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Determination of fenofibric acid in human plasma using automated solid-phase extraction coupled to liquid chromatography

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Abstract

The pharmacokinetic studies of fenofibrate require a rapid, selective and robust method to allow the determination of fenofibric acid, its active metabolite, in different biological matrixes (such as plasma, serum or urine). A new fully automated method for the determination of fenofibric acid in plasma has been developed, which involves the solid-phase extraction (SPE) of the analyte from plasma on disposable extraction cartridges (DECs) and reversed-phase HPLC with UV detection. The SPE operations were performed automatically by means of a sample processor equipped with a robotic arm (ASPEC system). The DEC filled with octadecyl silica was first conditioned with methanol and pH 7.4 phosphate buffer. A 0.8-ml volume of diluted plasma sample containing the internal standard (sulindac) was then applied on the DEC. The washing step was performed with the same buffer (pH 7.4). Finally, the analytes were successively eluted with methanol (1.0 ml) and 0.04 *M* phosphoric acid (1.0 ml). After a mixing step, 100 μl of the resultant extract was directly introduced into the HPLC system. The liquid chromatographic (LC) separation of the analytes was achieved on a Nucleosil RP-8 stationary phase (5 μm). The mobile phase consisted of a mixture of methanol and 0.04 *M* phosphoric acid (60:40, v/v). The analyte was monitored photometrically at 288 nm. The method developed was validated. In these conditions, the absolute recovery of fenofibric acid was close to 100% and a linear calibration curve was obtained in the concentration range from 0.25 to 20 μg/ml. The mean RSD values for repeatability and intermediate precision were 1.7 and 3.9% for fenofibric acid. The method developed was successfully used to investigate the bioequivalence between a micronized fenofibrate capsule formulation and a fenofibrate Lidose™ formulation.

Keywords: Fenofibric acid

1. Introduction

Fenofibrate (isopropyl ester of 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid) (Fig. 1) is a widely used hypolipidaemic drug. Its pharmacological activity consists in reducing triglyceride and cholesterol concentration in plasma [1]. Fenofibrate is essentially insoluble in aqueous solvents and can be considered as a prodrug. After absorption, fenofibrate is rapidly and completely metabolised, essentially to its major active metabolite, fenofibric acid (FEFA) by plasma and tissue esterases [2–6]. No unchanged fenofibrate can be detected in the plasma after an oral dose [6–8].

This lipid-lowering agent can be administered once daily and its half-life being about 20 h. After an oral administration of 100 or 200 mg of fenofibrate,

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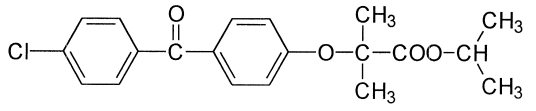
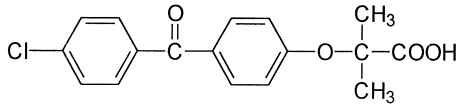
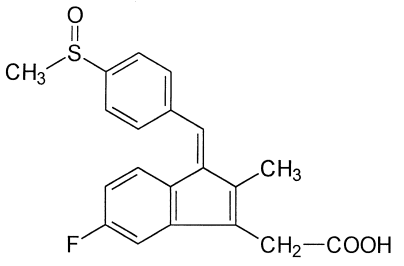


Fenofibrate



Fenofibric acid (FEFA)



Sulindac (IS)

Fig. 1. Chemical structures of fenofibrate, its active metabolite fenofibric acid and sulindac (internal standard).

the typical peak concentration (C_{max}) for the fenofibric acid is around 5–15 μg/ml [9–11].

