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Analytical issues in HPLC/MS/MS simultaneous assay of furosemide, spironolactone and canrenone in human plasma samples

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ABSTRACT

A new sensitive HPLC/MS/MS method for simultaneous determination of furosemide, spironolactone and canrenone in human plasma samples is presented. Electrospray ionization source (ESI) has been used. The tandem MS detection was performed under MRM conditions, in the negative ion mode for furosemide and indapamide (internal standard 1) and in the positive ion mode for spironolactone, canrenone and nitrazepam (internal standard 2). A simple plasma protein precipitation with acetonitrile was used as sample preparation technique. The chromatographic separation was carried out under the reversed phase mechanism, on a 250 mm length column packed with octadecyl modified silicagel and thermostated at 35 °C. The column was operated under isocratic conditions (3:7 aqueous 0.1% formic acid and methanol, v/v) at a flow rate of 0.8 mL/min. Quantitation intervals of 20–1600 ng/mL for furosemide and 2–100 ng/mL for spironolactone and canrenone have been concluded through validation. Precision and accuracy were situated within the accepted thresholds (maximum 15% relative standard deviation and ±15% percentage bias). The most sensitive aspects relating to the analytical method development and validation were highlighted and critically assessed in order to reach an objective opinion about the real performances and inherent applicability of the method in bioanalysis.

1. Introduction

Pharmaceutically speaking, the combination between spironolactone and furosemide is not a recent discovery. There are numerous pharmaceutical products, both innovative or generics approved to be marketed, which take advantage of the loop diuretic effect of furosemide (increases removal of sodium and potassium salts) and aldosterone antagonist effect of spironolactone. By combining effects of the two active substances, hypokalaemia is prevented and an efficient decrease in the volume of fluid circulating through blood vessels is obtained.

Analytical issues relating to the furosemide assay in pharmaceutical and biological matrices have been recently reviewed [1]. Although UV spectrometric detection has been used in the early nineties [2,3], it seems that fluorescence detection was preferred for furosemide together with the ion pair separation mechanism [4,5]. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) have also been used to determine furosemide in biological samples, more often by applying negative ion monitoring and atmospheric pressure chemical ionization (APCI) [6,7]. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) are generally cited as sample preparation methods for the assay of furosemide in biological fluids [8].

Spironolactone, along with its main metabolites, canrenone and 7-α-thiomethyl spironolactone, is usually detected through MS or MS/MS under APCI conditions [9], although UV detection is achievable if an adequate concentration technique (such as solid phase extraction) is used during sample preparation [10].

Best sensitivities obtained through MS/MS detection for furosemide, spironolactone and its metabolites in plasma samples, considering individual administration (40 mg for furosemide and 100 mg for spironolactone, respectively), place low limits of quantitation (LLOQ) at the 50 ng/mL and 2 ng/mL levels, respectively.

According to the literature, there are just two published works dealing with the simultaneous quantitation of furosemide, spironolactone and canrenone in human plasma samples [11,12]. Both works are focused on pharmacokinetics aspects and are not highlighting analytical aspects relating to method development and validation. The reason for finding only very few data on the simultaneous determination of furosemide, spironolactone and its related metabolites relies on two different aspects: (a) the lack of similarity between their analytical behavior (both chromatographic and ionization); (b) the therapy with spironolactone and furosemide is not

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considered so actual, so the interest on pharmacokinetic studies is constantly diminishing. However, at it results from the literature data and our experimental findings, absolute analytical solutions are not readily available and particularities need to be discussed.

Pharmacokinetic and pharmacodynamic aspects related to administration of furosemide and spironolactone as individual medications are also available in literature [13–17].

The aim of the present work consisted in the development and validation of an analytical method for assaying simultaneously furosemide, spironolactone and canrenone at pharmacokinetic concentration levels in plasma samples, based on a programmed bimodal functioning of the MS/MS detection (negative ion mode for furosemide, positive ion mode for spironolactone and canrenone).

The method was successfully applied to a bioequivalence study for immediate release pharmaceutical oral dosage forms (capsules) containing 20 mg of furosemide and 50 mg of spironolactone in fasting conditions. The most sensitive aspects of the analytical method were highlighted and critically assessed in order to reach an objective opinion about its inherent performance, when applied in bioanalysis.

