Large volume injection of 1-octanol as sample diluent in reversed phase liquid chromatography: Application in bioanalysis for assaying of indapamide in whole blood

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ABSTRACT

Large volume injection of samples in strong diluents immiscible with the mobile phases used in reversed phase liquid chromatography (RPLC) has been recently introduced in practice. In the present work, the potential of the technique has been evaluated for bioanalytical applications. The process consists of the liquid–liquid extraction of indapamide from whole blood into 1-octanol, followed by the direct injection from the organic layer into the LC. Detection was made through negative electrospray ionization (ESI) and tandem mass spectrometry (MS^2). The method was developed, validated, and successfully applied to a large number of samples in two bioequivalence studies designed for indapamide 1.5 mg sustained release and 2.5 mg immediate release pharmaceutical formulations. The performance of the analytical method is discussed based on data resulting from the validation procedure and the completion of the bioequivalence studies.

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1. Introduction

The main purpose of bioanalysis is the determination of selected compounds in biological matrices [1,2]. Two challenging problems relate to the sample preparation step in bioanalytical processes: elimination of the biological matrix to sustain method's selectivity and enrichment of the target compounds to achieve low quantitation limits. Protein precipitation methods and extraction processes are more often used to support the above mentioned goals.

Liquid–liquid extraction readily isolates analytes in water immiscible solvents. The sample transfer to the chromatographic system is usually preceded by the removal of the extraction solvent (under gas stream, eventually thermally assisted) and re-dissolution of the dry residue in a solvent compatible with the mobile phase, to further support a large injected volume. Evaporation step may add random errors to the experimental results and seriously lengthens the duration of the analytical process. It would be highly preferable to inject large volumes of the organic phase directly to the chromatographic column. However, it is generally accepted that if the injection solvent is stronger than the mobile phase, the chromatographic peaks will be broadened and/or dis-

torted [3–8]. Some practical solutions to accommodate stronger diluents to large volume injection – reversed phase liquid chromatography (RPLC) through application of pulsed elution gradients have been proposed [9]. Recent studies demonstrated that band broadening/peak distortion does not occur if the dilution solvent has an increased retention compared to target compounds [10]. This also applies for dilution solvents, which are not miscible with the mobile phase and exhibit enhanced affinity for the stationary phase compared to target analytes [11,12]. The reduction of the retention factors characterizing the target compounds should be considered, because the highly retained dilution solvent "saturates" a proportional amount of the stationary phase [11]. The use of water-immiscible solvents as diluents in RPLC has been recently highlighted for the assay of related impurities in active ingredients [13], antioxidants in pharmaceutical formulations [14] and ginkgolic acid in standardized extracts [15]. Additional phenomena related to similarity/dissimilarity of the viscosities characterizing injection solvent and the chromatographic eluent, namely the viscous fingering problem, should also be taken in consideration under these particular conditions [16]. Accordingly, if the injection solvent is less viscous than the eluent, chromatographic peaks tend to have fronting. By the opposite, a more viscous injection solvent should be fingered by the backward eluent, leading to peak tailing [17,18].

The basic phenomena relating to large volume injection of diluents non-miscible with the mobile phase, more precisely

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n-alkanes, were described earlier by our group [11], as a completion of studies carried out with strong miscible diluents (such as acetonitrile, methanol, i-propyl alcohol and tetrahydrofuran) in 100% aqueous mobile phases, discussed by Loeser and Drumm [10]. Some theoretical aspects are further discussed in the present work, in Section 3. These theoretical assumptions were previously verified for injection of relative polar compounds such as isosorbide-2 and -5 nitrates, pentoxifylline, tropicamide and methyl-p-hydroxybenzoate in n-hexane, n-heptane and i-octane [12]. We have also applied this approach for the assay of traces of butylated hydroxyanisole used as antioxidant in statines formulations [14]. Another interesting application referred to the assay of ginkgolic acids in standardized Ginkgo biloba extracts, which avoided the tedious sample preparation procedure described in the compendial specific monograph [15]. Recently, Loeser et al. [13] extended the application field through using ethyl acetate, i-propyl acetate and methyl-i-butylketone as diluents for the assay of polar related impurities in a relatively non-polar active pharmaceutical ingredient during the drug development phase. Although this approach has been used in these applications, its advantages in being used within the framework of large scale studies, where robustness is crucial, have not been yet verified. As liquid–liquid extraction in water immiscible solvents is largely used in sample preparation processes related to bioanalysis, we considered that the development, validation and application of this approach for an LC/(-)ESI/MS^2 method designed for a bioequivalence study would be specifically interesting. Our choice was oriented toward a difficult biological matrix, the whole blood. Such a matrix, if inadequately processed, may induce serious interferences in the ion source of the mass spectrometric detector, leading to reduced precision and accuracy. Indapamide, a moderate lipophilic molecule ($\log K_{ow} = 2.66$), was selected as a target compound, owing its particularity of binding on the surface of the red cells, inducing the objective need of its assay directly in blood (and not in plasma or serum). The isolation of the compound was achieved through liquid–liquid extraction, from whole blood to octanol. The extraction solvent was 1-octanol because it fulfills all appropriate conditions needed for large volume injection of samples in diluents non-miscible with the mobile phase (in relation to the target compound indapamide and internal standard in use), as it results from Refs. [10,11] and all argumentation presented later. A large volume (75 μl) from the organic layer was injected into the LC operated under reverse phase conditions. The method was successfully validated and used for two consecutive bioequivalence studies: a multidose bioequivalence study for a sustained release pharmaceutical formulation (coated tablets) containing 1.5 mg of indapamide and a single dose bioequivalence study for an immediate release pharmaceutical formulation (coated tablets) containing 2.5 mg of indapamide. The reliability of the method when used for such large scale applications and the intrinsic quality of the experimental results are discussed further in the present work.

2. Experimental

2.1. Reagents

All solvents (methanol, acetonitrile) were HPLC gradient 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 no. 4 for indapamide (4-chloro-N-[(2-methyl-2,3-dihydro-1H-indol-1-yl]-3-sulphamoylbenzamide) and batch no. 1c for 5-chloro-2-

methoxy-N-[2-(4-sulphamoyl phenyl)ethyl]benzamide, used as internal standard (IS). 1-Octanol and formic acid were extra pure grade from Merck. Sodium chloride from Merck was pro-analysis grade.

2.2. Equipment

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 mode. System control, data acquisition and interpretation were made with the Agilent Mass Hunter software version B 01.00 (B48). Alternative diode array detection (DAD) and refractive index detection (RID) modes used to asses effects arising on large volume injection of the non-miscible diluent were achieved by means of the Agilent 1200 SL series (G1315C) and Agilent 1100 G1362 modules, respectively.

