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Liquid chromatography–electrospray tandem mass spectrometry method for determination of indapamide in serum for single/multiple dose bioequivalence studies of sustained release formulations

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Abstract

Indapamide and internal standard (5-chloro-2-methoxy-*N*-[2-(4-sulphamoylphenyl)ethyl]benzamide) were isolated from plasma by a single step liquid–liquid extraction in *t*-butyl methyl ether. The chromatographic separation was achieved on a reversed-phase C_{18} monolithic column with a mobile phase consisting in a methanol/aqueous 0.1% formic acid mixture and a flow rate of 0.8 ml/min, in isocratic conditions, within 11 min. Target compounds were transferred in an ion trap analyzer via an atmospheric pressure electrospray interface (AP-ESI). The mass analyzer was used in a selected reaction monitoring (SRM) mode, in order to enhance on detection selectivity. Whole method produces quantitation limit for indapamide of 1 ng/ml. Method was successfully applied to assess bioequivalence of two sustained release marketed pharmaceutical formulations of indapamide 1.5 mg coated tablets, carried-out in a single/multiple doses, randomized design.

Keywords: Indapamide; Serum; Liquid–liquid extraction; Liquid chromatography; Electrospray tandem mass spectrometric detection; Bioequivalence; Sustained release formulations

1. Introduction

Indapamide, 4-chloro-*N*-[(2*RS*)-2-methyl-2,3-dihydro-1*H*-indole-1-yl]-3-sulphamoylbenzamide (CAS 26807-65-8) is an antihypertensive agent also acting as a diuretic, belonging to the new indolines class.

Although the assay of indapamide in pharmaceutical formulations and determination of the related substances profile is frequently referred in literature, using spectrometric [1–4], electrometric [5,6] and chromatographic [7–9] methods, only very few HPLC/UV methods were dedicated to its determination in biological fluids [10–15].

Indapamide is marketed as immediate release pharmaceutical formulations containing 1.25 and 2.5 mg active substance per dose and as sustained release coated tablets of 1.5 mg per dose. According to [16], expected maximum plasma concentration after a single intake dose of 2.5 mg indapamide formulation with immediate release should be three to five-folds higher than for a 1.5 mg single dose of a sustained release product (maximum plasma concentrations are reduced from 80 to 100 ng/ml to 10 to 30 ng/ml). In reference [17], a mean maximum blood concentration of 115 ng/ml indapamide was determined after about 2 h for a 2 × 1.25 mg immediate release tablet intake (study was carried out only on male subjects). The final results of the present work are in good agreement with data above cited.

Isolation of indapamide from plasma is a tedious task, because of low concentration levels and matrix induced interferences. More often, liquid–liquid extraction procedures require two or three successive steps (extraction, back-extraction, and re-extraction) [12,15] to eliminate matrix interferences. More over, ethyl acetate and diethyl ether were

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used as extracting phases. Solid phase extraction (SPE) was successfully used to isolate indapamide from plasma samples resulting after 5 mg active substance intake as an immediate release product [11,13]. However, recovery of indapamide strongly depends on the type of the hydrophobic adsorbent, variations from 30 to 100% being observed. It is worthwhile to note that UV detection can be considered as not selective and sensitive enough to assay indapamide in biological fluids. Recently, a SPE/HPLC/(AP-ESI) MS^2 method has been proposed for the assay of 35 diuretics (including indapamide) in urine [18]. The automated SPE approach was developed for doping control purposes. Recoveries for most analytes were greater than 80% for concentrations below 100 ng/ml. The liquid–liquid extraction approach developed within the present paper for isolation of indapamide from plasma samples in the view of bioavailability/bioequivalence (BA/BE) evaluation is characterized by identical recovery and quantitation limits around 1 ng/ml. Costs related to SPE automation as well as cartridge consumption during complete BA/BE studies should be also considered somewhat limitative (note that the multiple use of a SPE cartridge is not recommended and for a study completion, including validation purposes, around 2000 samples are run).

