN; Sidhu and Charles, 1993), methanol (Nava-Ocampo *et al*., 1999) or ultrafiltration through different porosity membranes (Okuda *et al*., 1996; Singh *et al*., 1989). A review focused on sample preparation procedures applied to the reversed-phase HPLC determination of several diuretics in human body fluids has been recently published (Zendelovska and Stafilov, 2006).

This paper describes a practical, high-throughput, highly selective, sensitive and robust reversed-phase LC-FLD method focusing mainly on the determination of furosemide in human plasma samples, using norfloxacin as internal standard. The advantage of this analytical method is its possible application to the determination of norfloxacin in plasma samples using furosemide as internal standard, the only improvement being related to the parameters of fluorescence detection in order to reach the highest sensitivity for norfloxacin. The literature has reported only a few studies on the determination of norfloxacin in biological matrices or application to the bioequivalence study (e.g. Anadon *et al*., 1992; Lim *et al*., 2002; Vybiralova *et al*., 2005).

The sample clean-up procedure is achieved by a simple ACN deproteinization, followed by a supernatant dilution to allow injection of high sample volume. The chromatographic separation of analyte and internal standard from each other and from the plasma endogenous compounds remaining in the sample was achieved in less than 5.3 min (injection to injection), using an overlapped injection cycle procedure in which the sample is introduced in the injection valve during the previous chromatographic run. The procedure allowed high-throughput analysis of samples from a furosemide bioequivalence study of 24 volunteers. The chromatographic separation was achieved by by ion-pairing reversed-phase mechanism. The ion-pairing mechanism was used in order to reduce the large difference between hydrophobicity of furosemide and norfloxacin (internal standard), quantified by their hydrobobicity index ($\log K_{o,w}$). Finally, the entire method was optimized and validated in order to be used for a large-scale bioequivalence study of two drugs containing furosemide.

EXPERIMENTAL

Instrument. In this study an Agilent 1100 liquid chromatograph (vacuum degasser, quaternary pump, autosampler, column thermostat and fluorescence detector) was employed. Chromatographic data were acquired by means of the Agilent Chemstation software (Agilent Technologies, Waldbronn, Germany).

Chromatographic conditions. A monolithic column, Chromolith Performance RP-18e (Merck KGaA, Darmstadt, Germany), was used, having 100 mm length and 4.6 mm internal diameter. The column temperature was maintained at 25°C, while the mobile phase consisted of 0.015 mol/L sodium heptane-sulfonate, 0.2% triethylamine and phosphoric acid to pH 2.5–acetonitrile–methanol in the ratio 70:15:15 (v/v/v); the elution regime was isocratic at a constant flow-rate of 3 mL/min. Detection was performed with a fluorescence detector (FLD) using 235 nm for excitation and 402 nm for emission; the photomultiplier (PMT) gain was set to 14, while detector response time was set to 1 s. The injection was performed automatically by the autosampler, each time a volume of 250 μL being injected. All solvents were HPLC grade and were purchased from Merck KGaA (Darmstadt, Germany).

Chemicals. All solvents were HPLC grade from Merck (Darmstadt, Germany). Sodium heptane-sulfonate for ion pair chromatography produced by Merck (Darmstadt, Germany) was used. Water for HPLC (minimum resistivity 18 MΩ and maximum content of total organic carbon 30 ppb) was produced within the laboratory with a TKA Lab HP 6UV/UF instrument and used during all experiments.

Furosemide and norfloxacin working standards were purchased from European Pharmacopeia, Council of Europe, Strasbourg, France (batch 1a).

Sample preparation procedure. Prior to HPLC analysis, isolation of furosemide from plasma was performed by a simple deproteinization procedure with acetonitrile as follows: 400 μL of acetonitrile solution containing 4 μg/mL norfloxacin as internal standard were added to 200 μL plasma sample; the mixture was vortexed for 5 min at 2000 rpm (with Heidolph Reax shaker) and then centrifuged for another 10 min at 12,000 rpm. Afterwards, the supernatant was quantitatively separated from the precipitated proteins and was diluted with 1.5 mL aqueous component of the mobile phase (0.015 mol/L sodium heptanesulfonate, 0.2% triethylamine and phosphoric acid to pH 2.5). The mixture was homogenized and injected (250 μL) in the chromatographic column.

