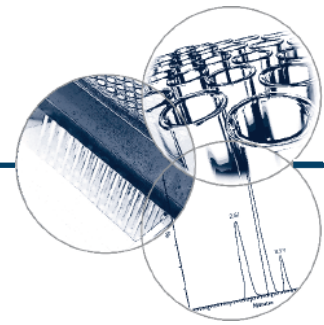
RESEARCH ARTICLE

SPECIAL FOCUS: BIOANALYTICAL SAMPLE PREPARATION



Large-volume injection of sample diluents not miscible with the mobile phase as an alternative approach in sample preparation for bioanalysis: an application for fenspiride bioequivalence

Background: Liquid–liquid extraction of target compounds from biological matrices followed by the injection of a large volume from the organic layer into the chromatographic column operated under reversed-phase (RP) conditions would successfully combine the selectivity and the straightforward character of the procedure in order to enhance sensitivity, compared with the usual approach of involving solvent evaporation and residue re-dissolution. Large-volume injection of samples in diluents that are not miscible with the mobile phase was recently introduced in chromatographic practice. The risk of random errors produced during the manipulation of samples is also substantially reduced. **Results:** A bioanalytical method designed for the bioequivalence of fenspiride containing pharmaceutical formulations was based on a sample preparation procedure involving extraction of the target analyte and the internal standard (trimetazidine) from alkalinized plasma samples in 1-octanol. A volume of 75 µl from the octanol layer was directly injected on a Zorbax SB C18 Rapid Resolution, 50 mm length × 4.6 mm internal diameter × 1.8 µm particle size column, with the RP separation being carried out under gradient elution conditions. Detection was made through positive ESI and MS/MS. Aspects related to method development and validation are discussed. **Conclusions:** The bioanalytical method was successfully applied to assess bioequivalence of a modified release pharmaceutical formulation containing 80 mg fenspiride hydrochloride during two different studies carried out as single-dose administration under fasting and fed conditions (four arms), and multiple doses administration, respectively. The quality attributes assigned to the bioanalytical method, as resulting from its application to the bioequivalence studies, are highlighted and fully demonstrate that sample preparation based on large-volume injection of immiscible diluents has an increased potential for application in bioanalysis.

The simplest and most straightforward way for enhancing the sensitivity of an analytical chromatographic method is through the increase of the amount of target compounds loaded onto the column. As samples are characterized by proper concentrations of the target analytes, this is equivalent to the increase of the injected sample volume. If the inherent sensitivity of the detection device is less than the threshold imposed to the analytical method, dedicated procedures for concentration of the analytes are required. If the target compounds are placed in complicated matrices (and this is specifically the case of bio-samples), sample preparation techniques are used prior to chromatographic separation for the isolation of analytes and/or to control for/avoid interferences. Liquid–liquid extraction (LLE) is one of the oldest techniques used for isolation of analytes from cumbersome matrices. Such an approach is efficient, easy to develop, does not require complicated and expensive laboratory equipment and automatically involves a concentration step through removal of the extraction solvent(s) by evaporation and redissolution of the dried extract in a convenient combination of other solvents, compatible with the mobile phase used in the consequent chromatographic separation technique. The extensive sample manipulation represents one of the main disadvantages of the LLE technique, directly affecting the final quantitative response variability through incorporation of random errors induced during the sample preparation procedure. Some of the variability induced by the sample preparation procedure and/or detection (i.e., ionization

Andrei Medvedovici[†1,2], Stefan Udrescu[2], Florin Albu[2], Florentin Tache[1] & Victor David[1]

[1]University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, #90 Panduri Av., Bucharest – 050663, Romania
[2]Bioanalytical Laboratory, SC Labormed Pharma SA, #44B Th. Pallady Blvd, Bucharest – 032266, Romania
[†]Author for correspondence:
Tel.: +40 214 102 279
E-mail: avmedved@yahoo.com

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Key Terms

Large-volume injection: injected sample volume loaded onto the chromatographic column overcoming the upper threshold which does not involve any special precautions to be taken in relation with the choice of the sample diluent with consideration of its compatibility with the mobile phase composition, producing no peak broadening and symmetry distortion.

Immiscible diluent: Organic solvent used for isolation/extraction of target analyte(s) from different (bio)matrices being immiscible with the liquid mobile phase used during the consequent chromatographic separation carried out under reversed phase conditions.

RPLC: Separation mechanism in liquid chromatography using a mobile phase exhibiting a higher polar character compared with the stationary phase, producing separation of analytes in the increased order of their hydrophobic character.

Validation: Documented evidence about quality attributes of an analytical method in order to meet its intended purpose.

Fenspiride: Non-steroidal anti-inflammatory agent with antitussive and antibronchoconstriction actions.

Bioequivalence: Measure of the therapeutic equivalence of two pharmaceutical formulations having identical active ingredients, assuming their interchangeability during use.

processes in the source of a MS detector) is compensated through the use of internal standards (IS).

Basically, biosamples are aqueous ones. Consequently, LLE is achieved in aqueous non-miscible organic solvents. Most of the chromatographic methods used in bioanalysis are based on the reversed-phase (RP) separation mechanism, requiring aqueous mobile phases. Elimination of the evaporation and re-dissolution steps from the common LLE procedure depends on the possibility of loading water-immiscible solvents onto the chromatographic column, operated with aqueous-rich mobile phases. To conserve the gain in terms of sensitivity, assumed through the appropriate use of the former sample preparation steps, **large-volume injection** of such **immiscible diluents** should be required.

The golden rule of the thumb for large-volume injection in liquid chromatography (LC) is that the sample diluent should be entirely miscible to, and weaker than, the mobile phase composition at the beginning of the separation [1,2]. Effects of poorly controlled large-volume injection conditions materialize in peak broadening and/or symmetry distortions [3,4]. Although the phenomena of peak focusing after large-volume injection are more often discussed in terms of the different solubilities of the analytes in the sample diluent and the mobile phase, additional factors such as pH, composition (i.e., presence or absence of an ion-pair agent) and relative viscosities should also be considered [5–7].

Aspects relating to injection under high-performance gradient elution were recently discussed [8]. Injection of diluents having higher elutropic strength than the mobile phase was first pioneered by Loesser [9]. The possibility of large-volume injection of diluents that are not miscible with the mobile phase was investigated by our team, and some theoretical aspects are addressed in [10], followed by some applications in the pharmaceutical field [11–13]. The possibility of using injection of non-miscible diluents in LC applied to drug discovery and development were highlighted in [14]. The first application in bioanalysis of the large-volume injection of immiscible diluents was recently reported by us [15].