Mass spectrometry combined with capillary gas chromatography was used for the sensitive and selective determination of indapamide and other 17 diuretics in human urine [14]. Isolation from urine was done by liquid–liquid extraction, followed by an additional derivatization procedure, microwave assisted. Mass spectrometric detection interfaced to HPLC by means of AP-ESI was recently reported in literature for monitoring reversed-phase chiral separation of indapamide enantiomers [19].

Therefore, our aim was to combine high separation capabilities of liquid chromatography with the selective/sensitive characteristics of mass spectrometry for determination of indapamide in plasma samples at ng/ml level. Isolation of indapamide was achieved by a single step liquid–liquid extraction in *t*-butyl methyl ether, followed by solvent evaporation, re-dissolution of the residue and injection onto the chromatographic column. The analytical procedure was fully validated and successfully used to assess bioequivalence of two marketed pharmaceutical formulations of 1.5 mg indapamide with sustained release.

2. Experimental

2.1. Instrument

Experiments were performed on Agilent 1100 series LC/MSD system composed of the following modules: degasser (G1379A), quaternary pump (G1311A), thermostated autosampler (G1329A), column thermostat (G1316A), AP-ESI standard interface (G1948A), ion trap mass spectrometric detector SL series (G24450), nitrogen generator (5183-2003). System control and data acquisition were made with the Agilent LC/MSD trap Software version 4.2. incorporating the MSD Trap Control software version 5.1. from Brucker Daltronics. The system was operationally qualified before and after the bioequivalence study. The repeatability ($n = 6$) of the MSD ion trap SL determined for 5 pg of reserpine loaded to interface was characterized during the study by relative standard deviations (R.S.D.%) of 10.9% (before) and 13.8% (after).

2.2. Chromatographic conditions

A monolithic Chromolith Performance RP-18e column (Merck, Germany), 100 mm length and 4.6 mm internal diameter fitted with a Chromolith Guard cartridge RP-18e (10 mm × 4.6 mm) was used. Column was thermostated at 40 °C. Column was validated before and after study completion, by computing the height equivalent to the theoretical plate (HETP) of fluoranthene peak (variation from 7.6 to 11.3 μm was noticed during the study including method validation).

Elution was isocratic, using methanol and aqueous 0.1% (v/v) formic acid as mobile phase constituents, mixed in the volumetric ratio of 42.5:57.5 and a flow rate of 0.8 ml/min. Injection volume was set at 100 μl.

2.3. Interface parameters

The parameters controlling the AP-ESI standard interface were: drying gas flow: 12 l/min; drying gas temperature: 365 °C; pressure of the nebulizer gas: 65 p.s.i.; capillary voltage: 3000 V; high voltage end plate offset: −500 V.

2.4. MSD ion trap SL operational parameters

Ion polarity was positive for both indapamide and internal standard (I.S.). SRM working mode was used. The trap parameters for indapamide were: chromatogram segment: 4–7.4 min; scanning interval: 125–370 *m*/*z*; accumulation time: 200 ms; ion current control: 20,000; eight averaged spectra per data point, isolation mass: 366.0; width: 4; fragmentation amplitude: 1.2 V. The trap parameters for I.S. were: chromatogram segment: 7.4–11 min; scanning interval: 155–375 *m*/*z*; accumulation time: 200 ms; ion current control: 30,000; eight averaged spectra per data point, isolation mass: 369.1; width: 4; fragmentation amplitude: 1.0 V.

2.5. Materials

All solvents were HPLC grade from Merck (Darmstadt, Germany). Formic acid (98–100%) was reagent Ph. Eur. grade, also from Merck. 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. Indapamide and the I.S., 5-chloro-2-methoxy-*N*-[2-(4-

sulphamoylphenyl)ethyl]benzamide, standard reference substances were purchased from European Pharmacopoeia, Council of Europe, Strasbourg, France (indapamide 97.7%, batch 2a, cat. no. EP I0150000 and internal standard, batch 1b, cat. no. EP G0325010).