Methodology and pharmacokinetic parameters. During the open-label, randomized, two-period, two-sequence, crossover bioequivalence study, 24 healthy male and female volunteers received one dose of furosemide from the tested and reference products, found on the Romanian market, with a 14 days wash-out period. The study medication was orally administered after overnight fasting. Blood samples were collected before dosing (0 h) and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 h post-dose. The pharmacokinetic parameters considered for evaluation of the bioequivalence between tested and reference products were: C_{max}, observed

DOI: 10.1002/bmc

maximum furosemide plasma concentration; t_{max}, sampling time of the maximum plasma concentration; $t_{1/2}$, terminal elimination half-life time; AUD, area under plasma concentration–time curve until last quantifiable value; $AUC_{0-\infty}$, AUD extrapolated to infinity.

Pharmacokinetic parameters were determined using Kinetica software (version 3.1./2000) from Innaphase Corp., USA.

RESULTS AND DISCUSSION

Method development

The chromatographic method described in the Experimental was optimized dependending on the retention behavior of both compounds (furosemide and norfloxacin) and the selectivity between these analytes and endogenous plasma components. Preliminary tests have shown that the highest sensitivity for furosemide with minimum interference is accomplished using 235 nm as excitation wavelength and 402 nm as emission wavelength. For these fluorescence parameters norfloxacin used as internal standard has a lower fluorescence yield than furosemide, and for this reason it was spiked in plasma samples in higher concentrations than the maximum concentration of furosemide studied by the calibration procedure. On the other hand, taking into consideration the reciprocity of this analytical method, the fluorescence parameters can be changed to maximum sensitivity for determination of norfloxacin and its application to bioequivalence of two norfloxacin containing formulations (excitation, 268 nm; emission, 445 nm), when furosemide is used as internal standard. This time, the fluorescence detection of furosemide is achieved with a lower sensitivity.

The chromatographic behavior in reversed-phase liquid chromatography of furosemide and norfloxacin is opposite, as can be estimated by computing the hydrophobicity index (as a 10-base logarithm octanol–water partition coefficient, denoted by $\log K_{o,w}$) for furosemide and norfloxacin using the fragment methodology (Meylan and Howard, 1995) showed a significant difference between their hydrophobicities.

Owing to this difference between their hydrophobicities, it is expected that in simple RP mechanism furosemide will elute at high retention time, while norfloxacin elutes at small retention time. A convenient solution is to apply an ion pairing mechanism in order to increase the retention of norfloxacine, because it contains two free amino groups. Addition of alkylsulfonatyes ion to the mobile phase will form ion pairs only with norfloxacin. On the contrary, furosemide cannot participate to the ion-pair formation owing to its acidic functional groups (pK_a = 3.9), namely sulfonamide and carboxylic. Therefore, furosemide will keep almost constant the interaction with the stationary phase during the ion-pairing retention mechanism, although a salting-out effect is expected to occur. This was found by studying the retention behaviour of both analytes for different ion-pairing agents (0.015 mol/L) at pH = 2.5, as shown in Fig. 1.

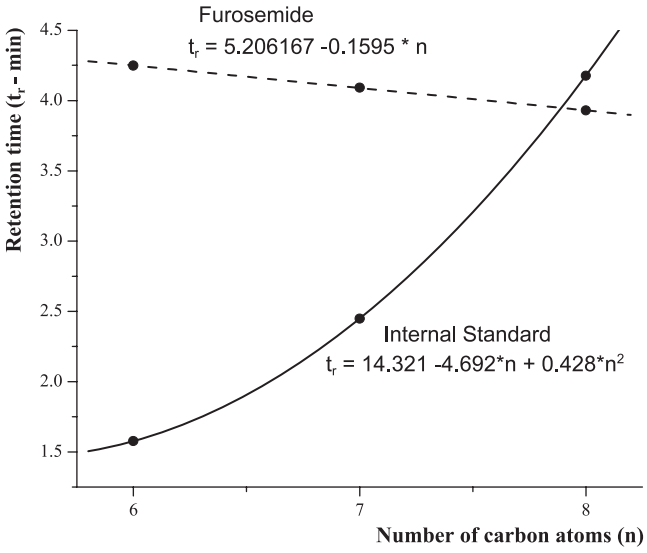

Figure 1. Dependence of retention time (t_r) of furosemide and norfloxacin on the nature of the ion-pairing agent (sodium hexane-, heptane- or octane-sulfonate).