In order to study the pharmacokinetic parameters in various situations in human, it is necessary to dispose of a method for the determination of fenofibric acid in plasma. Several analytical methods for the determination of fenofibrate or fenofibric acid have been reported including liquid chromatography [10,12–16], gas chromatography [17] or gas chromatography coupled to mass spectrometry [18]. Among these methods, protein precipitation with a strong acid or off-line liquid–liquid extraction (LLE) of the analytes with an organic solvent are the most frequently applied techniques for sample preparation [9,10,12,14,15,18]. Some other methods involve manual solid-phase extraction (SPE) using either octyl [16] or octadecyl [13] silica bonded phases as extraction sorbents. However, SPE on disposable extraction cartridges (DECs) can be easily automated and coupled to the LC system by using some robotic sample processors (i.e. ASPEC system), allowing the analysis of numerous biological samples [19–21].

In the present study, a sensitive and selective method for the determination of fenofibric acid in human plasma using solid-phase extraction on DECs as sample preparation coupled to liquid chromatography was developed. The SPE procedure was fully automated by means of an ASPEC system.

The LC system was investigated in order to obtain suitable conditions with respect to selectivity and retention of FEFA, and sulindac was used as the internal standard (I.S.). Moreover, the selection of the SPE sorbent and eluting solvents have been optimized to observe sufficiently high analyte recovery for FEFA from plasma.

Finally the fully automated method was validated and successfully used to perform the determination of fenofibric acid in actual human plasma samples. The method developed was used to investigate the bioavailability of two different oral formulations of fenofibrate: the first formulation consisted in a hard gelatin capsule containing micronized fenofibrate material while the second was a presentation of semi-solid dispersion (paste) of fenofibrate in hard gelatin capsules (Lidose™). This study was performed by comparing plasma concentration level profiles of FEFA from 24 healthy male volunteers.

2. Experimental

2.1. Chemical and reagents

Fenofibrate and fenofibric acid were obtained from the European Pharmacopoeia (Strasbourg, France). Sulindac, used as the internal standard (I.S.), was supplied by Sigma (St. Louis, MO, USA). Phosphoric acid (85%), sodium hydroxide and potassium dihydrogenphosphate were all of p.a. quality and obtained from Merck (Darmstadt, Germany). Methanol and water were of HPLC grade from Merck.

Isolute DECs (1-ml capacity) filled with 50 mg of octadecylsilica (C_{18}) were obtained from IST (Inter-

national Sorbent Technology, Mid-Glamorgan, UK). Other Isolute DECs filled with 50 mg of octyl (C_8), cyanopropyl (CN) or ethyl (C_2) silica were also tested.

The analytical and guard columns were respectively prepacked with 5 μm Nucleosil 100 RP-8 support from Macherey-Nagel (Düren, Germany).

2.2. Instrumentation

The automated sample preparation with extraction cartridges (ASPEC) system from Gilson (Villiers-le-Bel, France) consisted of an automatic sampling injector module, a model 401 dilutor-pipettor and a set of racks and accessories for handling DECs, plasma samples and solvents.

The LC system consisted of a Model 1100 series liquid chromatograph equipped with a quaternary pump, a vacuum degasser, a thermostatted column compartment, an autosampler and a diode-array detector, all from Hewlett-Packard (Palo Alto, CA, USA). A Manu-Cart system which consisted of an analytical column (125×4 mm I.D.) and a short guard column (8×4 mm I.D.) from Macherey-Nagel was thermostated at 35°C. The mobile phase consisted of a mixture of methanol and 0.04 *M* phosphoric acid solution (60:40, v/v). Before use, the mobile phase was degassed for 15 min in an ultrasonic bath. The flow-rate was 0.80 ml/min and UV detection was carried out at 288 nm. All data obtained were processed and stored on a Vectra XA computer from Hewlett-Packard using the HP Chemstation 6.01 software.

2.3. Standard stock solutions

The stock standard solutions of FEFA and I.S. were prepared by dissolving appropriate amounts of the compounds in methanol to give final concentrations of 2.0 mg/ml for FEFA and 5.0 mg/ml for I.S. The FEFA solutions were then successively diluted with phosphate buffer pH 7.4 to achieve concentrations of 2.0–400 μg/ml. The stock solution of I.S. was diluted with phosphate buffer pH 7.4 to reach a final concentration of 2.5 μg/ml.