2.6. Sample preparation procedure

One milliliter of plasma sample was mixed with 50 μl of a 250 ng/ml I.S. solution in methanol and 1 ml of water. The aqueous phase was wortexed 15 min at 350 rpm with 5 ml of *t*-butyl methyl ether. After phase separation, an aliquot of 4 ml from the organic phase was evaporated to dryness at 40 °C under nitrogen flow. The residue was re-dissolved with 200 μl of mobile phase.

2.7. Methodology and pharmacokinetic parameters

During the open-label, randomized, two-period, two-sequence, crossover bioequivalence study, 24 healthy male and female volunteers received five doses of 1.5 mg indapamide from the tested (T) and reference (R) sustained release coated tablets, at 24 h interval, with a 14 days wash-out between periods. Blood samples were taken before the administration of each of the five tablets, at 0.5; 1; 1.5; 2; 2.5; 3; 4; 5; 6; 8; 10; 12 h after the first and the fifth administration and 24; 48 h after the fifth administrations (31 samples per volunteer and per phase). The pharmacokinetic parameters used as primary evaluation criteria for bioequivalence assessment were: observed maximum concentration during first day (C_{max}); sampling time of C_{max} (T_{max}); area under plasma concentration/time curve up to 24 h after the first dose administration (AUD); C_{max} at steady state ($C_{max,SS}$); and area under curve at steady state (AUD_{SS}).

3. Results and discussion

3.1. Method development

3.1.1. Choice of the I.S.

Similarities between chemical structures of indapamide and I.S. are evident (see Fig. 1). Both compounds contain one chlorine atom substituted to an aromatic ring, aminosulfonyl and N-substituted amide moieties. Computed logarithms of the partition coefficient between *n*-octanol and water (log *P*) as descriptor of the molecular hydrophobicity, are 2.66 for indapamide and 2.51 for I.S. We can further assume that the extraction behavior of the target compounds in a polar solvents is similar. Chromatographic behavior also sustains the previous observation, as long as indapamide and I.S. are reciprocally separated with moderate resolution (∼4). The experimental reversed elution order (theoretically reversed phase mechanism achieves separation in the increased order of log *P* values) could be explained by a higher solubility of indapamide in methanol compared to I.S.

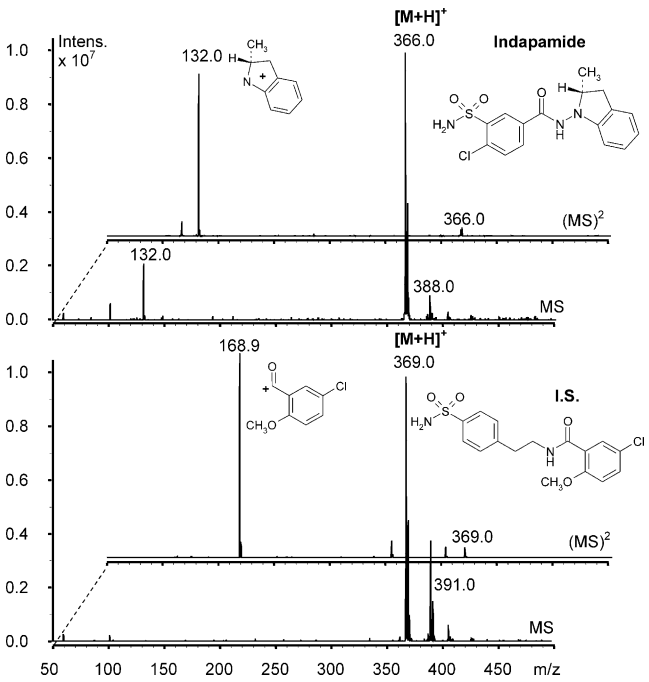

Fig. 1. Ionization pattern of indapamide and the I.S. in the ES interface; product ions resulting from the CID of the protonated molecular ions used for monitoring chromatograms.