According to data given in Fig. 1, only norfloxacin takes part in the ion-pairing mechanism. Its retention increases with the increase in hydrophobicity of the ion-pairing agent: hexanesulfonate (computed $\log K_{o,w}$ = 0.074), heptanesulfonate (computed $\log K_{o,w}$ = 0.565) and octanesulfonate (computed $\log K_{o,w}$ = 1.056) ions. The study was limited to these agents only, because for more than nine carbon atoms in the ion-pairing agent (IPA) molecule, the retention of the internal standard will increase without any benefit for the final application to the bioequivalence study. In conclusion, the difference in the intrinsic hydrophobicity between furosemide and norfloxacin molecules in acidic media correlated with their different behavior in the ion-pairing retention mechanism with alkyl sulfonates leads to the possibility of a very good and rapid separation of these compounds with respect to each other as well as with the endogenous components from the plasma matrix.

The influence of the nature of the IPA on the major chromatographic parameters is given in Table 1. The chromatographic parameters reach optimum values for sodium heptane-sulfonate. In addition, the selectivity of separation between target analytes and endogenous components from plasma matrix was highest using sodium heptane-sulfonate as IPA.

The dependences of the retention time (t_r, min) on the IPA concentration in mobile phase are shown in Fig. 2. According to these dependences the retention

DOI: 10.1002/bmc

Table 1. The influence of nature of ion-pair agent on main chromatographic parameters

	k' (capacity factor)		Efficiency (plates/column)		
Nature of IPA	IS	Furosemide	IS	Furosemide	Resolution
Solution 0.015 mol/L $C_6H_{13}SO_3Na$ and 0.2% TEA–H_3PO_4 (pH = 2.5)	2.17	7.55	2010	6120	14.90
Solution 0.015 mol/L $C_7H_{15}SO_3Na$ and 0.2% TEA–H_3PO_4 (pH = 2.5)	3.93	7.23	4846	6380	9.50
Solution 0.015 mol/L $C_8H_{17}SO_3Na$ and 0.2% TEA–H_3PO_4 (pH = 2.5)	7.40	6.91	5879	5675	1.16

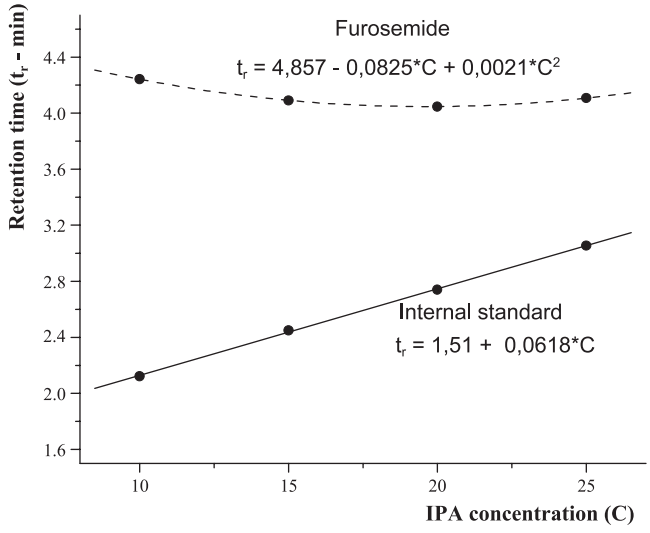

Figure 2. Dependences of the retention time (t_r) on ion-pairing agent (IPA) concentration (C as 10^{-3} mol/L) in the aqueous solvent.

time of the internal standard increases linearly with the concentration of ion pairing agent in the mobile phase, while the retention of furosemide decreases slightly with the increase in the ion pairing agent concentration. For a concentration of 0.01 mol/L sodium heptanesulfonate in the aqueous solvent the internal standard elutes with the endogenous compounds from the plasmatic pattern. An increase in the IPA concentration to 0.02 mol/L did not significantly improve the separation, and therefore the value of the IPA concentration was set at 0.015 mol/L as the optimum value for the final chromatographic method applied to samples resulting from the sample preparation procedure as discussed further.