The aqueous solutions were used to spike plasma samples either for calibration curves (0.25–20 μg/ml) for FEFA or for quality control during the pharmacokinetic study (0.25–20 μg/ml).

2.4. Sample preparation

Blood samples were collected in tubes containing heparin. After centrifugation at 3000 rpm during 10 min at 4°C, the separated plasma was collected and stored at −80°C. Before analysis, the plasma samples were thawed at 18°C. The automatic sample procedure starts by the washing of the needle and the external tubing of the injection valve with 2.0 ml of water. Between each step, the needle and the transfer tubing of the dilutor are rinsed with the same volume of water and a 10-mm air gap is generated inside the transfer tubing before the aspiration of other liquids in order to avoid cross-contamination.

A 0.30-ml volume of sample was transferred manually into a vial on the appropriate rack of the ASPEC system and a 1200-μl volume of I.S. solution (2.5 μg/ml) was automatically added to each plasma sample (diluted plasma). The DEC sorbent was first treated with 1.0 ml of methanol and then with 1.0 ml of phosphate buffer pH 7.4. A 0.80-ml volume of diluted plasma was aspirated by the autosampler needle from the corresponding vial and applied onto the DEC. The washing step was then performed by dispensing a 1.0-ml volume of phosphate buffer pH 7.4. The analytes are eluted by dispensing successively a 1.0-ml volume of methanol and 1.0 ml of 0.04 *M* phosphoric acid. The eluate was successively aspirated and dispensed twice in the collection tube (homogenization step). Finally, a 100-μl volume of the final extract was injected into the chromatographic system. All these operations are summarised in Table 1. Each plasma sample was prepared individually during the LC analysis of the previous sample (concurrent mode).

2.5. Pharmacokinetics study

The automated method developed was used to investigate the plasma profile after two oral doses of fenofibrate: a hard gelatin capsule containing 200 mg of micronised fenofibrate as a reference and a hard gelatin capsule containing a semi-solid dispersion (Lidose™) of fenofibrate (SMB Laboratories, Brus-

Table 1
Solid-phase extraction scheme

SPE steps	Liquid	Volume (ml)	Dispensing flow-rate (ml/min)
Conditioning	Methanol	1.00	6.00
	Buffer (pH 7.4)	1.00	6.00
Sample loading	Diluted plasma[a]	0.80	0.18[b]
Washing	Buffer (pH 7.4)	1.00	1.50
Elution	Methanol	1.00	1.50
Dilution	0.04 *M* Phosphoric acid	1.00	1.50
Mixing	Plasma extract	2.00	3.00
Filling of the injection loop (100 μl)	Plasma extract	0.60	0.75

[a] A 1.2 ml-volume of I.S. solution (concentration = 2.5 μg/ml) was added to 0.3 ml of plasma sample. After mixing, the resulting solution was then introduced onto the DEC.

[b] The plasma samples were applied onto the DEC at the miminum dispensing flow-rate (0.18 ml/min) in order to obtain high analyte recoveries [20].

sels, Belgium). A clinical study on 24 healthy male volunteers was conducted. The subjects received one capsule containing 200 mg of fenofibrate and blood samples were collected during 72 h. The plasma concentrations of fenofibric acid were determined for both formulations.

3. Results and discussion

3.1. Optimization of the HPLC system

In a bioanalytical HPLC procedure using UV detection, the retention of the analytes should be sufficiently high in order to avoid interferences with the front peak in the chromatogram [20]. For this reason, we have studied the influence of mobile phase pH on the analyte (FEFA) and I.S. retention. The mobile phase pHs tested were 2.5, 3.0, 4.0 and 5.0 because the optimal stability of the analytes was in the pH range from 2 to 5.