2. Experimental

2.1. Reagents

All solvents were HPLC grade from Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Reference standards were obtained from European Pharmacopoeia (Council of Europe, Strasbourg, France), batch 1d for furosemide, batch 1b for spironolactone, batch 3.1 for canrenone, batch 3a for indapamide and 1b for nitrazepam. Formic acid was extra pure grade from Merck. 7-α thiomethyl spironolactone was obtained from US Biological (Swampscott, MA, USA).

2.2. Equipments

Experiments were performed on a system built up from Agilent series 1200 modules (Agilent Technology, Waldbronn, Germany) as following: degasser (G1322 A); binary pump SL (G1312 B); thermostated autosampler (G1367 C); column thermostat (G1330 B). Detection was made through a MS/MS triple quadrupole detector (G2571 A) using an atmospheric pressure electrospray ion source (ESI), operated under negative and positive mode. System control, data acquisition and interpretation were made with the Agilent Mass Hunter software version B 01.00 (B48).

2.3. Chromatographic method

The chromatographic separation was carried out on a Luna 5u C18(2) 100A (Phenomenex), 250 mm length, 4.6 mm internal diameter and 5 μm particle size, thermostated at 35 °C. A Phenomenex C18 guard cartridge (2 mm length, 4 mm internal diameter) was used to protect the column inlet. Column qualifications made before and after completion of the study (more than 2600 loaded plasma samples, validation process included) reveal an increase of the reduced plate height ($\bar{h}$) calculated for the phenanthrene peak in a test mixture from 2.4 to 2.5. The column was operated under isocratic conditions, the mobile phase consisting in 3/7 (v/v) aqueous 0.1% formic acid and methanol, at a flow rate of 0.8 mL/min. The whole separation process takes 14 min. The injection volume is 400 μL in order to compensate the loss in sensitivity induced through dilution during the sample preparation procedure.

2.4. MS detection

MRM scan type was performed on two different time segments. The first scan segment with negative ion polarity was defined between minutes 3.2 and 7. It allows detection for furosemide and indapamide (IS1). The second, with positive ion polarity is needed for detection of spironolactone, canrenone and nitrazepam (IS2). The operational parameters of the ESI source were the following: vaporizing temperature 350 °C; pressure of the nebulising gas 60 psi; flow of the drying gas 13 L/min; capillary potential 4000 V. The fragmentor energy for the negative segment was set to 100 V and 140 V on the positive segment. The collision energy for furosemide was 20 V, 25 V for IS1 and 30 V for spironolactone, canrenone and nitrazepam. The electron multiplier was set to 600 V. The MRM transitions used for quantitative purposes (quantifier) were the following: m/z = 341 a.m.u. to m/z = 107 a.m.u. for spironolactone and canrenone, m/z = 329 a.m.u. to m/z = 205 a.m.u. for furosemide, m/z = 282 a.m.u. to 180 a.m.u. for IS2 and m/z = 364 a.m.u. to m/z = 189 a.m.u. for IS1. The MRM transitions used for qualitative purposes (qualifier) were the following: m/z = 341 a.m.u. to m/z = 187 a.m.u. for spironolactone and canrenone, m/z = 329 a.m.u. to m/z = 285 a.m.u. for furosemide, m/z = 282 a.m.u. to 132 a.m.u. for IS2 and m/z = 364 a.m.u. to m/z = 132 a.m.u. for IS1.

2.5. Sample preparation

Protein precipitation through organic solvent addition (acetonitrile, volume ratio 2/1) was used as sample preparation procedure. 200 μL of plasma sample was treated with 400 μL acetonitrile solution containing 150 ng/mL IS1 and 75 ng/mL IS2, followed by vortexing (1 min), centrifuging (9000 × g) and transfer of the supernatant to an injection vial. A volume of 600 μL of aqueous 0.1% formic acid was then added (dilution step is needed to prevent focusing of the target analytes on injection). To conserve final method sensitivity the dilution step was compensated through a large volume injection (400 μL). Plasma samples used for method validation as well as those being taken during the clinical trial (incurred samples) were collected over potassium edetate as anticoagulant.