A sample preparation procedure based on deproteinization was preferred over one based on extraction because it is simple and allows a larger number of plasma samples to be processed during the same time interval. The use of an organic solvent deproteinization instead of acid deproteinization was preferred because of the instability of the furosemide in highly acidic media. Although the sample preparation method avoids the extraction of target analytes, the use of an internal standard (IS) is imposed by the possible volume variation of the supernatant after the deproteinization step and by the incomplete transfer of the supernatant. The use of a monolithic column at high flow-rate of the mobile phase (3 mL/min) enabled us to obtain a short chromatographic run (injection to injection time interval of only 5.3 min). As can be seen from Fig. 3, the peaks corresponding to furosemide and norfloxacin are well separated from the peaks corresponding to the plasmatic pattern, in comparison with the situation when the ion-pairing mechanism is not applied.

In order to inject high volume of supernatant into the analytical column without having focusing effects, its composition must be adjusted to be almost identical to the mobile phase composition or, better, to that composition with a lower content of organic solvent than the mobile phase. The large volume of aqueous solvent added to the obtained supernatant allowed the injection of large volumes of sample without any focusing effects.

The final procedure used for the plasma sample preparation consists of the following main analytical operations:

- addition of 400 µL acetonitrile solution containing 4 µg/mL internal standard to 200 µL plasma sample and vortexing thoroughly (5 min, 2000 rpm);
- centrifugation (10 min, 12,000 rpm);
- separation of the supernatant in a polypropylene tube;
- addition of 1500 µL aqueous solvent;
- vortex (3 min, 1200 rpm);
- chromatographic analysis of the supernatant (injection volume is 250 µL).

The recovery yield for both analytes was estimated from parallel experiments, achieved on different concentration levels for furosemide and for a constant concentration value of IS (8000 ng/mL), in mobile phase

DOI: 10.1002/bmc

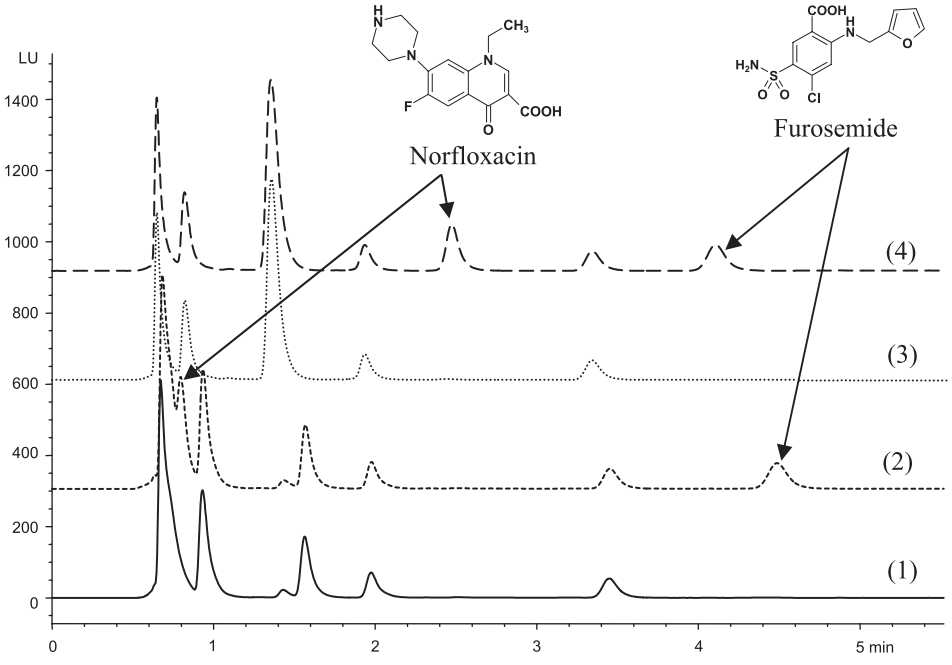

Figure 3. Four overlaid chromatograms corresponding to the following situations: (1) blank plasma sample in RP mechanism without ion-pairing; (2) spiked plasma sample with norfloxacin (8000 ng/mL) and furosemide (500 ng/mL) in RP mechanism without ion-pairing; (3) blank plasma sample in ion-pairing RP mechanism; (4) spiked plasma sample with norfloxacin (8000 ng/mL) and furosemide (500 ng/mL) in ion-pairing RP mechanism.