As shown in Fig. 2, the capacity ratios (k') of fenofibric acid and sulindac (I.S.) decrease with the increasing mobile phase pH, owing to the increase of the ionization of the carboxyl group. In addition, the selectivity between the two compounds also decreases with the increasing of pH, giving even a reversal of the elution order at pH higher than 4, probably because of a slightly higher pK_a value for sulindac. The final pH value selected with respect to resolution and retention (k') was 2.5.

3.2. SPE optimization

Four different kinds of DECs containing bonded silicas with various polarities were tested. Spiked plasma solutions were used as samples and the corresponding recoveries of FEFA and I.S. were determined (Table 2). The recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracts with those found by direct injection of aqueous solutions at the same concentration into the LC–UV system, using the same autosampler. As can be seen from Table 2, the fairly polar cyano phase gave very low recoveries. This can be explained by analytes losses during the loading and/or the washing steps. However, the analyte recoveries obtained with less polar phases were found to increase significantly. The recoveries obtained with the C_2, C_8 or C_{18} sorbents were high plasma recoveries. Taking into account the chromatogram profile obtained with the plasma samples, devoid of interferences from plasma components at the retention time of the analytes and the slightly higher recovery observed with DECs filled with C_{18} (98%), the latter phase was finally selected.

Moreover, the influence of the nature of solvent used for the elution step was investigated by testing the recoveries of FEFA and I.S. observed after elution either with pure methanol or with the LC mobile phase. As shown in Table 3, the recoveries observed when the elution step was performed with the LC mobile phase were 46 and 69% for FEFA and I.S., respectively, while they were 80 and 99% when

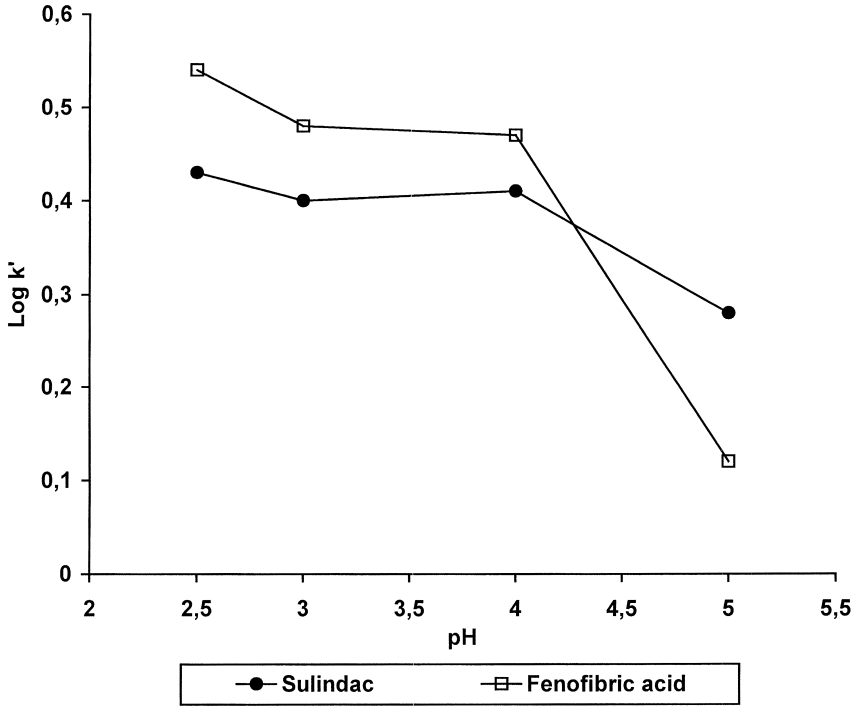


Fig. 2. Influence of pH on fenofibric acid and internal standard retention. Stationary phase: 5 μm Nucleosil 100 RP-8 (125×4 mm I.D.); mobile phase: methanol–phosphate buffer (60:40, v/v); UV detection: 288 nm; temperature: 35°C; flow-rate: 0.8 ml/min.

pure methanol and 0.04 *M* phosphoric acid were both passed through the DEC and 82 and 99% when phosphoric acid was added in the collection tube after elution with methanol. In spite of very similar recoveries obtained with the two elution mode (see Table 3), the delivery of 0.04*M* phosphoric acid on the DEC just after the methanol dispensing was given the preference in order to be ensured that all the volume of methanol was passed through the DEC.