Confirmation of the inherent potential of water-immiscible solvent injection in **RPLC** for bioanalytical applications is undoubtedly necessary. Thus, the aim of the present work relates to the development and **validation** of an analytical method for the assay of **fenspiride** in plasma samples designed for **bioequivalence** purposes, based on LLE of the target compound in 1-octanol and the direct injection of a large volume from the organic layer in the chromatographic column. The use of the method for two bioequivalence studies (a four arms, single-dose, fasting and fed conditions and multiple-doses administrations) confirmed once again the inherent quality attributes and, consequently, the intrinsic robustness of the large-volume injection of immiscible-diluents approach in bioanalysis. Chromatographic separation conditions, bioavailability data and pharmacokinetic parameters of fenspiride have also been reported [16–19]. The agreement between these data and our experimental findings may also be considered as a confirmatory tool.

Experimental

Reagents

Acetonitrile HPLC-gradient grade from Merck (Darmstadt, Germany) was used during experiments. Water for chromatography (resistivity of minimum 18.2 MΩ and TOC of maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument. Fenspiride hydrochloride (8-(2-phenylethyl)-1-oxa-3,8-diazaspiro[4.5]decan-2-one) was of characterized reagent grade from Sigma-Aldrich (through Redox Lab Supplies Com SRL, Bucharest, Romania). The IS was trimetazidine dihydrochloride (1-(2,3,4-trimethoxybenzyl) piperazine), as a certified reference substance from LGC GmbH (Germany). Lastly, 1-octanol and formic acid (extra-pure grade) and the sodium carbonate (pro-analysis grade) were all from Merck.

Apparatus

Experiments were performed with an Agilent 1200 SL series LC/MSD (Agilent Technologies, Santa Clara, USA) system consisting of the following modules: degasser (G1379B), binary pump (G1312B), automated injector (G1367C and the corresponding thermostat G1330B, respectively), column thermostat (G1316B), ESI standard interface (G1948B) and triple quadrupole MS detector (G2571A). System control, data acquisition and interpretation were made with the Agilent MassHunter software version B 01.00, incorporating both qualitative and quantitative packages. The

system was operationally qualified before and after the bioequivalence study. The vortex system was model Multi Reax from Heidolph (Schwabach, Germany) and the thermostated centrifuge was model Universal 320R from Hettich (Tuttlingen, Germany).

Sample preparation

Plasma sample (0.5 ml) was alkalinized with 0.05 ml of aqueous 5% (w/v) sodium carbonate. The sample is vortexed at 2000 rpm for 2 min. Extraction is made in 0.75 ml of 1-octanol already containing the IS (20 ng/ml). Phases are mixed for 10 min at 2000 rpm on a vortex and then centrifuged at 25°C for 5 min at 9000 × g. The octanol upper layer is easily and quantitatively transferred in the injection vial as the phases are well separated by a thin highly viscous film (probably of lipidic nature) aggregated at the interface between layers. A volume of 75 µl from the extractant is injected directly onto the chromatographic column. Vials were thermostated in the autosampler at 25°C. The following important aspects have to be mentioned: first, the sample preparation schema may be successfully applied to a reduced plasma volume (if sample availability concerns apply) by proportionally reducing all the other volumes of the solutions being used (i.e., 0.2 ml of plasma require 0.02 ml of aqueous alkaline solution and 0.3 ml of 1-octanol); second, for incurred samples having concentrations higher than the method's ULOQ, only half of the volume of plasma may be considered (0.25 ml) for preparation, while the volume difference is compensated with the aqueous 5% sodium carbonate solution (consequently, addition of 0.3 ml from the solution would be necessary); third, all the steps of the LLE procedure may be realized in a single injection vial by the adequate automated control of the needle insertion depth within the vial, injection may be realized without the transfer of the organic layer, making the whole process simpler, straightforward and free of sample manipulation-induced errors.

Chromatographic method

A Zorbax SB C18 Rapid Resolution column, 50 mm length, 4.6 mm internal diameter and 1.8 µm particle size (cat. no. 963967–902, Agilent Technologies) fitted with a Phenomenex Guard Cartridge C18, 4 × 2 mm (prod. no. AJO-4286) were used and thermostated at 50°C. The components of the mobile phase were acetonitrile and aqueous 0.2% formic acid solution. A gradient elution was applied, according to the profile in Table 1.

The flow rate increase at the end of the program is necessary for the elimination of the 1-octanol plug from the column. The stop time of the method is 7 min. Column equilibration at the flow rate of 0.8 ml/min is obtained during the period covering the consecutive injection since the drawing/dispensing speed of the autosampler was set to 200 µl/min, in order to compensate for the high viscosity of octanol.

MS parameters

The parameters controlling the ESI ion source were as follows: drying gas (N_2) temperature: 350°C; drying gas flow: 13 l/min; pressure of the nebulizing gas: 60 psi; and, capillary voltage: 4000 V. The fragmentor potential was set at 100 V. Collisional energy (CID) was 22 V for both analytes, using N_2 as collision gas. The resolution of the first mass analyzer was set to 'wide', while the resolution of the second mass analyzer was set to 'unit'. Dwell time was 200 ms. The MS/MS detection was carried out in the multiple reactions monitoring (MRM) mode.

Fenspiride produces the protonated molecular ion (m/z = 261) within the source, which is then isolated by the first mass analyzer. Through collisional-induced dissociation, two product ions are formed with m/z ratios of 105 and 169, due to the cleavage of the σ bonds around the second carbon atom from the phenylethyl moiety. Although the signal at m/z 105 exhibits an increased intensity, the resulting poor selectivity of the detector response (interferences from the residual plasma matrix) leads to the use of the m/z 261→169 mass transition for fenspiride quantitation.

The mass transition used for trimetazidine is m/z 267→166, representing the loss of a methyl-piperazine moiety from the protonated molecular ion.

Table 1. Gradient elution profile.

Time (min)	Acetonitrile (%)	Flow rate (ml/min)
0	2	0.8
5	30	0.8
5.01	100	0.8
5.50	100	0.8
6.0	100	1.2
6.01	2	1.2

■ Methodology & pharmacokinetic parameters

The method was used to analyze samples produced during two bioequivalence studies between film coated tablets containing 80 mg of fenspiride hydrochloride with modified release action. The reference product was Eurespal® 80 mg from Les Laboratoires Servier.

The first study, a single-dose, randomized, open-label, four-periods, four treatments, two sequences, cross-over trial with at least 7 days wash-out between periods I, II, III and IV was achieved under fasting and fed conditions, and enrolled 16 healthy volunteers. Blood samples were withdrawn before the drug oral administration and at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 48 and 72 h following its administration. One volunteer withdrawal was registered during the feed conditions, Phases III and IV of this study.

The second study, based on multiple dose oral administration, randomized, open-label, two-way, crossover comparative trial (two periods, two treatments and two sequences) enrolled 26 healthy volunteers. In each treatmetn period, each subject received 80 mg of fenspiride hydrochloride (one tablet from the tested or reference drug) every 12 h for 6 consecutive days and a last dose with 240 ml plain water on day 7. During the day 7, in each period, blood samples were withdrawn at 0 (before the last dose intake), 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h following the last administration. One volunteer withdrawal was documented.