In $(MS)^2$ detection, it is advisable that both analyte and I.S. produce precursor ions having similar *m*/*z* values, for narrowing the scan range and subsequently enhance on data acquisition rate and sensitivity. Indapamide (exact molecular mass of 365.06009 amu) leads under ES ionization mainly to the protonated molecular ion $[M + H]^+$ (*m*/*z* 366.0). Formation of the adduct ion $[M + Na]^+$ was also observed with low intensity. Molecular fragmentation during ES ionization is poor, the fragment ion *m*/*z* 132 exhibiting low intensity. If species *m*/*z* 366 is selected as precursor ion, after collisional induced dissociation (CID), fragment ion *m*/*z* 132 (whose structure is given in Fig. 1) is produced. As it can be observed, fragmentation of the precursor ion is almost quantitative. I.S. (exact molecular mass of 368.05978 amu) leads under ES ionization to the protonated molecular ion $[M + H]^+$ (*m*/*z* 369.0). Sodium adduct $[M + Na]^+$ is also formed with reduced yield. No fragmentation was observed under ES ionization conditions. After CID, the precursor ion *m*/*z* 369 is quantitatively fragmented to the positive ion *m*/*z* 169 (see Fig. 1). One can conclude that indapamide and I.S. form with high yield protonated molecular ions under ES ionization, characterized by close *m*/*z* values (366 and 369, respectively). These precursor ions are readily fragmented to product ions *m*/*z* 132 and 169, respectively, during CID.

3.1.2. Extraction procedure

Different extraction solvents were tested (chloroform, dichloromethane, 1,1,1-trichloroethane, 1,2-dichloroethane, diethyl ether, dibutyl ether, diisopropyl ether, *t*-butyl methyl ether, ethyl acetate). Among them, indapamide and I.S. were

recovered with yields higher than 80% in dichloromethane (bp 40 °C), diethyl ether (bp 34.6 °C), *t*-butyl methyl ether (bp 55.3 °C) and ethyl acetate (bp 77 °C) from spiked plasma samples at 10 ng/ml concentrations. The final solution was a compromise between higher boiling point solvents increasing evaporation time and lower boiling point solvents allowing accidental loss on manipulation due to vaporization. *t*-Butyl methyl ether was thus selected.

When controlling the pH in the aqueous phase at values of 3, 7 and 10 using phosphate buffers, no significant influence on recovery could be observed compared to the uncontrolled pH alternative. As indapamide behaves as a weak acid (pK_a is 8.8) similarities of results obtained for uncontrolled pH and buffered media at pH 3 and 7 values are fully understandable. pH 10 should be considered to be low enough to not drastically increase the solubility of indapamide in aqueous media. Addition of sodium chloride in plasma to increase ion strength reduces indapamide recovery. This effect could be explained by a reduction of detectability due to competitive formation of the molecular sodium adduct ions during ES ionization stage rather than a salting in effect appearing on extraction.

Volumetric ratio between the aqueous phase and the extraction solvent, when modified in the range 1:2 up to 1:10 do not significantly affect recoveries of the target compounds. It is also noticed that intervals of extraction between 5 and 30 min generate mean recoveries within the normal variation interval.

Recovery from spiked plasma samples in a concentration interval 10–50 ng/ml is 80.4 ± 6.8% (n = 10) for indapamide and 92.8 ± 6.7% (n = 15) for the I.S. When extraction procedure was applied on plasma samples spiked with indapamide at 1 ng/ml concentration, mean recovery of 85.4% (n = 5) falls within the variation interval mentioned above.

One can conclude that extraction procedure is simple, precise, and robust.

3.1.3. The chromatographic method

Reciprocal variations of ±0.1% of the components of the mobile phase produce retention data within the normal variation interval (5.8–6.2 min for indapamide and 8.0–8.6 for the I.S.). An increase of 5% in the methanol content of the mobile phase still generates baseline separation of the target compounds, while a same variation of the aqueous component leads to 30% longer duration of the chromatographic run. Replacing formic acid by acetic acid or trifluoroacetic acid (TFA) does not influence the retention behavior of the analytes. However, addition to the mobile phase with TFA reduces detection sensitivity by a factor of 10. The concentration of formic acid in the aqueous component of the mobile phase does not affect retention and resolution. Detection sensitivity decreases with a factor of 2 when doubling or halving the formic acid content. Variations of column temperature within ±2 °C interval produce retention data within normal variation intervals. A column temperature increase with 5 °C still generates chromatographic resolution higher than 3 between peaks, while a column temperature of 35 °C increase retention with 10%. According to these results, chromatographic method should be considered as robust.