Table 2. The values of recovery yield from plasma samples for furosemide and I.S.

Analyte	Concentration (ng/mL)	Recovery (η%)	RSD% (n = 5)
Furosemide	100	99.11	2.47
	1000	101.04	0.51
	3000	100.15	1.98
I.S.	8000	93.08	2.82

solvent and in plasma matrix. The experimental values of recovery (η) together with their precision (RSD% for n = 5) are given in the Table 2. Owing to their high solubility in a medium with high acetonitrile content, the recovery of both analytes were almost 100%.

Method validation

Linearity. Linearity was tested for 10 concentration values (0, 25, 50, 100, 300, 500, 700, 1000, 2000, 3000 and 4000 ng/mL) for furosemide in spiked plasma samples, prepared according to the sample preparation described previously. For this purpose a fresh initial spiked plasma sample (4000 ng/mL) was prepared by diluting an accurate 20 µL standard acetonitrile solution containing 1000 µg/mL furosemide to 5 mL with plasma under vortex. Then, each spiked plasma sample was obtained by dilution of the initial plasma sample after its strong vortex with plasma, in one or more corresponding steps. Calibration was done by plotting the ratio of the peak area corresponding to furosemide and IS against the concentration value of furosemide in spiked plasma samples, expressed in ng/mL. The calibration procedure was repeated on three different days. The slope (B) of the linear regression was 0.00194 ± 3.30×10^{-5}, and the intercept (A) was −0.0003 ± 0.0553. The mean correlation coefficient (r_{xy}) was 0.9994. The limit of quantitation (LOQ) deduced from calibration graph was estimated to the value of 26.95 ng/mL. By means of LOQ value, the limit of detection (LOD = 8.2 ng/mL) can be calculated as LOQ/3.3, and the value of LLOQ as LOQ/2 (about 13.5 ng/mL).

Precision and accuracy. Intra-day and interday precisions were studied for three concentration levels (100, 1000 and 3000 ng/mL furosemide and 8000 ng/mL in spiked plasma samples). The results obtained for the peak area values corresponding to furosemide and IS are given in Table 3.

Precision was also checked during the bioequivalence study of two furosemide-containing formulations. For this purpose, the trends of variation of the peak area and the retention time for the IS were studied (Fig. 4), leading to the conclusion that the entire process is precise. Both parameters had values within their accepted interval of variations.

The reversal application was focused on the determination of norfloxacin in plasma samples using

DOI: 10.1002/bmc

Table 3. The values of relative standar deviation (RSD%) characterizing the precision of the entire method for the determination of furosemide

Precision	20 ng/mL furosemide	100 ng/mL furosemide	1000 ng/mL furosemide	3000 ng/mL furosemide	8000 ng/mL IS
Intra-day precision (n = 10)	3.58%	1.46%	1.06%	2.26%	0.97%
Inter-day precision (n = 5)	5.21%	2.30%	2.99%	4.27%	2.86%