All volunteers signed an informed written consent before initiation of the screening procedure. Study protocols were formally accepted by the evaluation department of the Romanian National Drug Agency and received the approval of the Institutional Ethics Committee.

The rate and extent of absorption were compared after administration of a single oral dose of 80 mg fenspiride hydrochloride of each of the two formulations, under fasting and fed conditions, based on the logarithmically (ln) transformed primary target parameters AUC_{last} and C_{max}. Additional pharmacokinetic parameters such as AUC_{tot}, T_{max}, T_{half}, Lz, % AUC extra and MRT were calculated for informatory purposes only. Primary (C_{max} and AUC_{last}) and other (AUC_{tot}, % AUC_{extra}, Lz and T_{half}) pharmacokinetic parameters derived from measures of concentrations were analyzed using ANOVA with sequence, subject within sequence, period and treatment factors. ANOVA was performed on ln transformed C_{max}, AUC_{last} and AUC_{tot}, as well as on untransformed Lz and T_{half}. The ratio of means and 90% geometric confidence intervals of the ln transformed data were calculated for C_{max}, AUC_{last} and AUC_{tot}. The technique used for analysis of T_{max} was the Friedman rank sum test applied to untransformed data.

For the multiple dose study, the principal pharmacokinetic parameters were C_{max} and AUC_{tot} and were analyzed using ANOVA with sequence, subject within sequence, period and treatment factors. Additional parameters as C_{min} and % Ptf were also calculated. ANOVA was performed on ln transformed C_{max}, C_{min} and AUC_{tot}, as well as on untransformed % Ptf. The ratio of means and 90% geometric confidence intervals of the ln transformed data were calculated for C_{max} and AUC_{tot}. A non-parametric Friedman rank sum test was performed on the variables % Ptf and T_{max}. Pre-dose blood concentrations were also evaluated using repeated measures ANOVA to prove that the steady-state is achieved.

Acceptance range for concluding bioequivalence was the conventional 90% confidence interval of 80–125% around the geometric mean ratios test/reference of the primary pharmacokinetic parameters (ln transformed or not). Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1.) from Thermo Electron Corporation, USA.

Results & discussions

Injection of large volumes of diluents that are not miscible with the mobile phase may be successful if the following conditions are simultaneously fulfilled:

- The retention factor of the diluent front (k_{SF} [defined as $(t_R^{SF} - t_0)/t_0$, see [8]], where t_R^{SF} is the absolute retention time corresponding to the start of the diluent front in the chromatogram) is larger than the retention factors characterizing the target compounds;
- The solubility of the diluent in the mobile phase at the beginning of the chromatographic run should be very low in order to achieve its partition in the stationary phase and to produce local saturation;
- The diluent plug from a previous injection is eliminated from the column before a subsequent one;
- The difference between the viscosities of the diluent and the mobile phase does not induce fingering effects, potentially producing peak distortion [7];

- The chromatographic resolution between target compounds should be high enough to compensate the reduction of the retention produced through saturation of the stationary phase by the diluent;
- In addition, the diluent should extract the target compounds with high yields from the original matrix.

As it can be observed from Figure 1, the k_{SF} of the front, corresponding to a 100 µl 1-octanol injected volume, largely overcame the retention factors of fenspiride and trimetazidine (IS). It is worthwhile to mention that data in Figure 1 are obtained in isocratic elution conditions, with different percentages of acetonitrile in the mobile phase (as indicated in the *x* axis). Determination of k_{SF} for 1-octanol was possible through monitoring the retention time of the diluent front under isocratic elution conditions, using the refractive index detection. Consequently, large-volume injection in 1-octanol should be possible without peak focusing phenomena.

In accordance with the theoretical aspects presented in [10,15], retention of the target compounds linearly decreases with the increase of injection volume of the diluent, due to saturation of a proportional length of the stationary phase in the column. The retention decrease is reversely related to the content of the organic solvent in the mobile phase, as can be observed in Figure 2 for fenspiride; presence of a higher amount of the organic modifier in the mobile phase leads to an increased solubility of the diluent in the mobile phase, spreading it over a larger zone in the stationary phase, without saturation of the zone.

As can be seen in Figure 3, no major peak broadening and/or distortion effects could be observed on injection of 100 µl from solutions of the target compounds in 1-octanol under isocratic elution conditions, for mobile phases containing between 2 and 10% acetonitrile. As the retention of the IS is close to the column void time in mobile phases containing higher amounts of acetonitrile, the gradient elution mode has been preferred. It is to mention that the choice of a gradient profile would anyway be necessary, as the plug of 1-octanol has to be removed from the column before a subsequent injection.

As the chromatographic conditions allowed injection of high volumes of samples in 1-octanol without peak broadening and distortion phenomena, the attention was focused on the aspects related to the recovery of the target compounds. The assessment was based on the following sets of solutions containing 5, 15, 250 and 700 ng/ml of fenspiride hydrochloride and 20 ng/ml IS: set A – solutions made in 1-octanol (diluent spikes); set B – solutions made in 1-octanol previously used to extract blank plasma (post-spikes); set C – spikes made in blank plasma (plasma spikes). Only the plasma spikes were processed according to the sample

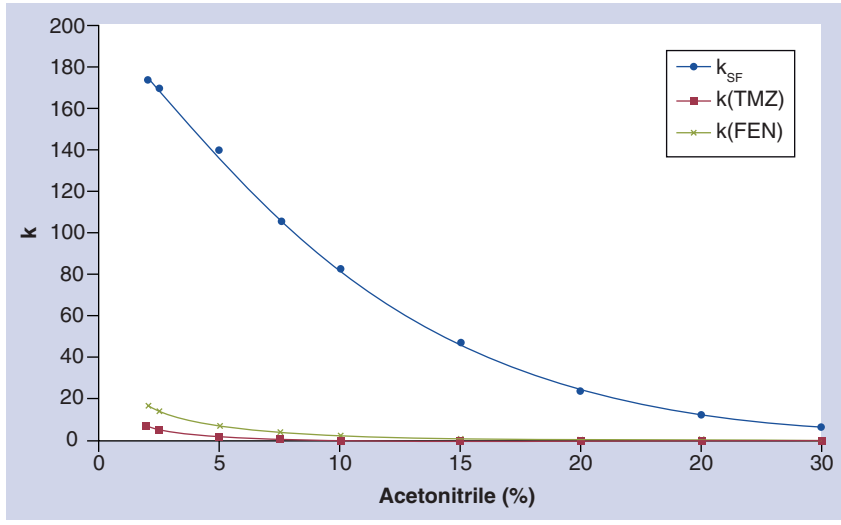

Figure 1. Variation of the retention factors (k) of the target analytes (FEN, TMZ) with respect of the retention factor of the diluent k_{SF} at different compositions of the mobile phase. FEN: Fenspiride; k_{SF}: Starting elution front; TMZ: Trimetazidine.