2.3. Chromatographic method

The chromatographic separation was carried out on a Zorbax SB C18 Rapid Resolution, 50 mm length × 4.6 mm internal diameter × 1.8 μm particle size column, thermostated at 40 °C. Such a short column was preferred to increase the method's throughput (considering the increased number of samples being analyzed). Elution conditions were optimized in order to conserve adequate chromatographic resolution and to control matrix effects arising within the ion source of the mass spectrometer. A Phenomenex C18 guard cartridge (2 mm length, 4 mm internal diameter) was used to protect the column inlet. The column was operated under gradient conditions, at a flow rate of 0.8 ml/min. The components of the mobile phase were aqueous 0.1% formic acid and a mixture acetonitrile/methanol in 1/1 volumetric ratio. The following gradient profile (including the re-equilibration step) was applied:

Time (min)	Organic modifier (%)	Flow rate (ml/min)
0	5	0.8
2	45	0.8
5.5	45	0.8
5.51	100	0.8
6.0	100	0.8
6.50	100	1.2
6.51	5	1.2
7.5	5	1.2

The flow rate increase at the end of the gradient is however needed for a faster elimination of 1-octanol from the column. A higher flow rate over the whole gradient profile would produce enhanced throughput through shortening the run (without a sensible loss in term of efficiency, as in such conditions, the van Deemter plot for a 1.8 μm particle size stationary phase is almost flat). However, flow rate limitation up to 0.8 ml/min was required by the proper functioning of the ESI source.

The injection volume was 75 μl. An accurate and reproducible injection process was obtained only through reducing the dispensing speed of the autosampler from the normal speed of 1000 down to 100 μl/min, to compensate for the high viscosity of 1-octanol. Additional column re-equilibration (at 0.8 ml/min and 5% organic modifier) is obtained during the injection process, which takes about 2 min.

Investigation of effects produced through injection of large volumes of octanol in the column was carried out under isocratic elution conditions, consecutively using acetonitrile, methanol or a mixture acetonitrile/methanol (1:1 (v/v)) as organic modifier in
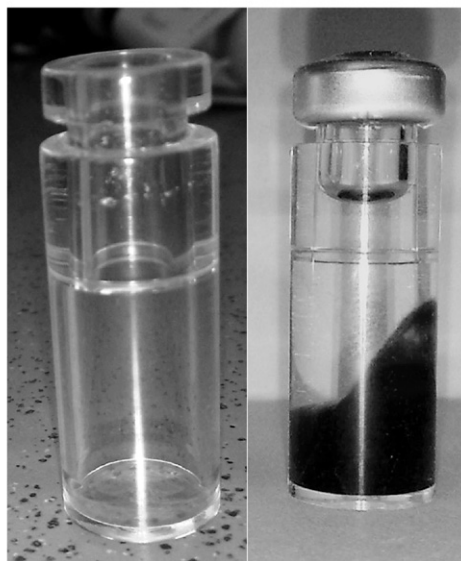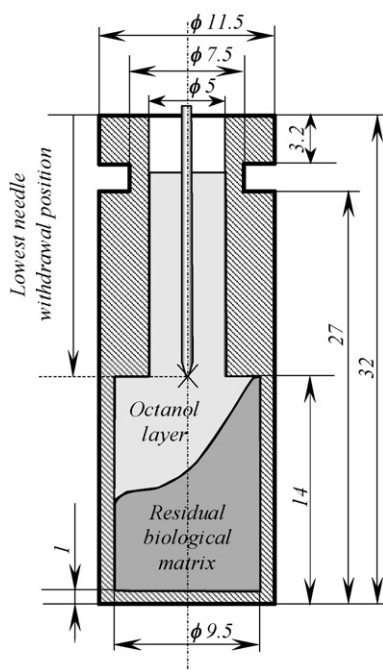
Fig. 1. Design of a vial allowing extraction, centrifugation and sample withdrawal through controlling the depth of the injection needle, making unnecessary the transfer to another vial of the organic layer after centrifugation.

the mobile phase. A solution of 0.1% formic acid was used as the aqueous component. The flow rate of the mobile phase was set at 0.8 ml/min. Injection volumes of 1, 10, 50, 75 and 100 μl were applied. The concentrations of the tested solutions in octanol were chosen in order to produce a loading of 1 μg amount from each analyte in the column. Resulting chromatograms were UV monitored at 235 nm. Retention and peak shapes were evaluated. To asses the behavior of the diluent in the chromatographic column, injections of different volumes (1, 10, 25, 50, 75 and 100 μl) of 1-octanol were monitored with a RID.

2.4. MS detection

A MRM scan type was performed under negative polarity. The operational parameters of the ESI source were the following: drying gas 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 was set to 150 V. Collision energy of 25 V was used for both compounds. The MRM transitions used for quantitative purposes (quantifier) were the following: m/z 364 to m/z 190 for indapamide, and m/z 367 to m/z 170 for IS. Qualifier transitions were the following: m/z 364 to m/z 132 for indapamide, and m/z 367 to m/z 127 for IS. The column effluent is diverted from the ion source (by means of a six ports valve) from minutes 0 to 4 and after minute 6, respectively.

2.5. Sample preparation

Spiked blank blood samples used during method validation were vortexed for homogenization at least 12 h at 4 °C. Whole blood aliquots of 0.5 ml were extracted with 0.75 ml 1-octanol containing 50 ng/ml IS. For blood samples resulting from the single dose bioequivalence study made for the 2.5 mg indapamide coated tablets, a dilution by a factor of 3.33 (v/v) with 0.9% aqueous sodium chloride solution was required. After 15 min of vortexing (2000 rpm), the Eppendorf tubes were centrifuged for 5 min at 9000 × g and 25 °C. A portion of about 0.6 ml from the supernatant was trans-

ferred in the injection vial. A volume of 75 μl was thus injected in the chromatographic column.