2.6. Methodology and pharmacokinetic parameters

The analytical method was applied for assessment of a two periods, cross-over, controlled, randomized, single dose, bioequivalence study of a generic immediate release capsule containing 20 mg of furosemide and 50 mg spironolactone against the reference formulation Lasilacton® 20 mg/50 mg-Kapseln (Sanofi Aventis), under fasting conditions, carried out on 35 healthy male and female volunteers, aged 18–45 and a body mass index within 19–29 interval. The protocol of the bioequivalence study was formally accepted by the evaluation department of the Romanian National Medicines Agency and received the approval of the National Ethics Committee. Venous blood samples were collected pre-dose (0 h) and the following post-dose intervals of time: 0.25; 0.5; 0.75; 1; 1.25; 1.5; 2; 2.5; 3; 3.5; 4; 4.5; 5; 6; 8; 10; 12; 14; 24; 48; 60 and 72 h. The wash-out period between phases was of 14 days.

The principal pharmacokinetic parameters considered for evaluation of the bioequivalence were: C_{max} – observed maximum plasma concentration; AUC_{last} – area under plasma concentration/time plot until the last quantifiable value; AUC_{total} – area under plasma concentration/time plot extrapolated to infinity. Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1.) from Thermo Electron Corporation, USA.

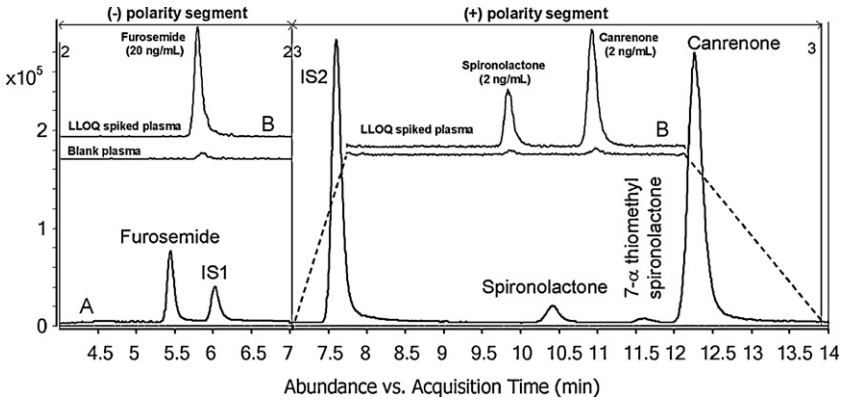

Fig. 1. (A) Typical separation of the target analytes under conditions described for the method, for an incurred sample collected from one volunteer. (B) Comparison between LLOQ signals and carry over effects.

3. Results and discussions

3.1. Method development

Target analytes (including IS1 and IS2) are medium hydrophobic compounds. The logarithm of the experimental partition coefficients between n-octanol and water ($\log K_{ow}$ or log P) are 2.03 for furosemide, 2.2 for IS1, 2.25 for IS2, 2.68 for canrenone and 2.78 for spironolactone. It is expected that under RP separation mechanism, elution takes place in the increasing order of log P values. This was found to be experimentally true, except for spironolactone and canrenone, exhibiting a reversed elution order. Explanation relies to their different solubility in methanol (as long as elution order follows the log P variation when the organic modifier in the mobile phase is a mixture acetonitrile/tetrahydrofuran).

As spironolactone is quantitatively cleaved within the ESI source to canrenone, the mass transitions used to detect both compounds are identical. Such behavior imposes full chromatographic resolution between the active substance and its metabolite [10]. Although the assay of 7-α-thiomethyl spironolactone was not required through the bioequivalence study protocol, its mutual chromatographic resolution against the parent compound and canrenone is necessary, because the ionization pattern follows the behavior of spironolactone.