Key Term

ESI–MS/MS: Detection technique based on the soft ionization of analytes from charged liquid droplets produced through gas-assisted nebulization (ESI), followed by isolation of a given ion species and its dissociation through collisional processes to product ions used at their turn for quantitation and structural confirmation.

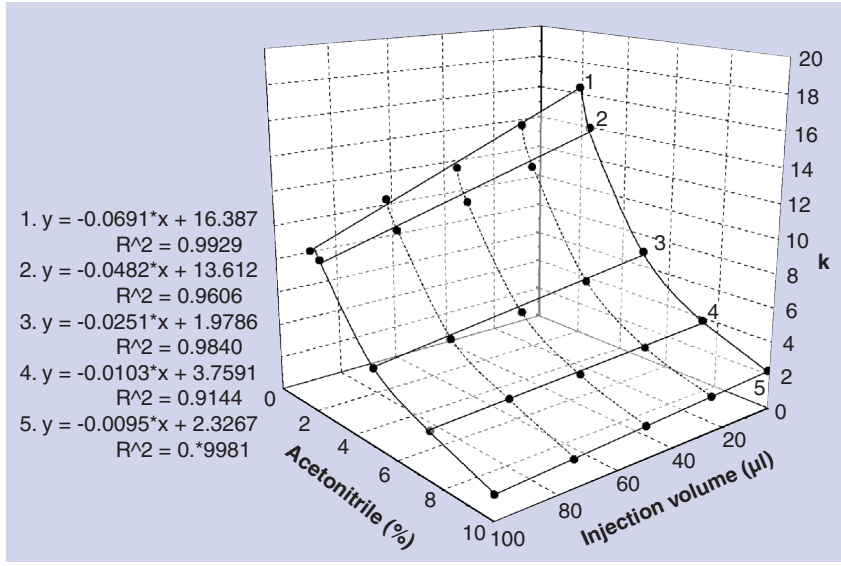

Figure 2. Variation of the retention factors of fenspiride at different injection volumes of its solutions in 1-octanol and different compositions of the mobile phase. Absolute amount of fenspiride loaded onto the column was 20 ng in all cases, detection was made by **ESI–MS/MS**, according to the conditions described in the 'Experimental' section.

Table 2. Evaluation of the extraction yields from plasma to 1-octanol as well as the residual matrix effects on ionization in the MS source for the target compounds.

Compound	Concentration (ng/ml)	n	Plasma spikes/post-spikes (set C/set B)		Post-Spikes/diluent spikes (set B/set A)		Plasma spikes/diluent spikes (set C/set A)	
			Recovery (%)	*RSD (%)*	*Recovery (%)*	*RSD (%)*	*Recovery (%)*	*RSD (%)*
Fenspiride	5	6	104.1	8.2	102.2	6.4	105.9	2.2
	15	6	104.2	2.6	109.7	3.3	114.2	1.4
	250	6	104.6	1.7	103.2	2.6	107.9	3.5
	700	6	96.9	2.5	101.8	2.0	98.7	3.3
Mean		24	102.4	3.6	104.2	3.6	106.7	6.0
IS	20	24	62.0	4.9	113.6	4.3	71.3	4.5

IS: Internal standard; RSD: Relative standard deviation.

preparation procedure, while diluent and post-spikes were directly injected onto the column. Six replicates were made for each of the concentration levels. Recoveries calculated through rationing absolute peak area values in plasma spikes and post-spikes produce information about the yields of the LLE process. Comparison between post-spikes and diluent spikes deals with matrix effects affecting the MS response. Finally, the comparison between plasma spikes and diluent spikes indicates the apparent recovery level over the whole analytical process (LLE yield and ionization yield). Results are given in Table 2. It can be observed that extraction of fenspiride in 1-octanol is quantitative and the residual matrix effects are not affecting ionization yields in the MS source. Trimetazidine is about 62% extracted from alkalinized plasma in 1-octanol and the residual matrix slightly enhances on the ionization yield (an increase of ~15%). Recoveries are consistent for fenspiride for all concentration levels being investigated.

The matrix factors (MF) for fenspiride and IS were determined according to the

Table 3. Quality characteristics of the validated method.

Stage	Characteristics
Linearity	Concentration levels = 5, 10, 50, 100, 250, 400, 500, 600, 800 and 2000 ng/ml; samples/level: n = 6; Instrumental LOD = 0.6 ng/ml (S/N = 3); instrumental LLOQ = 1 ng/ml (S/N = 5); LLOQ = 5 ng/ml (from calibration); ULOQ = 2000 ng/ml; response function = linear, weighted $1/x^2$; slope (B): 0.00337 ± 0.00006 (SD); intercept (A): 0.0043 ± 0.0008 (SD); correlation coefficient (r_{xy}): 0.9982; back-interpolated values: RSD% ∈ (0.9 ÷ 5.6)% ;% Bias ∈ (-10.4 ÷ 9.6)%; IS (RSD% on peak areas over the calibration stage) = 6.0%
Precision	QC levels = 5, 15, 100 and 250 ng/ml (single-dose study); QC levels = 10, 30, 300, 700 and 1500 ng/ml (multiple-doses study); repeatability: n = 10; intermediate precision: n = 6
	Repeatability: RSD% ∈ (0.7 ÷ 2.1)%;% Bias ∈ (-12.1 ÷ 6.8)% (single-dose study) Repeatability: RSD% ∈ (0.7 ÷ 2.3)%;% Bias ∈ (-11.4 ÷ 3.2)% (multiple-doses study)
	Intermediate precision: RSD% ∈ (1.8 ÷ 8.4)%; % Bias ∈ (-16.4 [at LLOQ] ÷ 10.9)% Intermediate precision: RSD% ∈ (1.8 ÷ 7.4)%; % Bias ∈ (-12.5 ÷ 9.2)%
Stability	Freeze–thaw: n = 5; concentration levels = 4 (5, 15, 250 and 700 ng/ml); RSD% ∈ (1.9 ÷ 6.4)%; % Bias ∈ (-11.9 ÷ 13.7)%
	Long-term (-40°C) = 4 months; samplings = 5; concentration levels = 4 (as above); RSD% ∈ (2.7 ÷ 8.2)%; % Bias ∈ (-14.0 ÷ 7.0)%
	Short-term (-70°C/dry ice: transfer period from the clinical center to the bioanalytical laboratory) = 1 day; samplings = 4; concentration levels = 5 (as above); RSD% ∈ (0.7 ÷ 4.3)%; % Bias ∈ (-7.7 ÷ 12.0)%
	Short-term (25°C) = 24 h; samplings = 5; concentration levels = 4 (as above); RSD% ∈ (2.4 ÷ 8.1)%; % Bias ∈ (-14.3 ÷ 10.5)%
	Post-preparative (25°C) = 113 h; samplings = 5; concentration levels = 4 (as above); RSD% ∈ (2.5 ÷ 6.7)%;% Bias ∈ (-11.8 ÷ 7.7)%
	Fenspiride (stock solution; 4°C) = 77 days; samplings = 5; concentration levels = 4 (as above); RSD% ∈ (0.5 ÷ 4.8)%; % Bias ∈ (-9.1 ÷ 7.5)%
	IS (stock solution; 4°C) = 70 days; samplings = 8; RSD% (peak area) = 10.1%
Dilution integrity	Dilution ratios = 1/10; 1/5; 1/2; dilution fluid: a) blank plasma; b) aqueous 5% Na_2CO_3; samples per case: n = 3
	1/10: a) mean RSD% = 4.8; mean% bias = 5.1; b) mean RSD% = 1.1; mean% bias = 11.8;
	1/5: a) mean RSD% = 3.5; mean% bias = -0.4; b) mean RSD% = 2.8; mean% bias = 0.1;
	1/2: a) mean RSD% = 3.6; mean% bias = -2.4; b) mean RSD% = 2.7; mean% bias = -0.3;