To reduce sample manipulation, the extraction, centrifugation, and injection of sample into the LC were performed by using a single vial. After centrifugation one withdraws an aliquot volume from the octanol upper layer while carefully controlling the depth of the needle when inserted into the vial. Thus the transfer of the supernatant to a new vial is omitted and sample manipulation is reduced, ultimately resulting in the elimination of potential errors. A special inner geometry of the vial used for extraction, centrifugation and injection makes possible the withdrawal of the octanol layer, and minimizes the risk of accidental blood carry over, as illustrated in Fig. 1. Experimentally, only few vials have been in-house crafted from polypropylene material to verify the previous assumptions. Such a design could successfully lead to a minimization of the manipulation of the sample, making sample preparation step more simple and straightforward.
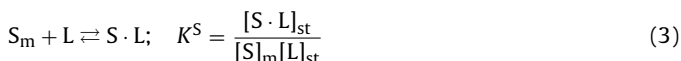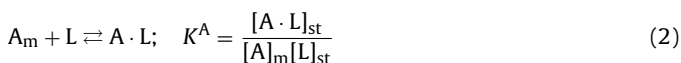
2.6. Methodology of the bioequivalence studies

The analytical method was applied for assessment of two bioequivalence studies: a multidose one, referring to controlled release formulations (coated tablets) containing 1.5 mg of indapamide, and a single dose study for immediate release formulations containing 2.5 mg of the active ingredient. The multidose study (two periods, cross-over, controlled, randomized, fasting conditions) enrolled 25 healthy volunteers, while the single dose one (two periods, cross-over, controlled, randomized, fasting conditions) 24 healthy volunteers. 17 sampling times (including the pre-dose sampling) were made during the multidose study, and 15 for the single dose one. The protocols of the bioequivalence studies were formally accepted by the evaluation department of the Romanian National Medicines Agency and received the approval of the National Ethics Committee. Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1) from Thermo Electron Corporation, U.S.A.

3. Results and discussion

Good chromatographic efficiency with acceptable peak shapes can be achieved by injecting large volumes of diluents non-miscible with the mobile phase if the following conditions are simultaneously fulfilled [11,13]: (i) the diluent has an increased chromatographic retention compared to the target analytes; (ii) the solubility of the diluent in the mobile phase is low enough to force the saturation of the stationary phase in the column head, immediately after injection; (iii) elimination of the diluent from the column before another injection. On the other hand, a special attention should also be paid to the difference of viscosity between the sample diluent and the mobile phase, potentially inducing peak symmetry distortion [17].

Additionally, changes in the chromatographic resolution measured between analytes should be evaluated, as the "apparent" column length is proportionally reduced with the increase of the injected volume (and consequently, a specific amount from the stationary phase is blocked through saturation with diluent). The principle of this approach is based on partition equilibriums taking place immediately after sample injection (V_{inj}). The analyte (A) diffuses out of the injection plug (the diluent S) in the mobile phase (m), according to equilibrium (1). The analyte and the diluent are competing for adsorption to the hydrocarbonaceous ligand in the stationary phase (L), according to following processes (2) and (3):

$$A_S \rightleftarrows A_m; \tag{1}$$

$$A_m + L \rightleftarrows A \cdot L; \quad K^A = \frac{[A \cdot L]_{st}}{[A]_m [L]_{st}} \tag{2}$$

$$S_m + L \rightleftarrows S \cdot L; \quad K^S = \frac{[S \cdot L]_{st}}{[S]_m [L]_{st}} \tag{3}$$

where st and m indexes refer to the stationary and mobile phases, respectively.

Due to the high amount of S loaded in the column and its higher hydrophobic character compared to A, equilibrium (1) is shifted toward right and equilibrium (2) toward left, as long as the entire amount of the diluent is adsorbed onto L. When the diluent has increased viscosity than the mobile phase, the low viscosity mobile phase penetrates into the viscous plug, sending "fingers" of greater mobility ahead of the normal position of the front. As a consequence, typical "waves" at the rear of the peaks may be observed. The process was exhaustively described in [18]. In this way, a portion from the stationary phase (ΔV) in the head of column is available only to the diluent. ΔV is proportional to V_{inj}, according to relation $\Delta V = \gamma V_{inj}$, where γ is a constant. Consequently, compound A participates to the retention mechanism in the chromatographic column based on a remaining available stationary phase volume equal to $V_s - \gamma V_{inj}$ (considering the initial volume of the stationary phase as V_s). Based on these assumptions, the dependence between the retention factor of compound A (k^A) and V_{inj}, for a mobile phase having the volume V_m, is illustrated by the following relationship:

$$k^A = K^A \frac{V_s}{V_m} - \gamma \frac{K^A}{V_m} V_{inj} \tag{4}$$

On the other hand, the sample diluent is also used as extraction solvent during the sample preparation step. Obviously, consistent extraction yields are necessary. The selectivity of the extraction process is also important, as the coextracted matrix may affect ionization yields of the target compounds in the MS source.

By choosing 1-octanol as extraction solvent and, consequently, as diluent for injected samples, the condition of an increased retention of the diluent compared to target analytes was fulfilled. The reversed phase mechanism achieves the separation of compounds according to the increase of their hydrophobic character. Therefore, the values of $\log P$ (or $\log K_{ow}$, representing the deci-
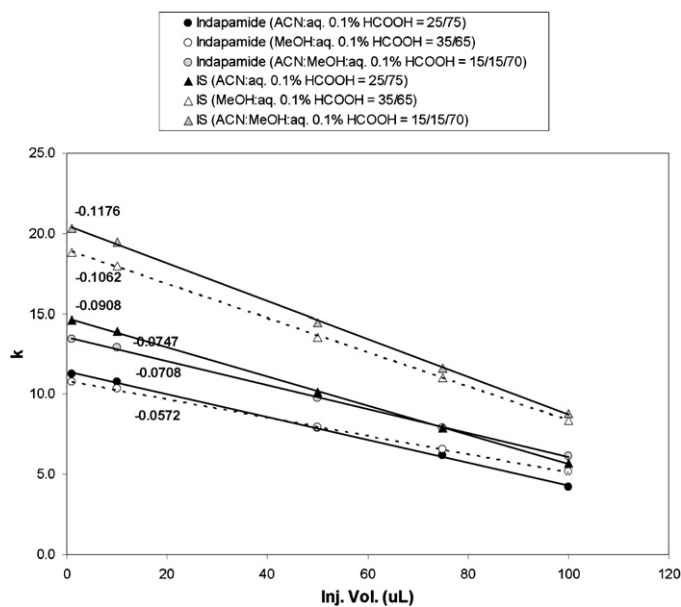

Fig. 2. Reduction of the retention times of the target compounds with the increase of the injected volume (the sample diluent is 1-octanol; working conditions are described in Section 2 and further discussed in the text).

mal logarithm of the partition coefficient of a compound between *n*-octanol and water) should be considered as relevant for the elution order. In this particular case, the calculated $\log K_{ow}$ values by means of the fragment theory [19] indicate the following elution order: IS ($\log K_{ow} = 2.51$), indapamide ($\log K_{ow} = 2.66$) and octanol ($\log K_{ow} = 2.81$; the shake-flask experimental value of 3.0 is also available). However, when formic acid is added to the mobile phase, the hydrophobicity descriptor governing the retention process becomes $\log D$, which considers all species of the analyte involved into partition between mobile and stationary phases. In acidic media, due to the protonation of the tertiary amino moiety from the structure of indapamide, the $\log D$ value is lower than its $\log K_{ow}$, which may explain the observed elution order.