Relatively fast gradients targeting an increased retention for furosemide failed to make baseline separation of the triplet spironolactone, 7-α thiomethyl spironolactone and canrenone. Slow gradients produce long chromatographic runs and add equilibration periods between runs. It has been decided to use an isocratic elution and to produce retention for furosemide through the use of an increased column length. Separation of the target compounds as showed in Fig. 1A has been achieved in 14 min by using a flow rate of 0.8 mL/min (highest flow tolerated by the ESI source), and represents the best compromise between chromatographic selectivity needed for spironolactone/metabolites and an increased retention of furosemide. However, considering a dead time of the column of about 3.1 min, capacity factors (k) of the target compounds (0.75 for furosemide, 1.0 for IS1, 1.45 for IS2, 2.36 for spironolactone, 2.75 for 7-α thiomethyl spironolactone and 2.95 for canrenone) are still in the interval where the residual plasma matrix from samples may induce serious ionization effects (enhancement or suppression), consequently affecting precision and accuracy [18–21].

Furosemide is readily ionized as a negatively charged molecular ion even from acidic solutions. Decarboxylation also arises in the ESI source (intensity of about 60% from the molecular ion signal) and formation of the negative ion dimer adduct with sodium is observable (about 15% relative intensity). Collisional induced dissociation (CID) follows the same pattern as for the ion source. As negative ionization is more selective and robust, ionization effects of the residual matrix should be lower.

Spironolactone does not produce the molecular $[MH]^+$ ion. Low intensity signal (below 5% relative abundance) of the sodium and potassium adducts may be observed. It is quantitatively transformed and ionized to protonated canrenone. Further CID of the ion species formed in the source leads to formation of a major product ion having $m/z = 107$ a.m.u.

3.2. Method validation

Another delicate aspect to be considered related to the known high inter-individual variability of the maximum plasma concentration values of furosemide, spironolactone and canrenone in healthy volunteers. Implicitly, the LLOQ produced by the analytical method for the target compounds should be low enough to accurately assess the elimination part of the concentration/time curves. This was practically translated in wide dynamic ranges of concentrations considered in the linearity study of the method (20–1600 ng/mL interval for furosemide and 2–100 ng/mL for spironolactone and canrenone) and, consequently, the use of weighted regressions ($1/x^2$, where x is the concentration) to obtain linearization of the MS response functions over the investigated concentration intervals. The need of sensitivity was supplied through the use of a high sample injection volume (400 μL), obtained by multiple loadings onto a loop placed between the needle seat and the column.

The high sensitivity being required together with the nasty habit of analytes to stick on the injector active parts imposed the use of a washing program (needle and needle seat) with acetonitrile to control the carryover effects. During the method selectivity evaluation on six different blank plasma samples, the averaged residual peak areas represented 4.2% for furosemide, 10.2% for spironolactone and 6.4% for canrenone from the corresponding LLOQ mean peak areas (see Fig. 1B). LLOQ values are determined predominantly through residual carryover effects than through signal to noise ratios considerations.

Linearity has been checked over wide concentration intervals, as above mentioned. Eight concentration levels were considered with six replicates per level. Weighed linear regressions ($1/x^2$) were applied between peak area ratios (analyte/IS) and concentration values. The results obtained from the linearity study are summarized in Table 1.

As major concerns were raised by the potential influence of the residual matrix on ionization yields (and consequently on the quantitative results), a comparison was made between the slopes of the regressions obtained during method validation ($n = 13$) produced through sample preparation of spikes made in the same blank

Table 1
Summary of the results obtained from the linearity study carried out on furosemide, spironolactone and canrenone in plasma samples.

Parameter	Furosemide	Spironolactone	Canrenone
No. of concentration levels (n)	8	8	8
Concentration levels (ng/mL)	20/80/200/400/600/800/1200/1600	2/10/14/30/40/60/70/100	2/10/14/30/40/60/70/100
Weighting of the linear regression (w)	$1/x^2$	$1/x^2$	$1/x^2$
Slope (B)	0.0102	0.0417	0.1301
Standard deviation of the slope (s_B)	0.0005	0.0028	0.0076
Intercept (A)	-0.0408	0.0071	0.0124
Standard deviation of the intercept (s_A)	0.0137	0.0095	0.0146
Correlation coefficient (r_{xy})	0.9975	0.9985	0.9994
Accuracy interval on backinterpolation of the experimental data (% bias)	−8.4% to 8.3%	−9.3% to 5.4%	−5.9% to 3.4%
LLOQ[a]	10.7	1.0	0.5
LLOQ[b]	4.4	0.7	0.4
LLOQ[c]	20.0	2.0	2.0