RSD: Relative standard deviation.

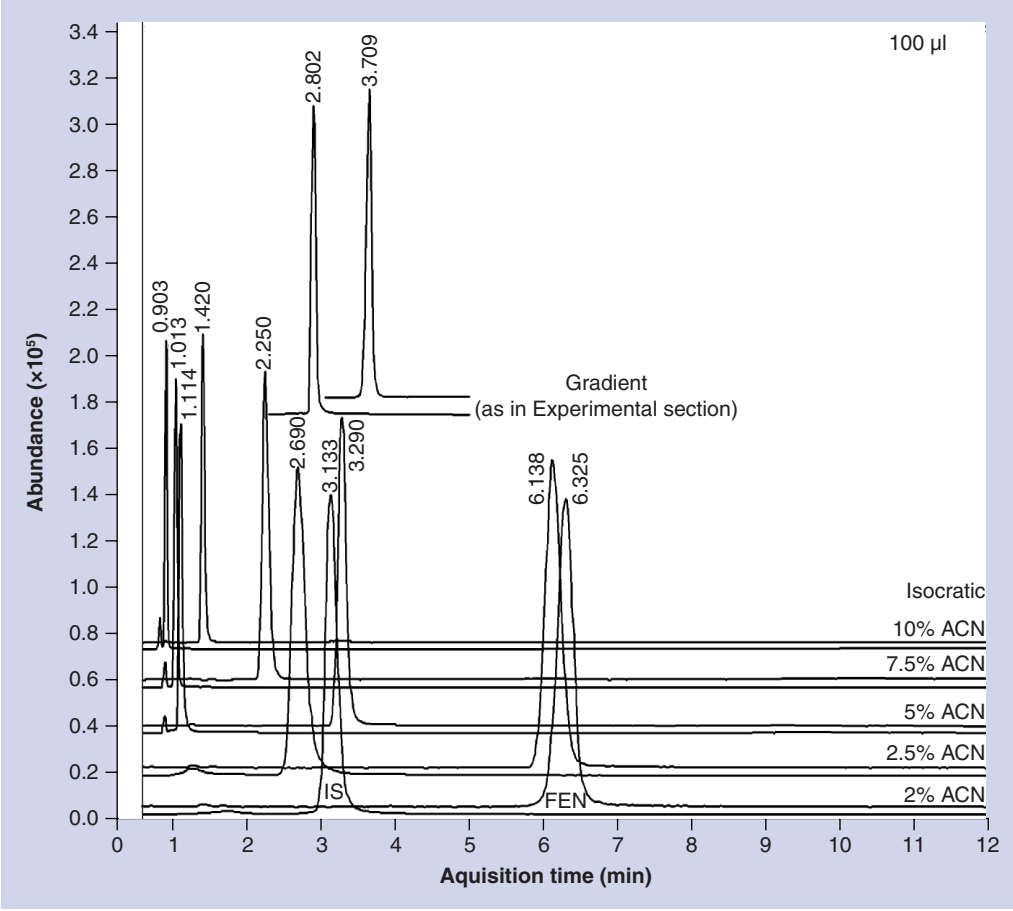

Figure 3. Chromatograms obtained after injections of 100 µl from solutions in 1-octanol containing fenspiride and trimetazidine, at different compositions of the mobile phase.
Absolute amounts of 20 ng from each individual compounds were loaded onto the column in isocratic elution conditions or according to the gradient profile described in the 'Experimental' section.
ACN: Acetonitrile; FEN: Fenspiride; IS: Internal standard.

recommendations existing in the European guidelines [20] for six different blank matrices, including hemolized and hyperlipemic ones, on spikes made at the 15 ng/ml level (three-times LLOQ) for the target compound and 20 ng/ml for the IS. The MF for fenspiride was 1.05 (RSD% = 2.4) and for the IS 1.04 (RSD% = 0.7). Consequently, the MF of fenspiride normalized to the IS was 1.01 (RSD% = 2.0).

The selectivity of the method was checked during method validation by comparing the residual peak area integrated at the retention time of fenspiride in the chromatograms of six different blank plasma samples to the peak areas resulting from analysis of the spikes at the LLOQ level in the respective matrices. The percentage of residual peak areas from LLOQ spikes ranged from 0 to 2.2%. The analysis of the samples collected before dose administration and comparison of the residual peak areas integrated at the retention time of fenspiride to peak areas at LLOQ (level one in the calibration associated with each analytical sequence, worked out during studies completion) confirmed the selectivity of the analytical method. Residual responses in pre-dose samples ranged between 0.8 and 31.2% from the peak area value at the LLOQ level for the 16 volunteers enrolled during the single-dose study over the four administration phases. Residual responses in pre-dose samples collected from the 25 volunteers enrolled during the multiple-doses study represented between 3.1 and 89.3% from LLOQ values over the two administration phases.

The quality characteristics of the analytical method are listed in Table 3. It is important to mention that the dilution integrity procedure revealed the possibility of making sample dilution, not only with blank plasma matrix but also with aqueous 5% sodium carbonate solution, without altering of the quantitative results.