Optimization of the chromatographic method was based on the study of retention and peak symmetry obtained under isocratic elution conditions. Acetonitrile, methanol and the 1/1 (v/v) mixture between acetonitrile and methanol were successively considered as organic modifiers in the mobile phase. The aqueous component was 0.1% formic acid and the flow rate was 0.8 ml/min. Isocratic experiments were made successively for 25% acetonitrile, or 35% methanol or 30% acetonitrile/methanol mixture in the mobile phase. In each of the elution conditions, 1, 10, 50, 75 and 100 μl volumes from the solutions of the target compounds in octanol and the pure diluent were injected. The amount of each analyte loaded to column, regardless of the injected volume, was 1 μg. Separations were UV monitored, at 234 nm (in case of analyte's solutions) and through RID (for the diluent only). Between successive chromatograms, after injection of solutions of target compounds, the column was flushed with 100% organic solvent for 5 min, and then re-equilibrated at the mobile phase composition for another 5 min (for elimination of the octanol plug). According to our earlier assumptions (see Eq. (3)) and to experiments described in [11,12], retention factors linearly decrease with the increase of the injected volume, as illustrated in Fig. 2. Negative numbers in Fig. 2 are the slopes (b) of the linear regression functions $k = a + bV_{inj}$.

In the case of an asymmetrical and/or a distorted profile, the retention time was considered as corresponding to the maximum of the registered peak. For a better insight on the chromatographic results, Table 1 enlists peak characteristics of the target analytes

Table 1
Peak characteristics for studied analytes under different elution conditions and different injection volumes in 1-octanol.

Elution conditions	Peak parameters	Injected volume (μl)									
		1		10		50		75		100	
		Indapamide	IS	Indapamide	IS	Indapamide	IS	Indapamide	IS	Indapamide	IS
25% ACN	Peak width (min)	0.206	0.278	0.269	0.305	0.241	0.459	0.235	0.644	0.246	0.591
	Symmetry (10% height)	1.11	1.07	1.50	1.17	6.44	2.06	2.73	1.78	1.20	2.72
	Symmetry (integrator)	0.92	0.94	0.71	0.89	0.25	0.42	0.53	0.52	0.9	0.33
	Skew	0.22	0.20	0.77	0.31	0.67	0.45	0.95	0.56	0.01	0.29
35% MeOH	Peak width (min)	0.19	0.316	0.184	0.279	0.25	0.256	0.165	0.25	0.138	0.209
	Symmetry (10% height)	1.17	1.13	1.28	1.08	3.14	1.19	5.63	1.68	7.87	2.41
	Symmetry (integrator)	0.88	0.93	0.82	0.96	0.37	0.88	0.26	0.67	0.22	0.53
	Skew	0.55	0.31	0.81	0.26	0.64	0.37	0.65	0.59	0.67	0.75
15% ACN + 15% MeOH	Peak width (min)	0.238	0.376	0.233	0.351	0.333	0.296	0.216	0.286	0.191	0.255
	Symmetry (10% height)	1.13	1.10	1.16	1.05	2.57	1.11	4.52	1.36	5.68	1.78
	Symmetry (integrator)	0.91	0.94	0.89	0.96	0.42	0.92	0.30	0.78	0.27	0.64
	Skew	0.20	0.14	0.48	0.11	0.53	0.17	0.64	0.35	0.65	0.50
Gradient	Peak width (min)	0.054	0.065	0.059	0.071	0.049	0.056	0.045	0.046	0.037	0.044
	Symmetry (10% height)	1.23	1.17	1.33	1.28	1.33	1.23	1.43	1.15	2.63	1.18
	Symmetry (integrator)	0.84	0.87	0.78	0.81	0.78	0.84	0.76	0.86	0.62	0.84
	Skew	0.60	0.45	0.10	0.42	1.05	0.42	1.00	0.36	1.07	0.37

in the above mentioned elution conditions as well as in the optimized elution gradient (described in Section 2). For a meaningful characterization of peak shapes, the following characteristics have been considered: the peak width at half height; the asymmetry factor computed at 10% from the peak height; the integrator asymmetry factor (according to USP, ⟨6 2 1⟩ general chapter); the skew, calculated from the third central statistical moment (an alternative expression of peak asymmetry, as positive values indicates tailing and negative values, fronting). Experimental peak data treatment was made using the commercially available peak performance macro from Agilent.

In all cases, injection of 10 μl volumes from octanol solutions of analytes produces fair symmetry and undistorted peak shapes. For larger injection volumes, distortion effects may affect the first eluting peak (indapamide) or both peaks, as shown in Fig. 3A (examples are given for 75 μl injected volume). RID monitoring of octanol elution from column (Fig. 3B) unambiguously demonstrates that the retention factor k_{SF} of the solvent front, as defined in [10], is placed beyond the elution interval of the target compounds. The first condition to achieve large volume injection of non-miscible diluents is thus fulfilled in the case of octanol (diluent must have increased retention compared to target compounds).

However, in some cases, profiles of both peaks in the chromatogram are seriously distorted, such phenomena being produced due to the relative miscibility of the diluent with the mobile phase and a subsequent local inadequate saturation of the stationary phase with octanol, representing an infringement of the second condition exposed at the beginning of this section (the solubility of the diluent in the mobile phase is low enough to force the saturation of the stationary phase in the column head, immediately after injection).

When only the first eluting peak is affected, it makes more sense to involve viscous fingering effects as responsible (it is to note that viscosities at 25 °C for octanol, methanol, acetonitrile and water are 7.21, 0.54, 0.38 and 1.0 cP, respectively [20]).

From functional dependences in Fig. 2, we may observe that the most important decrease of the retention factors with the injected volume was obtained when the organic modifier in the mobile phase is the mixture acetonitrile/methanol, suggesting that the diluent (octanol) better saturates a larger bulk of the stationary phase. Consequently, a gradient profile was applied, starting with an increased content of the aqueous component in the mobile phase (95% – see conditions in Section 2). As observable in Fig. 3, the gradient successfully eliminates the distortion of the indapamide peak and provides increased apparent efficiency.