[a] LLOQ = $(5 \times s_A - A)/B$, according to [22,23].
[b] LLOQ = $[t \times (s_A + s_B \times x_m)]/(B + 2 \times t \times s_B)$, according to [24], where t is the Student coefficient tabulated for 6 degrees of freedom ($n-2$) and 90% level of certainty and $x_m = \sum_{i=1}^{n} w_i \times x_i / \sum_{i=1}^{n} w_i$.
[c] LLOQ considered as the concentration corresponding to five times the average residual peak area in the chromatograms of blank samples.

plasma and the slopes of the regressions obtained through preparation of spikes in ten different blank plasmas. Results are shown in Table 2.

When comparing data from Tables 1 and 2, a decreasing trend may be observed for the slopes characterizing the linear response function of the mass spectrometric detector. When results obtained from samples spiked in the same blank plasma are compared, the decreasing trend (probably produced through accumulation of the residual matrix in the detection system) is less significant and their variability is placed within the normal threshold. When such data are compared to results obtained from samples spiked in different blank plasmas, the higher variability and the decreasing trends are obvious. Despite its reduced chromatographic retention, potentially allowing increased interference risks, furosemide seems less affected. The specificity and robustness of the negative ionization mode used for the detection of the analyte may represent a coherent explanation of the facts. Although exhibiting considerable retention compared to furosemide, spironolactone and canrenone are sensibly influenced by the specific residual matrix produced by the plasma. The influence on ionization of the common anticoagulants (citrate, potassium edetate and lithium heparin) used for blood sample collection has been also investigated. No influences were observed for furosemide (concentration level of 700 ng/mL, R.S.D.% values between 0.6 and 3% and % bias between 11.5 and 13.8%, $n = 5$). Citrate and potassium edetate are not influencing ionization of spironolactone and canrenone, tests made on samples spiked at 50 ng/mL level producing response variations in the R.S.D.% range of 0.6–6.6% and % biases from −1.6 to 4.2% ($n = 5$). In the presence of lithium heparin, signal suppression effects were observed for both compounds (% biases ranging from −16% to −21%), although precision is unaffected (R.S.D.% values from 4 to 6.6%).

Table 2
Statistic evaluation of the slopes (B) of the regressions obtained from different linearity studies carried out on samples made in the same blank plasma or in different blank plasmas.

Parameter	Furosemide	Spironolactone	Canrenone
$n = 13$, same blank plasma			
Mean B	0.0097	0.0406	0.1292
s_B	0.0008	0.0033	0.0165
R.S.D.%	8.6	8.2	12.8
$n = 10$, different blank plasma			
Mean B	0.0107	0.0316	0.1059
s_B	0.0010	0.0050	0.0226
R.S.D.%	9.2	15.7	21.3

Recoveries were evaluated at three concentration levels (60, 700 and 1400 ng/mL for furosemide and 6, 50 and 80 ng/mL for spironolactone and canrenone) for the target compounds and at 300 ng/mL level for IS1 and 150 ng/mL for IS2 (concentrations of internal standards are expressed with respect to the plasma aliquot volume). Five replicates were considered per concentration level. Recovery was computed as ratio (expressed as percentage) of peak area values integrated in chromatograms from spiked plasma samples and samples spiked to corresponding bulk protein precipitated blank plasma having identical concentrations of the target compounds. Found recovery values are 98% for furosemide, 101.9 for spironolactone, 99.6% for canrenone, 97.4% for IS1 and 99.4% for IS2. Precision (expressed as R.S.D.%) ranged from 1.8 to 5.3%.