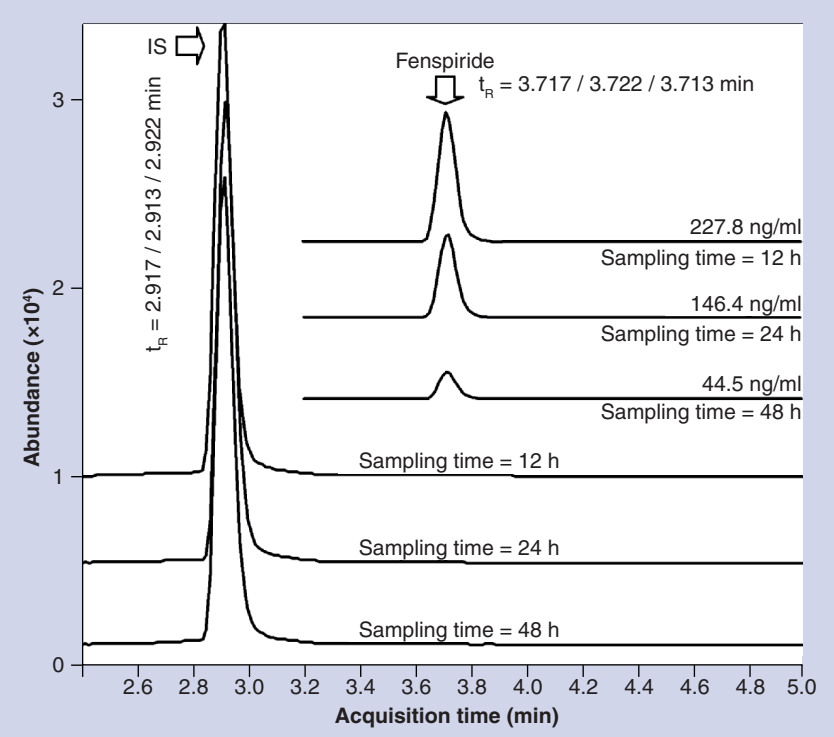

Figure 4. Chromatograms corresponding to consecutive incurred samples collected from one volunteer through completion of the bioequivalence studies, based on injections of 75 µl aliquots from the extracting 1-octanol layer.
IS: Internal standard.

During 31 analytical sequences involved in the completion of the single-dose study, none of the calibration samples failed on back-interpolation from a total of 248; 11 QC samples from a total of 496 were placed outside the ±15% accuracy threshold (four concentration levels, four independent replicates per level within a sequence); six of the QC outliers were placed at the 15 ng/ml concentration level and five at the 250 ng/ml level; in no cases were two QC samples at the same concentration level placed outside the accepted limits; mean absolute retention time values for target compounds were 2.84 min for IS (RSD% = 2.2; n = 1736) and 3.71 min for fenspiride (RSD% = 1.64, n = 1612); the RSD% for peak area values of fenspiride at the LLOQ (first calibration level; n = 31) was 17.2; the RSD% for peak area values of IS in calibration and QC samples was 14.9.

During completion of the multiple-doses study, one calibration sample failed to back-interpolate during 25 analytical sequences (from a total of 250 calibration samples); one QC sample at 15 ng/ml and three QC samples at 1500 ng/ml, respectively, were placed outside the accepted accuracy limits (from a total of 250 QC samples, at five concentration levels, two independent replicates per level within a sequence); mean absolute retention time for fenspiride was 3.65 min with a RSD% of 0.63 (n = 1250); mean absolute retention time characterizing IS was 2.86 min with a RSD% of 1.52 (n = 1350); peak areas at the LLOQ from calibrations are characterized by a RSD% of 10.9 (n = 25); a RSD% of 15.2 was calculated for peak area values of the IS in calibrations and QC samples. All these features taken together sustain the reproducibility and reliability of the analytical method.

To illustrate the results obtained through applying the large-volume injection of samples extracted in 1-octanol, three consecutive chromatograms corresponding to incurred samples (volunteer 2/Phase 1/sampling times 12, 24 and 48 h) are plotted in Figure 4. The reproducibility of the retention time values indicate that the diluent plug was successfully eliminated through the gradient profile, leaving the chromatographic column ready for receiving the consecutive sample.

Last but not least, incurred sample reanalysis (ISR) was performed for both studies to obtain a better insight about method's reproducibility. For the single-dose study, samples withdrawn at 8 and 72 h after administration during the four phases were reanalyzed. The first sampling corresponds to high plasma levels of fenspiride (at or near T_{max}), while the second sampling is the last withdrawal from the elimination stage (concentration levels should be close to LLOQ). A total number of 119 samples were reanalyzed, as one volunteer had not completed Phase III and IV, and samples at 72 h for volunteers 4 (one sample) and 10 (four samples) were initially placed below the method's LLOQ. For the multiple-doses study, sampling at 120 and 172 h after administration over both phases was selected. The first sampling corresponds to the steady-state concentration (before the fifth dose (producing the lowest concentration level reported over the study), while the second sampling corresponds to a maximum concentration level reached in steady-state conditions, after the last dose administration. A total of 100 samples were reanalyzed for the multiple-doses study.

Data resulting from ISR were treated through the Bland–Altman approach, according to the procedure described in [21]. The interpretation deals with calculation of the neat difference (repeat - original) between paired determinations, followed by normalization of the difference to

the average of the paired results. The mean of the normalized differences and the corresponding standard deviation are used for the calculation of the confidence limits as well as the tolerance limits (TLs). The TLs are expressed as $D \pm k^*s_D$, where k is the tolerance factor for a normal distribution with a proportion p = 66.7%, D is the mean normalized difference and s_D is the standard deviation of the mean normalized difference. Acceptance criteria (in accordance with the 4/6/20 rule), 33.3% of the mean normalized differences may be placed outside the -0.2–0.2 interval. Results of ISR are shown in Table 4.

For the single-dose study, the average of the mean normalized differences between repeated and original results (slightly negative, but very close to 0) and their corresponding confidence interval (not including 0) indicate a negative systematic error; confidence limits are included in the -0.2–0.2 range, sustaining the accuracy of the approach; the lower limit of the tolerance interval is placed slightly below the -0.2 threshold; eight data pairs are spread outside the acceptance limits in the concentration interval from LLOQ to 50 ng/ml; 19 other data pairs placed outside the acceptance limits in the concentration range 180 to 620 ng/ml; 27 (22.7%) of the pairs fell outside the -0.2–0.2 limits specified by the 4/6/20 rule; if the small negative systematic bias was inexistent (normalized differences are corrected with the mean), random errors would keep only 15 pairs outside the ±20% limit, and these would be evenly distributed above and below the thresholds.

For the multiple-dose study, the mean (slightly negative, but very close to 0) and the confidence interval (including 0) indicate the lack of any systematic error; both confidence and tolerance limits are included in the -0.2–0.2 range; one single pair falls outside the -0.2–0.2 interval.

One can conclude that the method's reproducibility was sustained by the experimental data obtained from the ISR study.