Elution of both analytes in a narrow retention window of 4.8–5.4 min may produce, in some way, the equalization of the residual matrix profiles reaching ion source simultaneously to the target compounds. Obviously, the effect has low efficiency compared to the case of using isotope labeled IS (when co-elution between analyte and IS is achieved), when a rigorously similar pattern of residual matrix occurs during ionization, but major discrepancies between the ionization behavior of the target compounds is somehow avoided.

The increased water content in the mobile phase at the beginning of the chromatographic run forces the saturation of the stationary phase with octanol in the head of column. The gradient profile (composition and flow rate, as described in Section 2) applied after elution of the target compounds accelerates the elution of the octanol plug from the column, and meanwhile, is favorable to elution of the residual matrix brought by the samples prepared from human blood.

The analytical method was validated according to the guidelines in force [21,22]. The general quality characteristics of the validated method are enlisted in Table 2. The LLOQ of the method was found at 0.5 ng/ml concentration level and the linearity interval of the response function ranges up to 100 ng/ml. The response function fitting model was the $1/x^2$ weighted linear regression ($R^2 = 0.9918$). Precision of the experimental validation data was placed below of the 15% RSD threshold, as well as accuracy, expressed as %bias and situated within the ±15% interval (for the LLOQ level, accepted interval is ±20%). The determined LLOQ level is lower compared to data reported earlier [23]. However, our previous experimental approach for determination of indapamide in plasma was based on liquid–liquid extraction in t-butyl-methyl ether, using a large sample volume (1 ml) and needing organic solvent evaporation and a four folds sample concentration. It is not the main purpose of this work to present a detailed approach relating to the validation step, but only to highlight some specific features relating with the extraction of the target compounds and the direct large volume injection of the diluent non-miscible with the mobile phase into the chromatographic column. A special focus will be made on the intrinsic quality of the analytical results obtained after application of the method on incurred samples resulting from two different bioequivalence studies (different designs, different strengths of the pharmaceutical formulations considered for bioequivalence).

Selectivity, recovery and matrix effects merit a distinct discussion, as these characteristics are strongly related to the method's intrinsic quality. The recovery over the entire analytical process and the influence of the residual co-extracted blood matrix were
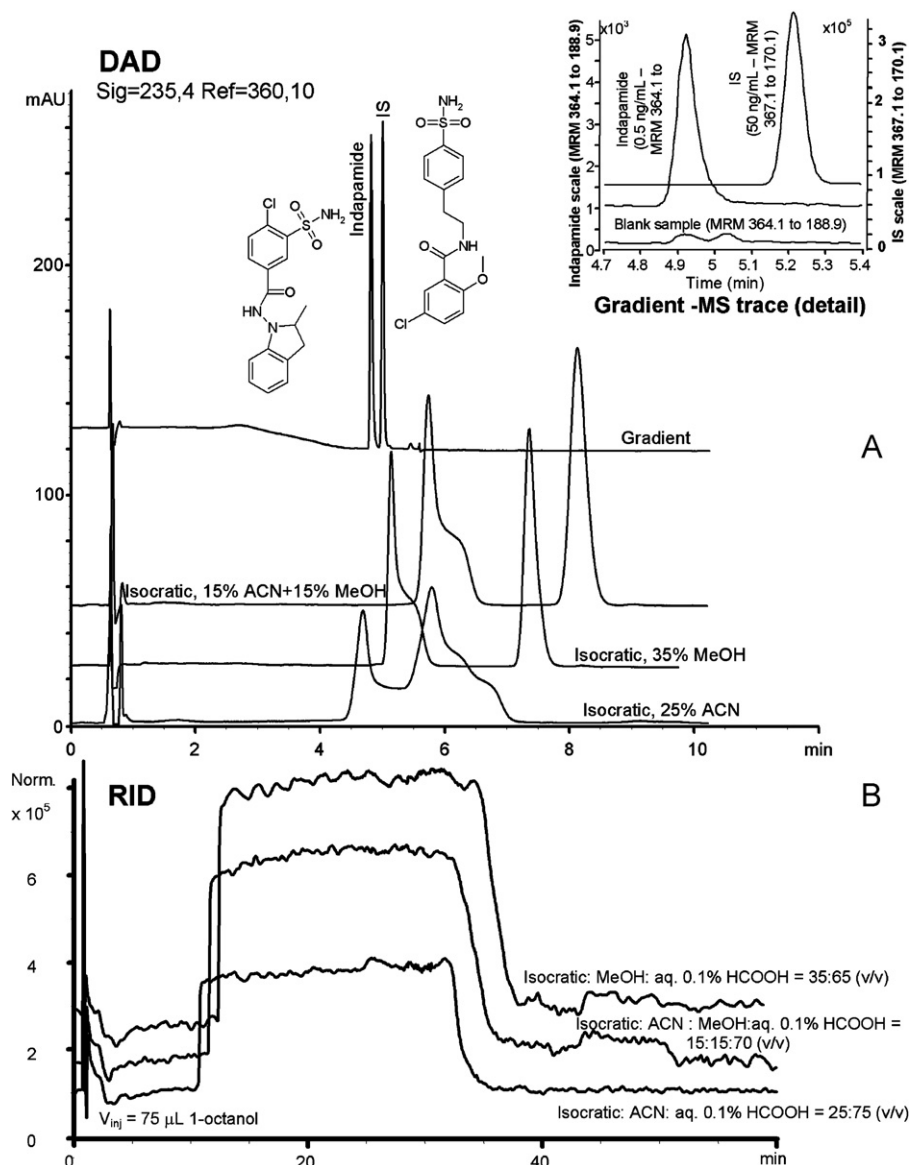
Fig. 3. Retention behavior of analytes and related peak distortion effects appearing on injection of samples dissolved in octanol, in relation with the mobile phase composition and elution conditions (A). The MS/MS trace obtained for an indapamide blood spiked sample at LLOQ (A-detail). The elution behavior of the octanol plug through the chromatographic column (B).

assessed through comparison of results obtained during analysis of the following spiked solutions: (set 1) solutions of indapamide in octanol having concentrations of 1.5, 7.5, 35 and 75 ng/ml and 50 ng/ml IS; (set 2) solutions of indapamide in bulk octanol previ-

ously used to extract a blank blood sample having concentrations of 1.5, 7.5, 35 and 75 ng/ml and 50 ng/ml IS; (set 3) solutions of indapamide spiked in aqueous 0.9% sodium chloride having concentrations of 1.5, 7.5, 35 and 75 ng/ml and 50 ng/ml IS; (set 4)

Table 2
Quality characteristics of the analytical method, as resulting from the validation procedure.