3.2. Method validation

3.2.1. Linearity, limit of quantitation (LOQ), limit of detection (LOD)

Linearity was tested at nine concentration points (0; 0.25; 0.50; 1; 2.5; 5.0; 10.0; 25.0 and 50 ng/ml, respectively) for indapamide and at 12.5 ng/ml concentration of the I.S. Calibration was done by plotting the ratio of the peak area corresponding to indapamide and the I.S. against the concentration of indapamide in spiked plasma samples, expressed in ng/ml. Calibration was repeated during 5 different days. The slope (B) of the linear regression was 0.0317 ± 0.0026 and the intercept (A) 0.0009 ± 0.0160. Mean correlation coefficient (r_{xy}) was 0.9986. LOQ was computed according the relationship $\mathrm{LOQ} = 2t(s_A + x_m s_B)/(B + 2ts_B)$, where t is the Student coefficient for a degree of certainty of 90% and 7 degrees of freedom (1.397); s_A the standard deviation of the intercept; s_B the standard deviation of the slope and x_m is the mean value of the concentrations of the solutions used for calibration. Calculations on experimental data lead to a LOQ for indapamide of 0.75 ng/ml. It results that LOD for indamide is situated at 0.2 ng/ml level (LOD ≈ LOQ/3.33). Note that peak areas of the I.S. during experiments were characterized by an R.S.D.% of 8.11% (n = 45). In Fig. 2, chromatograms of spiked plasma samples containing 0.25 ng/ml (close to LOD), 1.0 ng/ml (close to LOQ) of indapamide and 12.5 ng/ml I.S. are presented.

3.2.2. Precision

Repeatability was studied at three concentration levels (1, 10 and 50 ng/ml) for indapamide and 12.5 ng/ml level for the I.S. Ten aliquots were prepared from the stock solution at each concentration level. The relative standard deviations of the ratios between indapamide and I.S. peak areas were 14.3% at

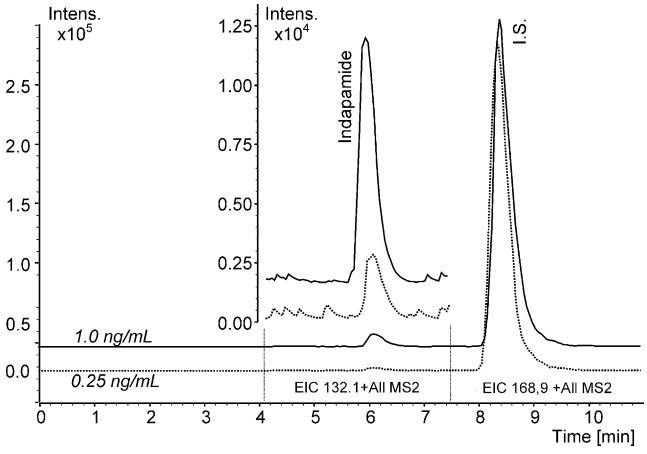

Fig. 2. Extracted ion chromatograms resulting after processing spiked plasma samples containing indapamide at LOD and LOQ levels.

1 ng/ml, 5.4% at 10 ng/ml and 4.6% at 50 ng/ml. The R.S.D.% calculated for the values of the peak area corresponding to the I.S. during the study was 6.7% ($n = 30$). R.S.D.% for retention time values characterizing chromatographic peaks is 0.53% for indapamide and 0.22% for the I.S.