A comparison with the standard protein precipitation method has been also emphasized. The 'classical' approach consisted in protein precipitation of a 200 µl plasma aliquot spiked with fenspiride through addition of 400 µl of acetonitrile (containing 15 ng/ml IS) followed by centrifugation and separation of the supernatant that was injected in the chromatographic column. The separation conditions were kept unchanged, except the injected volume. It has been determined that in such conditions, the maximum injection volume not inducing peak focusing phenomena

Table 4. Characteristics of the results obtained after the incurred sample reanalysis for the two bioequivalence studies involving fenspiride.

Parameter	Study	
	Single-dose	Multiple-doses
Number of data pairs [O/R concentration values]	119	100
% of reanalyzed samples from the total number of incurred samples	11.3	11.1
Range of the means [M = (R+O)/2]	Min: 4.3 Max: 611.8	Min: 393.8 Max: 1467.6
Average of the mean normalized differences [(R – O)/M]	-0.05	-0.03
Standard deviation of the average of the mean normalized difference	0.16297	0.08832
CL of the mean normalized differences	Min: -0.08166 Max: -0.02309	Min: -0.05163 Max: -0.01701
TL of the mean normalized differences	Min: -0.20997 Max: -0.10522	Min: -0.13165 Max: 0.06301
AL for the mean normalized differences	Min: -0.2000 Max: +0.2000	Min: -0.2000 Max: +0.2000
No./(%) of data pairs outside the acceptance limits	27/(22.7%)	1/(1.0%)
No./% of data pairs having R values outside ± 20% from O values	23/(19.3%)	2/(2.0%)
No. of data pairs below AL min	21	0
No. of data pairs over AL max	6	1
Coefficient of variation of measured data (%)	11.5	6.3
Distribution of errors	Normal (B* = 0.965)	Normal (B* = 0.955)
Status of the ISR approach	Pass	Pass

†B is the slope of the functional dependence between the ordered mean normalized differences and a normal inverse cumulative distribution of values and should be closest to unity for normal distribution of errors.
AL: Acceptance limits; CL: Confidence limits; M: Mean; O: Original; R: Repeated; TL: Tolerance limits.

is 2 µl. From a simple theoretical calculation, the absolute amounts of the analytes loaded onto the column when extracting in 1-octanol are approximately 62.5-times higher compared with the amounts used in the protein precipitation approach. Experimental findings confirmed the basic calculations (FIGURE 5), comparatively presenting chromatograms resulting from analysis of a blank plasma sample spiked with fenspiride at 15 ng/ml level, processed according to both preparation alternatives (extraction in 1-octanol and protein precipitation with acetonitrile).

As expected, retention times for both analytes were higher in the chromatogram of the sample precipitated with acetonitrile (no diluent is saturating the stationary phase in the column's head). It could be observed that mass transitions used for quantitation of both fenspiride and IS revealed some interferences arising from the residual matrix left after protein precipitation with acetonitrile (additional peaks preceding IS and fenspiride) – hopefully chromatographically resolved. Through studying the ionization effects brought by the residual matrix into the MS source by means of the classical setup (monitoring baseline on injection of samples processed though both preparation alternatives from blank plasma, while the MS source is continuously fed with a constant mass flow of 5 ng/min of analytes), the similarity between the tested methods was clearly shown. When the protein precipitation method is applied for sample preparation, the 15 ng/ml concentration level may be considered the method's LLOQ, as it results from the S/N ratio.

For making it more evident that large-volume injection of samples in 1-octanol does not induce any major peak shape alteration, FIGURE 6 compares results obtained for the fenspiride peak in chromatograms resulting after processing of a 15 ng/ml spiked plasma sample according to the LLE approach and a 500 ng/ml spiked plasma sample prepared through protein precipitation with acetonitrile (comparable absolute amounts of analyte were loaded onto the column). It appears that no major peak broadening effect or symmetry deterioration arises on large-volume injection of samples having 1-octanol as the diluent.

Some of the pharmacokinetic parameters determined for fenspiride in the pharmaceutical formulations tested for bioequivalence, during the single-dose fasting conditions, single-dose fed conditions and multiple-dose administration studies are presented in TABLE 5. These results are in fair agreement with reported data [16] during the early bioavailability studies made on the innovator formulation containing 80 mg of fenspiride (note that data in the cited

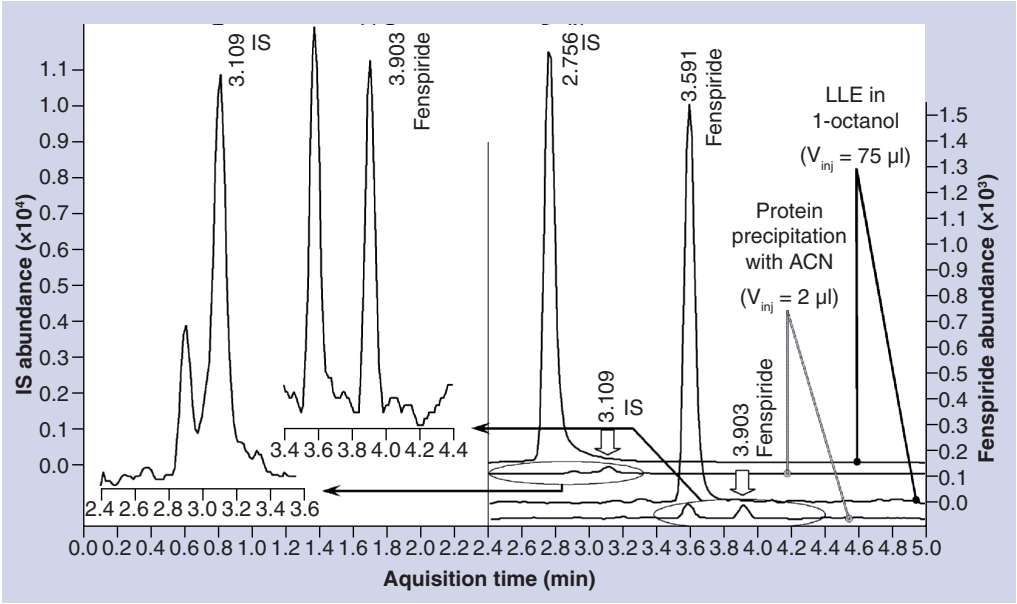

Figure 5. Comparative overview between chromatograms resulting after injection of a 15 ng/ml fenspiride-spiked plasma. Processed through **(A)** LLE in 1-octanol, followed by the direct injection of an aliquot from the organic layer (detailed conditions are given in the 'Experimental' section); or **(B)** protein precipitation through addition of acetonitrile, followed by injection of 2 µl volume from the supernatant (details are given in text).
ACN: Acetonitrile; IS: Internal standard; LLE: Liquid–liquid extraction.