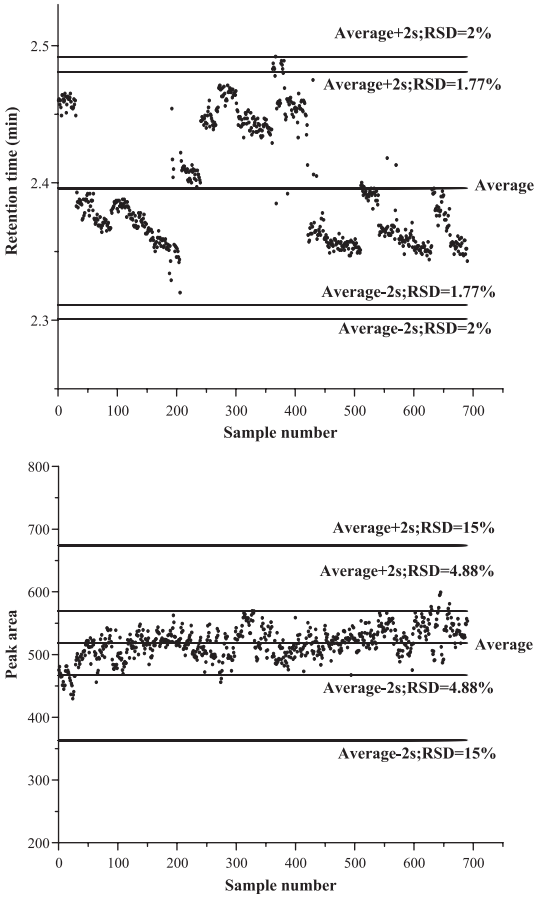

Figure 4. The trends of variation for the retention time and peak area of the internal standard. (RSD = 1.77%, the experimental value corresponding to retention time; RSD = 2%, maximum accepted limit for variation of retention time; RSD = 4.88%, experimental value corresponding to peak area; RSD = 15%, maximum accepted limit for variation of peak area).

furosemide as IS, by changing the detection parameters to 268 nm for excitation and 445 nm for emission. The linearity domain was checked for nine concentration levels over a wide range of concentration, i.e. 50–2000 ng/mL norfloxacin, using a concentration of the IS (furosemide) of 1000 ng/mL. The precision (intra-day and interday) was studied for three levels of norfloxacin concentration: 75, 750 and 1500 ng/mL. The RSD% values for these levels of concentrations are given in Table 4. The quantitation limit for norfloxacin was lower (19.5 ng/mL) owing to its better fluorescence yield.

Accuracy was controlled during the bioequivalence study using four QC spiked plasma samples (25, 120, 600 and 1500 ng/mL) for each set of samples provided from one volunteer. Interpolation of peak area obtained for QC samples was done on the linearity checked during each sequence (samples belonging to two volunteers) at the following concentration levels: 50, 100, 300, 500, 700, 1000, 2000 and 3000 ng/mL. The normal interval of variation (average ± 2s, n = 12) for slope was situated within [0.00201; 0.00168], while the normal interval of variation for intercept was [0.0406; −0.0153]. The experimental values obtained for QC were situated within their accepted interval of variation (theoretical concentration ± 15%), proving that the entire method was accurate. Different studies were carried out in what concerns the stability of furosemide during the bioequivalence study and they will be discussed as follows.

Freeze and thaw stability. Stock spiked plasma samples having concentrations of 100, 1000 and 3000 ng/mL furosemide were stored at −40°C for 24 h, then thawed unassisted at room temperature. An aliquot from each stock plasma sample was then processed. Stock plasma samples undergo five successive freeze and thaw cycles. RSD% values calculated for furosemide after five cycles were 1.64, 1.42 and 2.24%, respectively. During such studies, RSD% of 20% at LLOQ and 15% at higher concentration are considered as acceptable for proving stability of the analyte.

Long-term stability. Stock spiked plasma samples having concentrations of 100, 1000 and 3000 ng/mL furosemide were divided in separate vials and stored at −40°C. At the beginning of each daily session, one vial for each concentration level was thawed unassisted at room temperature and then processed. RSD% calculated for recovered furosemide concentration at each level for samples processed during 6 days were 8.93, 3.70 and 3.12%, respectively.

Short-term stability. Stock spiked plasma samples having concentrations 100, 1000 and 3000 ng/mL furosemide were stored for 24 h at −40°C, then thawed

DOI: 10.1002/bmc

Table 4. The values of relative standar deviation (RSD%) characterizing the precision of the entire method applied to the determination of norfloxacin

Precision	75 ng/mL norfloxacin	750 ng/mL norfloxacin	1500 ng/mL norfloxacin
Intra-day precision (n = 10)	0.66	0.22	0.54
Inter-day precision (n = 5)	7.09	2.26	3.67

unassisted at room temperature and kept at this temperature for 24 h. Aliquots from each stock plasma sample (0.2 mL) were processed immediately after thaw, and 4, 6, 12 and 24 h later. The RSD% calculated for recovered furosemide concentration at each level for processed samples were 2.33, 1.2 and 1.21%, respectively.