Table 2
Types of sorbents used in the disposable extraction cartridges (DECs)[a]

Sorbent	Recovery of I.S. [mean±SD (%); $N=3$]	Recovery of fenofibric acid [mean±SD (%); $N=3$]
CN	13±3	5±1
C_2	78±2	87±5
C_8	80±2	95±2
C_{18}	82±2	98±3

[a] DECs: Isolute (50 mg); conditioning: methanol–buffer pH 7.4 (1.0 ml of each); washing: 1.0 ml buffer pH 7.4; elution: 1.0 ml of methanol; buffer addition: 1.0 ml of phosphoric acid solution (0.04 *M*); sample: spiked plasma solution of FEFA and I.S. (20 μg/ml).

3.3. Validation

3.3.1. Stability

Because of its influence on the other criteria, stability of the analytes investigated has been mainly studied during the development step of the method and further checked during the validation step. Table 4 shows the stability of the sample processing, chromatography and storage of processed spiked samples. In the present study, the stability of stock solution (7 days), autosampler eluate (18 h), plasma sample (48 h), plasma storage −80°C (30 days) and after three freeze–thaw cycles was demonstrated. No significant degradation of both compounds of interest was observed.

3.3.2. Selectivity

The selectivity of the analytical method was investigated in order to assume that the method can

Table 3
Selection of the eluent[a]

Elution step	Recovery of I.S. [mean (%); $N=3$]	Recovery of FEFA [mean (%); $N=3$]
Mobile phase (2 ml)[b]	69	46
Methanol (1 ml)[b] + 0.04 *M* phosphoric acid (1 ml)[c]	82	99
Methanol (1 ml)[b] + 0.04 *M* phosphoric acid (1 ml)[d]	80	99

[a] DECs: Isolute (50 mg); conditioning: methanol–buffer pH 7.4 (1.0 ml of each); washing: 1.0 ml buffer pH 7.4; sample: spiked plasma solution of FEFA and I.S. (20 μg/ml).
[b] Applied on the DEC (Bondelut C_{18}, 50 mg).
[c] Dispensed directly in the collection tube.
[d] Applied on the DEC just after the dispensing of methanol.

be used to quantitate FEFA and I.S. in presence of other constituents in the sample. Potential interfering substances in a biological matrix include endogenous matrix components, related substances (fenofibrate), metabolites and concomitant medication drugs such as OTC drugs (aspirin, caffeine, acetaminophen, ibuprofen). The selectivity was studied by analysing six individual blank plasma samples and by injecting aqueous solutions of fenofibrate (RT≈19 min), aspirin (RT≈2.8 min), caffein (RT≈2.4 min), acetaminophen (RT≈1.8 min) and ibuprofen (RT>25 min) in the chromatographic system. No endogenous sources of interference were observed at the retention times of the analytes. The chromatograms of blank plasma and spiked plasma samples are presented in Fig. 3.