Stage	Characteristics
Linearity	LOD = 0.3 ng/ml (S/N = 3); LLOQ = 0.5 ng/ml (S/N ≥ 5); ULOQ = 100 ng/ml; Conc. levels = 0.5/1/5/10/25/50/80 ng/ml; samples/level: $n = 6$ RSD% ∈ [4.4/9.7]%; %bias ∈ [−7.4/8.0]%; Response function = linear, weighted $1/x^2$
Precision	QC levels = 1.5/7.5/35/75 ng/ml; Repeatability: $n = 10$; Intermediate precision: $n = 6$ Repeatability: RSD% ∈ [0.8/1.3]%; %bias ∈ [−7.9/8.1]% Intermediate precision: RSD% ∈ [7.3/8.5]%; %bias ∈ [−3.0/3.3]%
Stability	Freeze/thaw: $n = 5$; Conc. levels = 4 (as per precision); RSD% ∈ [0.7/3.5]%; %bias ∈ [−8.0/12.5]% Long term (−40 °C) = 3 months; $n = 4$; Conc. levels = 4 (as per precision); RSD% ∈ [0.9/2.2]%; %bias ∈ [−14.3/12.8]% Short term (25 °C) = 8 h; $n = 5$; Conc. levels = 4 (as per precision); RSD% ∈ [0.8/3.9]%; %bias ∈ [−8/14.6]% Post-preparative (25 °C) = 48 h; $n = 6$; Conc. levels = 4 (as per precision); RSD% ∈ [0.5/4.9]%; %bias ∈ [−9.6/12.8]% IS (stock solution; 4 °C) = 30 days; $n = 12$; RSD% (peak area) = 7.8%
Dilution Integrity	Dilution ratios = 1/10; 1/5; 1/2; Dilution fluid: a) whole blood; b) aqueous 0.9% NaCl; samples per case: $n = 3$ 1/10: whole blood; mean RSD% = 5.4%; mean %bias = 13.9%; aq. 0.9% NaCl; mean RSD% = 0.8%; mean %bias = 7.9%; 1/5: whole blood; mean RSD% = 2.8%; mean %bias = 5.8%; aq. 0.9% NaCl; mean RSD% = 0.8%; mean %bias = 2.7%; 1/2: whole blood; mean RSD% = 0.1%; mean %bias = −2.9%; aq. 0.9% NaCl; mean RSD% = 2.6%; mean %bias = −5.9%;

Table 3
Evaluation of the octanol/blood extraction yield and effect of the residual co-extracted blood matrix on the MS response of target compounds.

	Peak areas				
	Indapamide				IS
	1.5 ng/ml	7.5 ng/ml	35 ng/ml	75 ng/ml	50 ng/ml
Set 1	121,787	696,629	2,883,654	5,569,383	1,527,922
	RSD = 1.9%, n = 6	RSD = 0.7%, n = 6	RSD = 0.3%, n = 6	RSD = 0.7%, n = 6	RSD = 1.1%, n = 24
Set 2	122,764	662,025	2,775,036	5,654,088	1,155,967
	RSD = 1.7%, n = 6	RSD = 0.6%, n = 6	RSD = 0.9%, n = 6	RSD = 0.7%, n = 6	RSD = 0.9%, n = 24
Set 3	124,920	680,971	2,867,846	5,532,825	1,585,842
	RSD = 4.0%, n = 6	RSD = 1.2%, n = 6	RSD = 1.6%, n = 6	RSD = 0.9%, n = 6	RSD = 0.5%, n = 24
Set 4	94,997	540,420	2,375,364	5,004,375	1,199,021
	RSD = 1.4%, n = 6	RSD = 0.7%, n = 6	RSD = 0.7%, n = 6	RSD = 0.5%, n = 6	RSD = 1.2%, n = 24
Residual co-extracted matrix effects (S2 × 100/S1)	100.8% 98.4% (RSD = 3.3%)	95.0%	96.2%	101.5%	75.7%
Octanol/aqueous 0.9% NaCl extraction yield (S3 × 100/S1)	102.6% 99.8% (RSD = 2%)	97.8%	99.5%	99.3%	103.8%
Octanol/blood extraction yield (S4 × 100/S2)	77.4% 83.3% (RSD = 5.8%)	81.6%	85.6%	88.5%	103.7%

solutions of indapamide spiked in blank blood having concentrations of 1.5, 7.5, 35 and 75 ng/ml and 50 ng/ml IS. Solutions from sets 1 and 2 were directly injected in the chromatographic column (75 µl), while samples from sets 3 and 4 were processed according to the sample preparation procedure described in Section 2. Comparison of peak areas for indapamide and IS determined in solutions from sets 2 and 1 (expressed as percentage) shows the effects of the residual co-extracted matrix, but not the effects of extraction. Comparison of peak areas for indapamide and IS determined in solutions from sets 3 and 1 (expressed as percentage) illustrates the extraction yields from aqueous 0.9% sodium chloride to octanol (no residual co-extracted matrix effects appear). Comparison of peak areas for indapamide and IS determined in solutions from sets 4 and 2 (expressed as percentage) illustrates the extraction yields from blood to octanol (the residual co-extracted matrix effects equally appear in both sets). Results of the experiments are presented in Table 3.

Both analytes are almost quantitatively extracted from aqueous 0.9% sodium chloride in octanol. Indapamide is about 80% extracted from whole blood in octanol and the co-extracted residual matrix is practically not affecting its ionization yield in MS source. The internal standard is quantitatively extracted from whole blood, but suppression of ionization arises within the MS source in the presence of the residual co-extracted matrix. However, the global recoveries of the target analyte and IS are substantially similar.

Matrix factors (MFs) for the target compound and IS were determined according to the protocol described in the latest guideline in force [22], including hyperlipaemic blood sample. Determinations were made at spiked concentrations of 1.5 ng/ml indapamide and 50 ng/ml IS. MF for indapamide (different blank blood samples = 6, n = 3 for each type) was found 0.77 (with a RSD% of 8.1%). MF for IS (n = 18) was found 0.79 (with a RSD% of 7.1%). Accordingly, the mean normalized MF was 0.98 (with a RSD% of 6.7%, with variation interval between 0.94 and 1.1).

Residual peak areas integrated at the retention time of indapamide in the chromatograms of the six different batches of the blank blood samples being processed (including the hyperlipaemic one) represented 1.5–7.9% from the peak areas integrated in chromatograms of spiked samples at the LLOQ level in the respective matrixes. The residual peak areas integrated in the chromatograms corresponding to the incurred samples collected pre-dose (blanks) from each of the clinical phases and from both studies are placed below the method LOD level, as illustrated in Fig. 4. The two exceptions (from 98 samples) are still placed below LLOQ.