Taken into consideration the experimental findings, the following decisions have been made on bioequivalence study completion: (a) the analytical sequence should contain a calibration set with eight concentration levels (same as for the linearity study in method validation), a quality control (QC) set with four concentration levels (60/300/700/1400 ng/mL for furosemide and 6/20/50/80 for spironolactone and canrenone), two replicates per level, and samples obtained from one volunteer (two phases, each phase containing the pre-dose sample, IS spiked pre-dose sample and 22 sampling times; samples corresponding to the same sampling time and different phases are consecutive in the sequence); (b) calibration and QC samples are produced for each volunteer through spiking the corresponding pooled pre-dose plasma portions collected during the two phases (consequently, matrix effects are basically the same for calibration, QC and analyzed samples); (c) cleaning of the ESI source should be made before starting every analytical sequence; (d) dilution of the samples exceeding upper quantification limits (based on successfully dilution integrity results achieved during method validation for 1:2, 1:5 and 1:10 volumetric ratios) should be made with the blank plasma belonging to the respective volunteer. For validation of the analytical sequence, the general conditions referred by the guideline in force [25] were applied: a maximum of two QC samples (belonging to different concentration levels) from a total of eight may be placed outside the accuracy threshold of ±15%, and a maximum of two from eight calibration samples may be excluded during calculation of the weighted regression parameters with fulfillment of the condition that the remaining six experimental data produce through backinterpolation results within the accuracy threshold. Due to an increased duration of a working sequence (around 16 h), an additional condition addressing precision was imposed: the R.S.D.% computed for IS peak areas along the analytical sequence should be placed within the 15% limit.

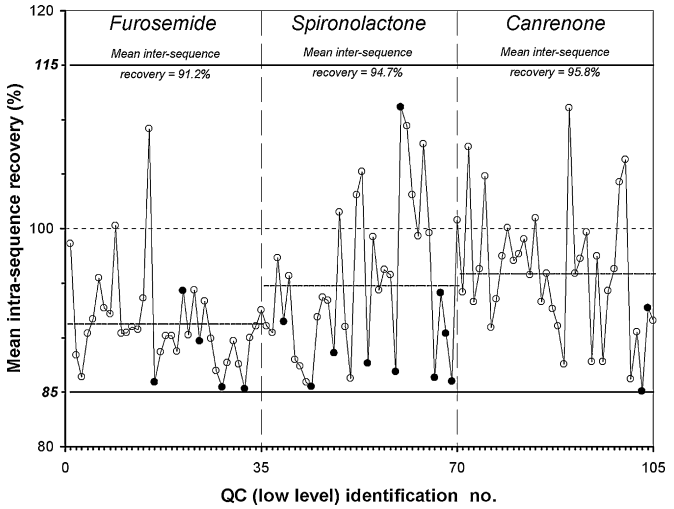

Fig. 2. Variation of the mean intra-sequence recoveries obtained for the target compounds in QC samples at the lowest concentration level (filled points indicates that the value is not resulting from averaging, as one of the experimental results was placed outside the imposed accuracy threshold).

As the analysis of the 2240 individual samples organized under 35 sequences took around 24 working days, interpretation of data resulting from calibrations and QC sets run over study completion represents an important tool for evaluation of accuracy and precision of both analytical method and MS instrument. The variation of the mean intra-sequence recoveries (%) determined for the target compounds over the study completion (35 sequences) at the low QC level is presented in Fig. 2. As the low QC is close to the quantification limit (3 × LLOQ), data from Fig. 2 illustrate the "worst case" situation. Obviously, the requirements of the guidances in force have been fulfilled.

From 280 calibration samples, 21 (7.5%) were excluded from calculation of the corresponding regression parameters in the case of furosemide, 24 (8.6%) in the case of spironolactone and only 12 (4.3%) in the case of canrenone. The concentrations of the QC samples were computed based on these regression parameters resulting from calibration data sets. Table 3 summarizes experimental data obtained for the QC samples analyzed during study completion.

Precision of the method has been studied at 4 concentration levels (60/300/700/1400 ng/mL for furosemide and 6/20/50/80 ng/mL for spironolactone and canrenone). Intra-run precision was verified through 10 replicates made from the same spiked sample at each of the concentration levels during a single analytical sequence taking around 7 h (calibration included). Inter-run precision was evaluated using independent samples prepared and analyzed during six analytical sequences delayed by at least 24 h and run over a total time interval of 13 days. R.S.D.% values ranged (for both procedures and for all analytes) between 2.2 and 10.9%, while % biases cover the interval between −11% and 8.2%.