Table 4
Stability of fenofibric acid and sulindac in plasma control samples

	Storage conditions	Fenofibric acid		Sulindac 2.50 μg/ml
		0.25 μg/ml	20.0 μg/ml	
Stock solution ($N=3$)	8 h, 18±2°C			
μg/ml		0.28	19.53	2.48
% of initial		106.1	101.6	99.4
Stock solution ($N=3$)	7 days, 4±2°C			
μg/ml		0.27	21.36	2.50
% of initial		104.2	111.1	100.0
Autosampler stability eluate ($N=3$)	18 h, 18±2°C			
μg/ml		0.25	19.84	2.42
% of initial		101.1	99.2	97.0
Plasma sample ($N=3$)	48 h, 18±2°C			
μg/ml		0.26	21.50	–
% of initial		104.1	107.5	–
Freeze–thaw ($N=3$)				
2nd cycle (% of initial)		–	102.5	–
3rd cycle (% of initial)		–	101.5	–
Plasma sample storage ($N=3$)	30 days, −80±5°C			
μg/ml		0.26	20.30	–
% of initial		104.6	101.4	–

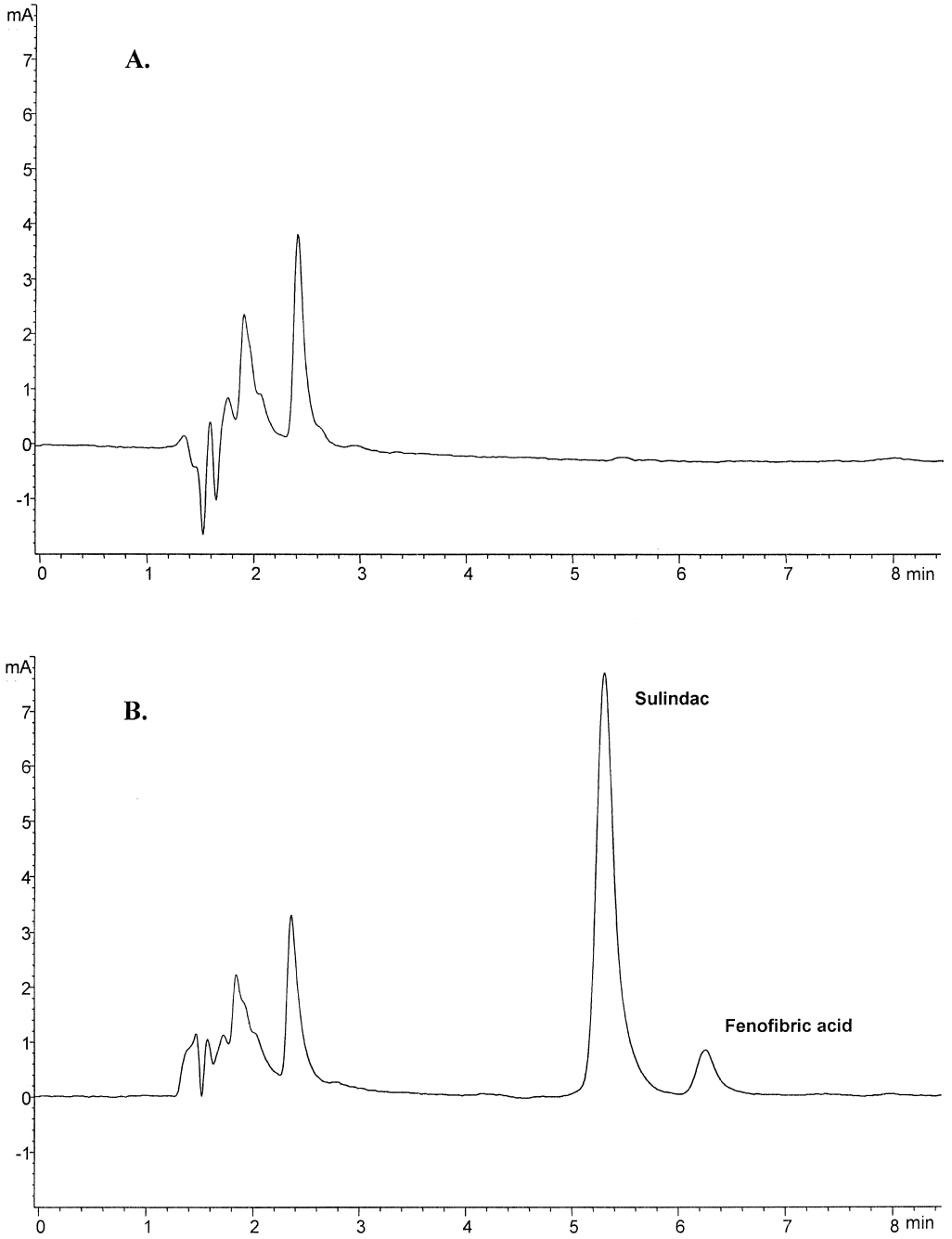


Fig. 3. Typical chromatograms obtained by using SPE coupled to LC. (A) Chromatogram of blank plasma; (B) chromatogram of plasma spiked with fenofibric acid (1.0 μg/ml) and I.S. (2 μg/ml). SPE and LC conditions as described in Section 2.

3.3.3. Extraction efficiency

The absolute recoveries of FEFA over the whole concentration range were calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous standard solutions at the same concentration, using the same autosampler equipped with the same loop [22]. The mean recovery was about 98% and the extraction efficiency was relatively constant over the range considered in view of the relative standard deviation value obtained (2%; $N=6$) (cf. Table 5).

3.3.4. Linearity

The calibration curve was performed in the concentration range 0.25–20 μg/ml ($N=3$; $k=6$) and the following regression equation was found by plotting the peak area ratio (y) versus analyte concentration (x) in μg/ml: $y = 0.1523x - 0.00161$ $r^2 = 0.9998$.