The intermediate reproducibility was carried out in five different days at the same concentration levels for spiked plasma samples freshly prepared daily. R.S.D.% for ratios between indapamide and I.S. peak areas were 6.7% at 1 ng/ml level, 9.1% at 10 ng/ml level and 5.8% at 50 ng/ml level, respectively. The R.S.D.% calculated for peak area values characterizing I.S. during the study was 11.2% ($n = 15$).

3.2.3. Accuracy

Accuracy was determining by measuring quality control plasma samples spiked at 1; 2.5; 5; 10; 25 and 50 ng/ml levels during 7 different days. I.S. is always spiked at 12.5 ng/ml. The values of the ratio between indapamide and I.S. peak areas were then interpolated in the linear regression equation found during the *linearity* study, to calculate experimental concentration values. Results are given in Table 1.

3.2.4. Freeze and thaw stability

Stock indapamide spiked plasma samples having concentrations of 1, 10, and 50 ng/ml, respectively, and 12.5 ng/ml I.S. 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. R.S.D.% calculated for indapamide recovered concentration at each during five cycles were 14.95% (1.02 ± 0.3 ng/ml), 12.6% (10.0 ± 2.5 ng/ml), and 6.65% (49.3 ± 6.6 ng/ml), respectively. During such studies, R.S.D.% of 20% at LOQ and 15% at higher concentration were considered as acceptable for proving stability.

3.2.5. Long term stability

Stock indapamide spiked plasma samples having concentrations of 1, 10, and 50 ng/ml, respectively, and 12.5 ng/ml I.S., were divided in separate vials and were stored at −40 °C. At the begging of each daily session, one vial for each concentration level was thawed unassisted at room temperature and then processed. R.S.D.% calculated for recovered indapamide concentration at each level for samples processed during 22 days were 17.1% (1.07 ± 0.4 ng/ml), 6% (10.25 ± 1.2 ng/ml), and 0.9% (50.1 ± 0.9 ng/ml), respectively.

3.2.6. Short term stability

Stock indapamide spiked plasma samples having concentrations of 1, 10, and 50 ng/ml, respectively, and 12.5 ng/ml I.S., were stored 24 h at −40 °C, then thawed unassisted at room temperature and kept at this temperature for 48 h. Aliquots from each stock plasma sample were processed immediately after thaw, 4, 12, 24 and 48 h later. R.S.D.% calculated for recovered indapamide concentration at each level for processed samples were 6.2% (1.07 ± 0.14 ng/ml), 6.3% (9.6 ± 1.2 ng/ml), and 7.5% (48.04 ± 7.2 ng/ml), respectively.

3.2.7. Stock solution stability

Stock solution of the I.S. (0.01 mg/ml) in methanol was stored at room temperature during 22 days. From this solution, dilutions at 50 ng/ml level in the mobile phase were made during the 1st, 5th, 8th, 12th, 19th, and 20th day. Resulting samples were injected as such. R.S.D.% calculated for I.S. peak areas is 8.7%.

3.2.8. Post-preparative stability

Processed stock samples obtained from 1, 10 and 50 ng/ml indapamide spiked plasma samples were stored at room temperature, bench top or in the autosampler. Samples were assayed immediately, 1, 3, 6, 12, 18 and 24 h after preparation. R.S.D.% calculated for recovered indapamide concentration at each level for processed samples kept at room temperature 24 h were 6.5% (1.1 ± 0.14 ng/ml), 8.0% (9.45 ± 1.5 ng/ml), and 7.6% (46.3 ± 7.0 ng/ml), respectively.

3.3. Pharmacokinetic parameters

The previously presented method was used to assess bioequivalence of two indapamide 1.5 mg sustained release tablets found on the Romanian market (one is referred as tested, the other as reference). For the first 24 h interval, C_{max} was similar for both drugs (6.70 for R and 6.45 for T, respectively). The T_{max} was practically identical for both drugs (11.9 h for T and 11.8 h for R). The ratio between determined AUD for tested and reference products is 0.967 (115.6 ng/ml h for R and 113.7 ng/ml h for T, respectively).