Results obtained during validation, under the dilution integrity stage (see Table 1), are of a special interest. One can observe that

blood dilution with aqueous 0.9% NaCl (isotonic solution) may be applied without altering the resulting quantitative data. This feature is particularly interesting, if considering the limited availability of blank blood samples belonging to each volunteer participating to the clinical trials necessary for sample dilution when determined nominal concentrations were exceeding the linearity range. Many of the samples resulting from the single dose bioequivalence study of 2.5 mg indapamide immediate release formulations sensibly overcome the method's ULOQ. Consequently, the decision was taken to apply a dilution by a factor of 3.33 for all incurred samples, according to findings made under the dilution integrity study. Dilution was made with aqueous isotonic solution, without affecting precision and accuracy of the experimental results.

Some carry over effects could be observed on the mass transition of indapamide (see in Fig. 3A the MS trace shown as a detail). However, the extent of the carry over effect, compared to the response obtained at the LLOQ concentration level for indapamide, did not require additional measures for its elimination.

During analysis of incurred samples, the working sequence consisted in a calibration set (8 concentration levels), a quality control (QC) set (4 concentration levels, two replicates per concentration level, one series from replicates is analyzed immediately after calibration and the second one before the end of the sequence) and
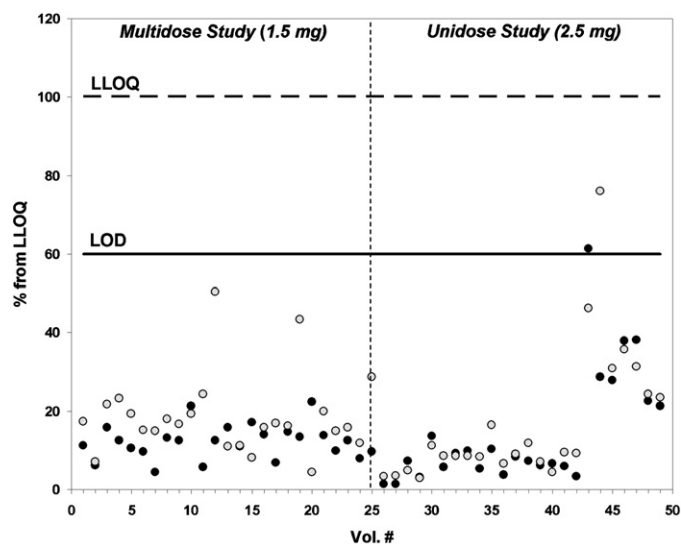

Fig. 4. Residual responses at the retention time of indapamide (according to its specific mass transition) observed in samples withdrawn pre-dose administration from volunteers enrolled in the bioequivalence studies, during both phases.
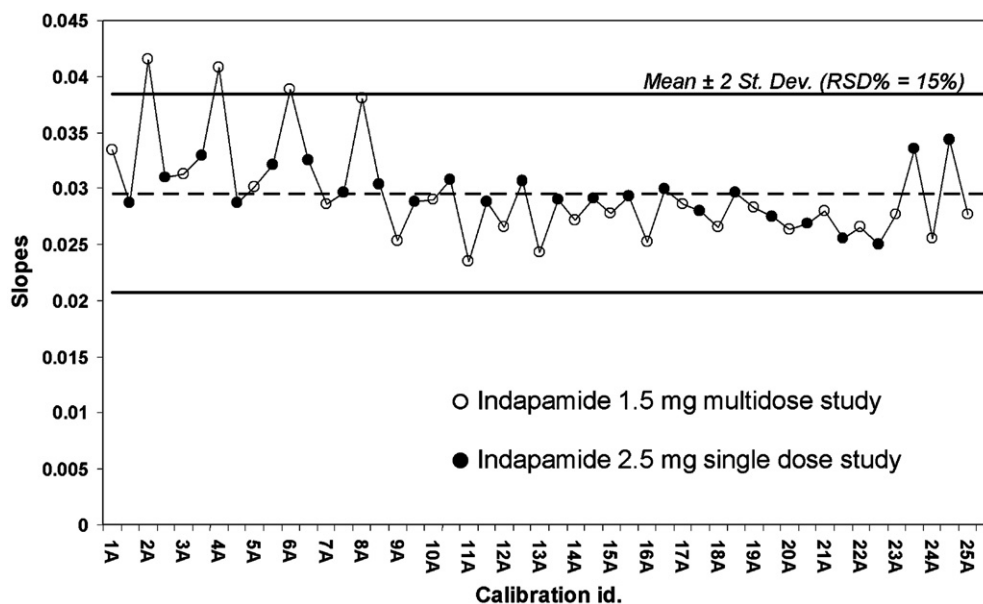
Fig. 5. Variation of the slopes of the regressions calculated from calibration sets associated to the working sequences processed during completion of the two bioanalytical studies (the multidose study designed for 1.5 mg indapamide controlled release formulations and the single dose study designed for 2.5 mg indapamide immediate release formulations), illustrating the reproducibility features of the proposed analytical method.

incurred samples from one volunteer, introduced in the increased order of the sampling time for both phases.

The slopes of the regressions characterizing the calibration sets processed during all working sequences resulting from the multidose 1.5 mg indapamide study and the single dose 2.5 mg indapamide one are plotted together in Fig. 5. Data are presented in the sequential order, noting that the multidose study was preceding the single dose one. Presented data sustain the reproducibility characteristics of the proposed analytical method over a large period of time and a great number of analyzed samples.

Only one QC sample (at the highest concentration level) from 200 (0.5%) was placed outside the ±15% accuracy interval during completion of the multidose study. Eleven QC samples (10 at the lowest concentration level) from 192 (5.73%) were placed outside the accuracy limit during completion of the single dose study. In none of the working sequences, two QC samples at the same concentration level were simultaneously placed outside the allowed accuracy interval.

The relative standard deviations computed for the retention time values of indapamide and IS peaks in chromatograms of all incurred samples ($n = 1668$) analyzed during the two bioequivalence studies on a single chromatographic column (having previously supported the validation stage) are 1.48 and 1.52%, respectively (4.95 ± 0.073 min for indapamide and 5.25 ± 0.079 min for IS). During the multidose study, the mean peak area of IS in all incurred samples was $399,871 \pm 101,376$ (RSD% = 25.4%), while during the single dose study, the mean peak area was $431,282 \pm 53,671$ (RSD% = 12.4%). Sample dilution with aqueous isotonic solution improved stability of the MS response. It is to mention that the MS source was not cleaned during completion of a whole bioequivalence study. The mean normalized response (the normalized responses were calculated by dividing the ratios between the analyte and IS peak areas with the calculated concentration values resulting from interpolation in the corresponding calibrations, and roughly illustrate the response per concentration unit) determined for all incurred samples in a study was: 0.0294 ± 0.005 (RSD% = 17%) for the multidose study and 0.0296 ± 0.0024 (RSD% = 8.0%) for the single dose one.