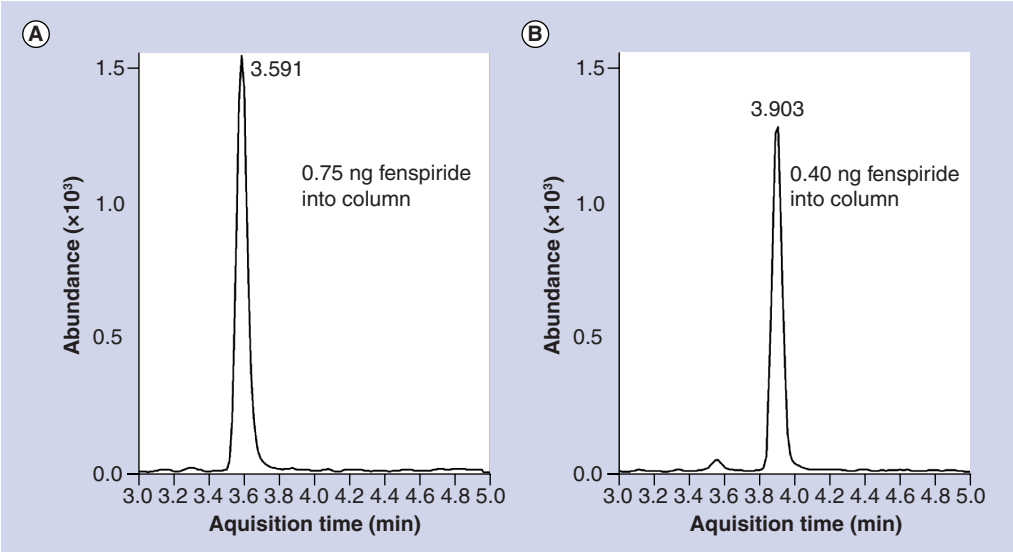

Figure 6. Peak shapes obtained after injection of comparable absolute amounts of fenspiride onto the chromatographic column, from samples processed in two different ways. (A) 15 ng/ml fenspiride spiked plasma, extracted in 1-octanol and injection of 75 µl aliquot from the organic layer; **(B)** 500 ng/ml fenspiride spiked plasma, followed by protein precipitation through acetonitrile addition and injection of 2 µl aliquot from supernatant onto the chromatographic column.

reference resulted from a clinical study made on 12 volunteers and the analytical assay was made alternatively with RPLC coupled to electrochemical and UV detection).

Conclusions

Injection of large volumes of 1-octanol as sample diluent directly onto the chromatographic column was possible without producing analyte peak broadening and/or distortion. This was mainly due to the fact that the retention of the diluent is much higher compared with the retention of the target compounds, namely fenspiride and trimetazidine (IS). Consequently, the sample preparation procedure could be significantly simplified: the target analytes being

Table 5. Principal and some of the secondary pharmacokinetic parameters determined for fenspiride over the bioequivalence studies carried out under single-dose fasting, single-dose feed and multiple-dose administration conditions.

PK parameter	R	T	Geo mean ratio (T/R)	Confidence interval	Within subject CV%
Single-dose: fasting conditions					
AUC_{last} (ng/ml*h)	10199.67	10259.90	1.0095	0.9669–1.0539	6.92
C_{max} (ng/ml)	345.89	329.35	0.9528	0.9039–1.0042	8.45
AUC_{tot} (ng/ml*h)	10701.30	10751.02	1.0078	0.9628–1.0549	7.33
T_{max} (h)	7.06	8.31	Friedman – the difference between means is not significant		
Single-dose: feed conditions					
AUC_{last} (ng/ml*h)	9940.23	9576.89	0.9704	0.9372–1.0047	5.38
C_{max} (ng/ml)	400.90	381.58	0.9563	0.9236–0.9902	5.38
AUC_{tot} (ng/ml*h)	10442.34	10066.87	0.9714	0.9362–1.0081	5.71
T_{max} (h)	6.14	7.80	Friedman – the difference between means is not significant		
Multiple-dose					
AUC_{tot} (ng/ml*h)	11105.06	11208.73	1.0194	0.9623–1.0799	11.90
C_{max} (ng/ml)	1023.40	1034.09	1.0214	0.9640–1.0822	11.93
% Ptf	25.95	26.47	1.0275	0.9483–1.1125	4.55

PK: Pharmacokinetic; R: Reference product; T: Tested product.

extracted in 1-octanol from alkalinized plasma, followed by the injection of an aliquot from the organic layer directly onto the chromatographic column. Such an approach avoids organic solvent evaporation as well as re-dissolution of the dried residue in an adequate solvent. The procedure is straightforward, injection being possible directly from the vial in which LLE was achieved, simply by controlling the position of the needle during withdrawal of the sample volume. The characteristics determined during the validation of the method fully support the increased potential of this approach. Comparison of the results provided through the application of this sample preparation alternative to those produced by the 'classic' protein precipitation technique also revealed advantages in terms of sensitivity and selectivity. The method was successfully applied for two bioequivalence studies concerning fenspiride-containing pharmaceutical formulations. The principal pharmacokinetic parameters determined during bioequivalence are also presented and are in good agreement with the literature. Additional quality characteristics resulting from the method's application were highlighted, as well as the reproducibility insights produced through ISR.

The approach successfully combines the advantages of LLE: reduced sample manipulation and increased sensitivity produced through a large injected volume. Its potential in bioanalysis seems very promising, other candidates (target analytes as well as water non-miscible extractants/diluents) need to be considered.

Future perspective

Phenomena relating to injection of samples made in diluents immiscible with the mobile phase should be attentively studied due to their increased application potential in bioanalysis. Extension of the study to other diluents and extracted analytes would not only enlarge the field of applications, but will also reveal specific aspects relating to the fundamentals aspects of the phenomena. Similar approaches may be developed for other separation mechanisms used in chromatography.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

- The principle of large-volume injection of diluents that are not miscible with the mobile phase was applied for extraction of fenspiride and trimetazidine (as internal standard) from alkalinized plasma samples in 1-octanol, followed by the direct loading of an aliquot from the organic layer in the chromatographic column.
- Features relating to injection of samples dissolved in 1-octanol on octadecyl chemically modified silica gel as the stationary phase and aqueous-rich mobile phases are discussed in order to optimize separation conditions and to avoid production of broad and/or distorted chromatographic peaks.
- The method, based on liquid–liquid extraction of target compounds in 1-octanol followed by the direct injection of a large volume from the organic layer with reversed-phase chromatographic separation and ESI-MS/MS detection, has been validated and successfully used for the evaluation of the therapeutic equivalence of two controlled-release pharmaceutical formulations containing 80 mg of fenspiride hydrochloride, during single-dose administration (feed/fasting conditions) and multiple-doses administration clinical studies.
- The quality characteristics of the analytical method, as well as its reproducibility resulting from reanalysis of incurred samples, are discussed in detail. The conclusions reached fully support the increased potential of the analytical procedure, that is, injection of solvents that are not miscible with the mobile phase used during the chromatographic separation.

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