The determination coefficients (r^2) obtained for the regression line of FEFA demonstrate the excellent relationship between peak area ratio and concentration. During routine analysis, the calibration equation was computed by least-squares regression as mentioned, and the concentration of each calibration sample was calculated. If the back-calculated concentration of a calibration sample did not fall within ±15% of nominal, that sample was discarded and the equation was recalculated. However, for the calibration and the run to be valid, no more than two calibration samples were discarded and at least five accepted calibration samples had to be kept.

3.3.5. Limits of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) for FEFA were estimated from the intercept ($\bar{a}$) of the regression line and the corresponding residual standard deviation ($s_{y/x}$) [23]. The responses at the LOD and LOQ were estimated by the following expressions, respectively:

$$f(\mathrm{LOQ}) = \bar{a} + 3s_{y/x} \quad (1)$$

$$f(\mathrm{LOQ}) = \bar{a} + 10s_{y/x} \quad (2)$$

Table 5
Validation of the method for the determination of FEFA

Validation criterion		Fenofibric acid
Absolute recovery (mean±SD, $N=6$)		98±2%
Linearity ($k=6$, $N=3$)	0.25–20 μg/ml	$y = 0.15231x - 0.00161$ $r^2 = 0.9998$
LOD		0.036 μg/ml
LOQ		0.12 μg/ml
Precision		
Repeatability [RSD (%); $N=6$]		
	0.25 μg/ml	1.4
	5.0 μg/ml	2.2
	20.0 μg/ml	1.6
	Mean	1.7
Intermediate precision [RSD (%); 3 days; $N=18$]		
	0.25 μg/ml	1.5
	5.0 μg/ml	6.4
	20.0 μg/ml	3.8
	Mean	3.9
Accuracy [recovery±IC (%); $N=6$]		
	0.25 μg/ml	99.8±1.5
	5.0 μg/ml	100.3±1.5
	20.0 μg/ml	99.8±1.3

By applying this method, the LOD and LOQ for FEFA of the developed method were found to be 36 and 120 ng/ml (cf. Table 5).

3.3.6. Precision

The precision of the bioanalytical method was estimated by measuring repeatability and intermediate precision for FEFA at three different concentrations levels ranging from 0.25 to 20 μg/ml. The variances of repeatability and time-dependent intermediate precision as well as the corresponding relative standard deviations (RSD) were computed from the estimated concentrations. The RSD values presented in Table 5 were relatively low, less than 7% for the medium concentration of the range, and illustrated the good precision of the proposed method.