Long term (−40 °C) stability for all analytes was confirmed for a period of 3 months and short term stability (room temperature) for a period of 24 h. The freeze and thaw stability for five consecutive cycles was studied and confirmed. Stability at 25 °C in the autosampler of processed plasma samples have been demonstrated over an interval of 48 h. IS1 and IS2 stock solutions were found stable over a month period.

For evaluation of the robustness of the method, the following operational parameters and variation intervals were considered: (1) mobile phase composition (solvent A from 29 to 31%; (2) formic acid content in the aqueous component of the mobile phase (from 0.09 to 0.11%; (3) source drying gas temperature (from 300 to 350 °C); (4) source drying gas flow rate (from 11 to 13 L/min); (5) source nebulizer pressure (from 50 to 60 psi); (6) capillary voltage (from 3500 to 4500 V). Quantitative results were compared for three discrete concentration levels for each of the target com-

Table 3
Summary of the results obtained from QC sets on bioequivalence study completion (35 analytical sequences).

Concentration level	Furosemide			Spironolactone			Canrenone		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
QC1	5	91.2	5.0	10	94.7	7.6	2	95.8	6.3
QC2	5	95.8	6.9	5	106.7	4.8	1	97.5	6.2
QC3	2	98.7	6.9	3	102.9	6.1	4	103.1	6.5
QC4	5	104.8	6.1	7	106.5	5.8	6	104.9	6.0

(1) Number of QC samples placed outside the accuracy threshold.
(2) Mean inter-sequence recovery (%).
(3) R.S.D.% of mean intra-sequence recoveries.

Table 4
Principal pharmacokinetic parameters determined for furosemide (F), spironolactone (S) and canrenone (C), from reference (R) and tested (T) pharmaceutical formulations considered for the bioequivalence trial in fasting conditions.

Pharmacokinetic parameter	F/R	F/T	S/R	S/T	C/R	C/T
C_{max} (ng/mL)	526.06	607.8	29.54	27.76	61.75	69.57
s[a]	232.9	327.6	21.4	14.6	22.9	26.4
AUC_{last} (ng/mL h)	1101.53	1253.17	55.44	58.27	873.79	915.83
s	395.0	537.0	36.5	31.3	372.2	384.2
AUC_{tot} (ng/mL h)	1189.20	1332.00	60.57	63.37	968.07	1006.38
s	404.3	537.5	36.7	32.3	389.5	396.3
R.S.D.% inter-subject for						
C_{max}	35.6		40.4		15.1	
AUC_{last}	20.5		25.1		7.6	
AUC_{tot}	18.4		23.9		7.7	
90% confidence limits for						
C_{max}	0.934–1.246		0.847–1.193		1.054–1.193	
AUC_{last}	1.012–1.194		0.977–1.209		1.018–1.084	
AUC_{tot}	1.010–1.172		0.968–1.186		1.011–1.108	

[a] s = standard deviation.