Stock solution stability of IS. The stability of the stock solutions of norfloxacin was evaluated on 4 µg/mL stock solution in acetonitrile stored at room temperature for 6 days. The RSD% calculated for peak areas was 3.11%.

Post-preparative stability. Processed plasma samples containing 100, 1000 and 3000 ng/mL furosemide were stored at room temperature, on the bench top or in the autosampler. The processed samples were analyzed immediately after preparation, and 2, 6, 8, 12, 24 and 48 h later. RSD% calculated for recovered furosemide concentration at each level for processed samples kept at room temperature 48 h were 1.26, 0.84 and 0.97%, respectively.

Application to a bioequivalence study. The entire analytical method has been applied successfully to the bioequivalence study of two formulations containing 40 mg furosemide, found on the Romanian market (one is denoted the reference, and the other the tested drug). The time profiles of the concentration of furosemide in plasma samples provided from a healthy volunteer for both formulations are given in Fig. 5. Owing to the high throughput of the present method, the entire analytical process applied to the plasma samples from 24 healthy volunteers and the additional checking samples (more than 700) took no more than one week (except for the initial part of method development and validation). The pharmacokinetic parameters obtained for both formulations are given in Table 5, proving their bioequivalence.

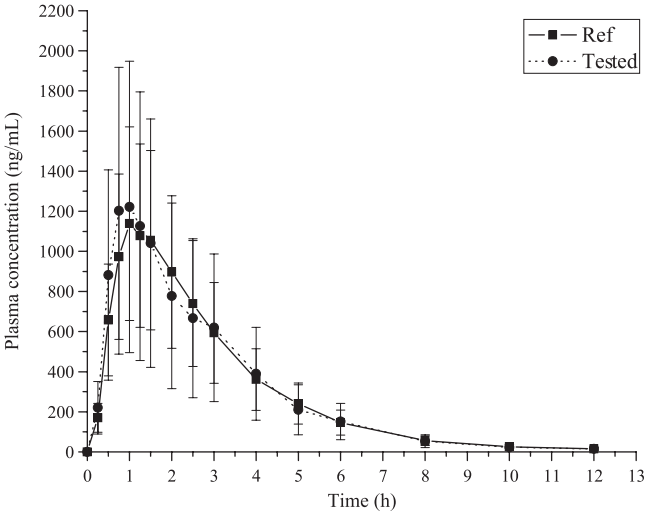

Figure 5. Mean plasma concentration profiles of two formulations (tested and reference) containing 40 mg furosemide, after bioequivalence study, together with their confidence intervals.

Table 5. Pharmacokinetic parameters determined during bioequivalence assessment for two 40 mg furosemide containing formulations

Pharmacokinetic parameter	Product	Mean value	RSD%
C_{max} (ng/mL)	Reference	1574.9	46.2
	Tested	1461.9	69.3
t_{max} (h)	Reference	1.277	79.7
	Tested	1.304	81.6
$t_{1/2}$ (h)	Reference	1.853	35.6
	Tested	1.872	36.8
AUD (ng/mL h)	Reference	3435.8	36.1
	Tested	3458.2	40.8
$AUC_{0-\infty}$ (ng/mL h)	Reference	3491.1	35.3
	Tested	3512.3	39.9

DOI: 10.1002/bmc

CONCLUSIONS

A new, selective and high-throughput (injection to injection time of only 5.3 min) analytical method for reciprocal determination of furosemide or norfloxacin in plasma sample is presented. Sample preparation is based on a simple and quantitative procedure using acetonitrile for protein precipitation. The chromatographic method is based on an ion-pair mechanism in order to enhance the retention of the norfloxacin. The entire analytical process was validated and applied succesfully to the bioequivalence study of two formulations containing 40 mg furosemide. According to this study, this method can also be applied for the determination of norfloxacin in plasma samples using furosemide as internal standard. The only modification is given by changing the fluorescence detection parameters in order to reach the highest sensitivity for the determination of norfloxacin in plasma samples.

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DOI: 10.1002/bmc