Incurred sample reanalysis was performed after both bioequivalence studies, at two weeks distance from their ending moments. From each volunteer, two samples per phase were reanalyzed, one corresponding to a lower concentration value, the other one corresponding to an increased blood concentration level (immediately after t_{max}). More precisely, for the multidose study, sampling times 3 (144 h, steady state) and 13 (177 h) were considered, while for the single dose study, sampling times 9 (4 h) and 16 (48 h) were reanalyzed. Consequently, for the multidose study, 100 incurred samples were reanalyzed, while for the single dose one 96 samples. Data from incurred sample reanalysis were treated according to the Bland–Altman approach [24,25]. According to the Bland–Altman approach, 33% from the total number of the reanalyzed incurred samples may be placed out-side the accuracy interval of ±20%.
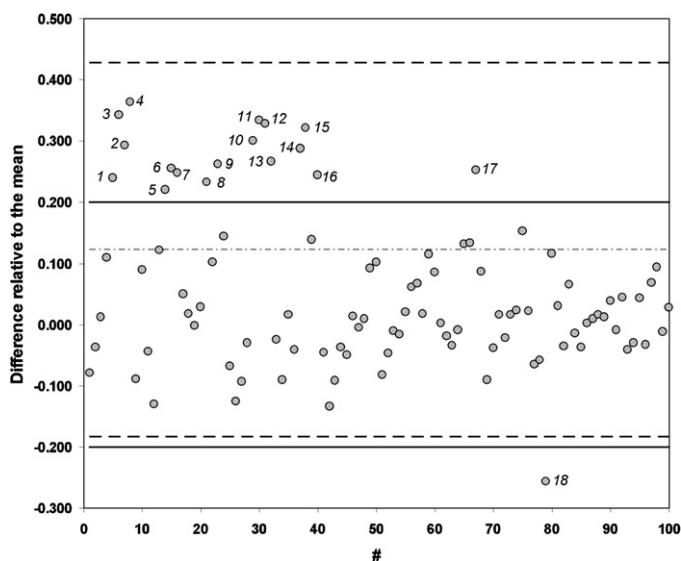

Fig. 6. Bland–Altman plot illustrating results obtained through incurred sample reanalysis after completion of the multiple dose bioequivalence study.

Table 4
Comparison between the principal pharmacokinetic parameters determined during the single dose bioequivalence study of 2.5 mg indapamide immediate release pharmaceutical formulations under fasting administration conditions, and data from literature.

Reference	Present work	[26]	[27]
Matrix	Blood	Blood	Blood
Extraction solvent	1-Octanol	t-Butyl methyl ether	Diethyl ether
Sample preparation	Direct large volume injection of the extract	Evaporation to dryness, residue dissolution in the mobile phase	Evaporation to dryness, dissolution of the residue in the mobile phase
LLOQ	0.5 ng/ml	5 ng/ml	10 ng/ml
Administered dose	2.5 mg	2.5 mg	5 mg
Pharmaceutical formulation	Reference	Reference	Reference
Pharmacokinetic parameters (mean ± SD)			
C_{max} (ng/ml)	154.5 ± 28	135 ± 24	358 ± 43
AUC_{last} (ng/ml h)	2964 ± 860	2027 ± 339	6290 ± 899
AUC_{total} (ng/ml h)	3222 ± 818	2161 ± 383	7529 ± 1323

Results obtained after reanalysis of incurred samples selected from the multidose bioanalytical study are graphically illustrated in Fig. 6.

For the multidose study, 18 reanalyzed incurred samples from a total of 100 produced results out-side the allowed accuracy interval. For the single dose study, 21 reanalyzed incurred samples from a total of 96 (21.9%) produced results falling out the accepted accuracy limits. Apparently, results obtained through reanalyzing incurred samples from the single dose study are somehow worst compared to the multiple dose study. However, a justification may be advanced, as each of the experimental results was corrected by a 3.33 multiplication factor (compensating sample dilution with an isotonic solution). This operation leads also to amplification of experimental errors by the same factor.

The pharmacokinetic parameters determined by means of the data resulting from application of the proposed analytical method on the incurred samples were compared to similar values recently reported in literature [26,27]. The available literature data refers to pharmacokinetic parameters produced by 2.5 mg indapamide formulations, during single dose bioequivalence studies carried out under fasting administration conditions made on Chinese population. Comparison is given in Table 4. Pharmacokinetic data are in good agreement, which sustain the intrinsic quality attributes of the proposed analytical method based on large volume injection of samples using octanol as diluent.

4. Conclusions

The approach of large volume injection of octanol as sample diluent non-miscible with the mobile phase was successfully applied in large scale routine procedures in bioanalysis. Its principle was demonstrated by making extraction of indapamide from whole blood samples in octanol, followed by the direct injection of a large volume from the organic layer directly to the chromatographic column. A specific geometry of the vial to avoid the organic phase transfer after extraction and centrifugation steps was also proposed. The experimental approach sensibly shortens the duration of the analysis and readily enhances on the sensitivity of the assay. Despite the complex composition of the blood, the residual matrix transferred in the chromatographic column is reduced. Further elimination of residual matrix effects is possible by strictly controlling the admission of the column effluent in the MS source (through the divert valve placed before the ion source) and through application of fast gradient elution profile at the end of each run (simultaneously used to achieve elimination of the bulk of the diluent from the stationary phase). The column life time is significantly increased (validation and two successive bioequiva-

lence studies have been performed on a single column, meaning around 3000 injected samples). The quality attributes of the analytical method were evidenced by means of the results obtained during validation and analysis/reanalysis of the incurred samples. The proposed method is accurate, precise and rugged. Pharmacokinetic parameters calculated from the single dose bioequivalence study achieved for 2.5 mg indapamide immediate release formulations were compared with similar data from literature and they are in good agreement, which additionally confirm the quality of the proposed analytical method.

The present approach, as an extension of the basic principle relating to large volume injection of non-miscible diluents in the bioanalytical field should only be considered as a model study. Although the present work illustrates an application for indapamide (as target compound) and 1-octanol (as extracting agent and non-miscible sample diluent), when considered together with the previously published results [12–15] make us confident about the spreading potential of this analytical technique to other analytes and diluents. Further work will be undoubtedly necessary to generalize the statements and to diversify the application fields.

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