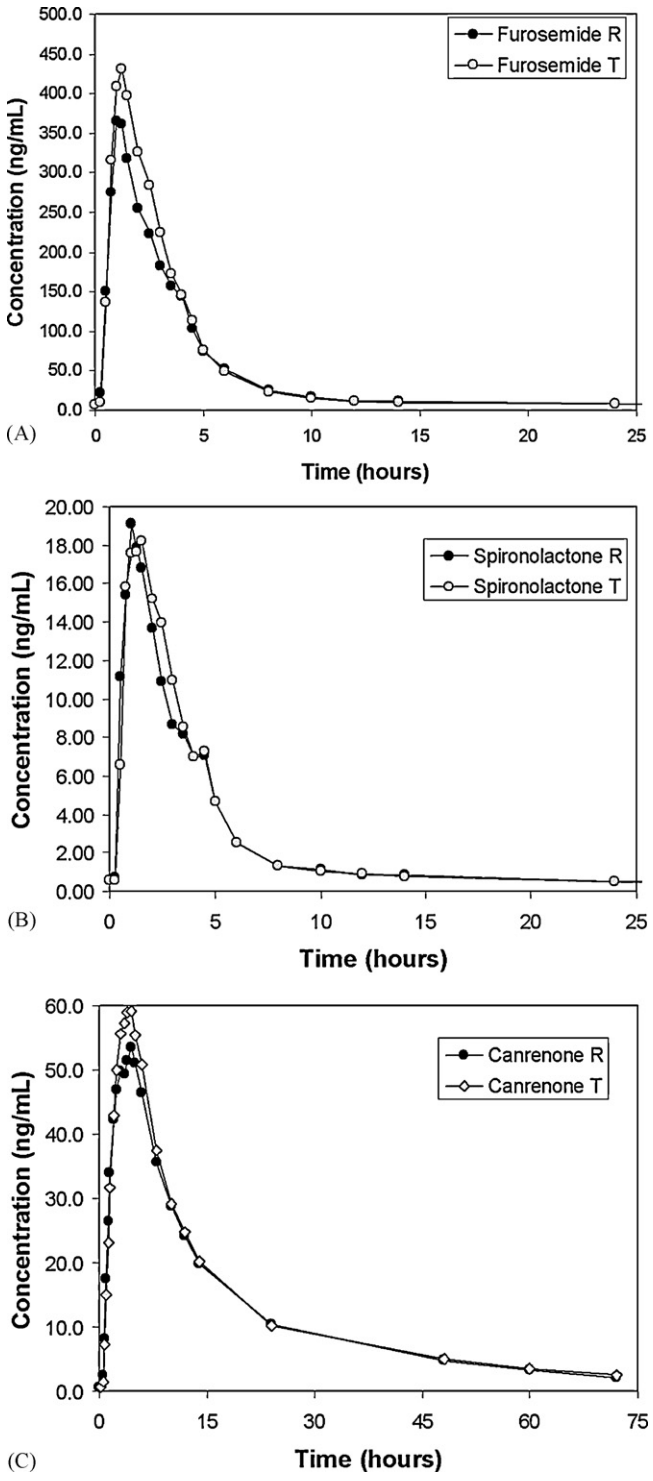

Fig. 3. Mean plasma concentration/time profiles resulting from the bioequivalence study for the target compounds (A: furosemide, B: spironolactone and C: canrenone).

pounds. None of the combinations of the operational parameters above cited are producing quantitative results outside the required thresholds of precision and accuracy.

3.3. Application to a bioanalytical study

Mean concentration/time profiles obtained for the reference and tested pharmaceutical products over the bioequivalence study completion are given in Fig. 3.

Pharmacokinetic parameters obtained for furosemide, spironolactone and canrenone during the fasting single dose clinical trial are given in Table 4. Large inter-individual variations of the principal pharmacokinetic parameters were observed for furosemide and spironolactone, as already indicated in literature. The bioequivalence of the two products could be satisfactorily concluded based on the experimental data. Determined pharmacokinetic parameters are in fair agreement with previously published data [26–31].

4. Conclusions

A new HPLC/MS/MS method has been developed and validated to assay simultaneously furosemide, spironolactone and canrenone in human plasma samples. The method was successfully applied to the analysis of more than 2000 real samples issued from a bioequivalence study of two pharmaceutical formulations containing 50 mg of spironolactone and 20 mg of furosemide.

The simple sample preparation procedure (plasma protein precipitation with acetonitrile) induces low analytical variability but decreases the sensitivity of the method (through the intrinsic dilution and through water addition to the supernatant to prevent focusing of the analytes on injection).

For preserving sensitivity, a large volume (400 μL) injection was applied, resulting in LLOQ of 20 ng/mL for furosemide and 2 ng/mL for spironolactone and canrenone. Carryover effects were controlled by an appropriate acetonitrile washing procedure for the needle and the needle seat.

The response function of the instrument was affected by the presence of the residual matrix reaching the interface. The increased duration of the chromatographic run (needed for baseline separation of spironolactone, canrenone and 7-α thiomethyl spironolactone and to induce some retention for furosemide) imposes the adequate choice of the analytical sequence length, to control precision over the study completion. Method development and validation were carefully assessed and specific analytical problems issuing from the application on a large number of samples were largely discussed.

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