Table 1
Illustration of the method accuracy at six indapamide concentration levels during seven separate experimental sessions

Spiked concentration (ng/ml)	Concentration found (ng/ml)							Mean	Standard deviation	R.S.D.%
	1	2	3	4	5	6	7			
1.0	0.9	1.1	1.1	0.9	1.1	1.1	1.0	1.04	0.088	8.5
2.5	2.0	2.2	2.1	2.1	2.1	2.1	2.5	2.27	0.353	15.6
5.0	4.3	4.8	4.4	5.2	5.3	5.3	4.0	4.56	0.537	11.3
10.0	11.1	9.6	9.7	9.8	9.6	9.6	10.4	10.17	0.649	6.4
25.0	25.1	23.8	24.6	25.6	26.3	26.3	25.1	25.28	0.919	3.6
50.0	52.2	47.8	49.4	46.5	51.3	51.3	49.1	49.98	2.496	5.0

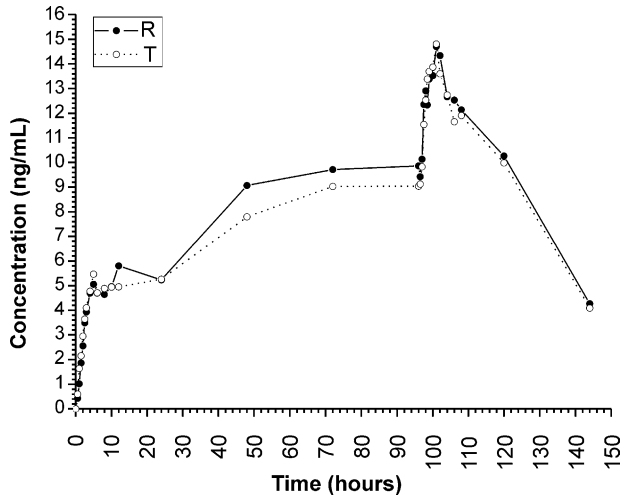

Fig. 3. Mean plasma concentration–time plots obtained during assessment of the bioequivalence of two marketed sustained release pharmaceutical formulations containing 1.5 mg of indapamide carried out on 24 healthy volunteers, in a randomized, two-period, two-sequence, crossover, single/multiple dose design (notations: T for the tested formulation, R for the reference formulation).

For the steady state interval, $C_{max,SS}$ and AUD_{SS} were also very similar for the considered pharmaceutical formulations ($C_{max,SS}$ were 16.5 ng/ml for R and 16.23 ng/ml for T, while AUD_{SS} were 277.7 ng/ml h for R and 274.1 ng/ml h for T). Variability of the pharmacokinetic parameters was very similar for the products under study. Bioequivalence was assessed by the statistical evaluation of the pharmacokinetic parameters and is not detailed in the present material. The mean indapamide plasma concentration–time plots, obtained for tested and reference pharmaceutical formulation, on 24 healthy volunteers, during the open-label, randomized, two-period, two-sequence, crossover, single/multiple dose bioequivalence study is given in Fig. 3.

4. Conclusions

An analytical method for determination of indapamide in plasma samples at the low ng/ml level was presented. 5-Chloro-2-methoxy-*N*-[2-(4-sulphamoylphenyl)-ethyl]benzamide was used as internal standard. Sample preparation is based on liquid–liquid extraction using *t*-butyl methyl ether. Organic layer is evaporated; residue is retaken and loaded to the chromatographic column. The sample preparation procedure is relatively simple and robust. Chromatographic method is based on a reversed phase mechanism carried out under isocratic conditions. Tandem mass spectrometric detection was used; ionization was realized with an electrospray interface. The ion trap mass analyzer isolates first the protonated molecular ions of the target compounds as precursor ions while after the CID, product ions 132 *m*/*z* for indapamide and 168.9 for I.S. are monitored. Whole analytical method was validated and used to assess bioequivalence of two marketed sustained release formulations containing 1.5 mg indapamide. During the study, 1488 real plasma samples were processed and analyzed within 18 working days, demonstrating the high throughput characteristics of the method.

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