3.3.7. Accuracy

The accuracy of the procedure was assessed by calculating the ratio between the analyte amount found versus the amount spiked in the plasma at three concentration levels ranging from 0.25 to 20 μg/ml. The accuracy, defined as mean%±interval of confidence ($P>0.05$), shows that the automated LC procedure developed for the determination of fenofibric acid can be considered as accurate within the concentration range investigated (Table 5). Mean values are very close to the theoretical concentrations, showing method accuracy ranging from 99.8 to 100.3%.

3.4. Pharmacokinetics

The automated method developed was used to investigate the bioavailability parameters of FEFA. A clinical study on 24 healthy male volunteers was performed to demonstrate the bioequivalence between a micronised fenofibrate (200 mg capsule) and a Lidose™ formulation. The bioequivalence was assessed by measuring plasma concentrations of FEFA as the first criteria. Plots of the plasma FEFA levels (μg/ml) versus post-dose sampling time (h) for the micronized and the Lidose™ formulations are presented in Fig. 4. Pharmacokinetic parameters ($AUC_{0\rightarrow 72}$, $AUC_{0\rightarrow\infty}$, C_{max}, T_{max}) calculated from these data are presented in Table 6 and clearly

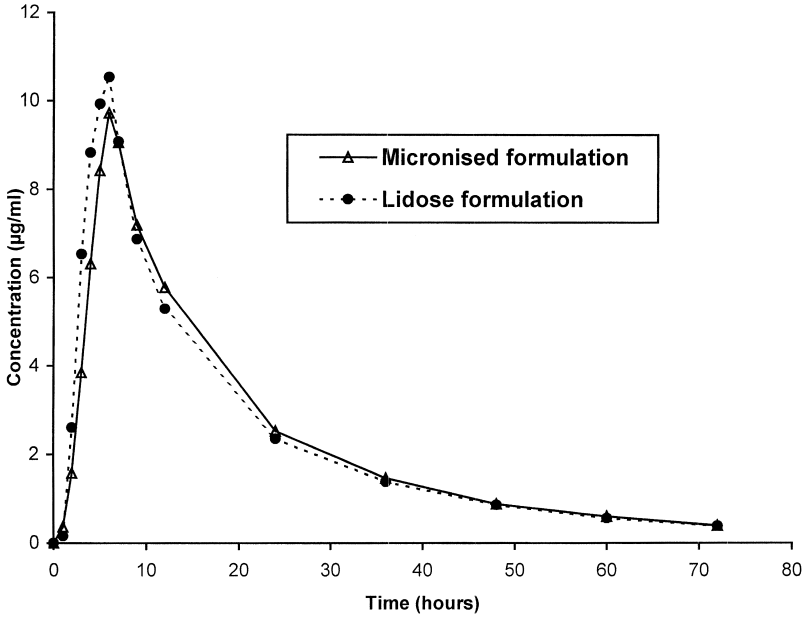


Fig. 4. Plasma concentration–time profiles of FEFA following a single oral dose of 200 mg fenofibrate. (▲) Reference; (●) Lidose™ formulation.

Table 6
Pharmacokinetic parameters

Parameters	Micronized formulation	Lidose™ formulation
$AUC_{0\rightarrow 72}$ ($\mu g\ ml^{-1}\ h$)	173.2±49.0	173.2±41.3
$AUC_{0\rightarrow \infty}$ ($\mu g\ ml^{-1}\ h$)	185.0±56.5	185.0±47.5
C_{max} ($\mu g\ ml^{-1}$)	10.4±2.8	11.3±2.3
T_{max} (h)	5.8±1.4	5.2±1.0

demonstrate the bioequivalence of the Lidose™ and the micronized formulation.

4. Conclusions

A fully automated, accurate and precise procedure based on a solid-phase extraction coupled on line with a LC–UV determination has been developed for the assay of FEFA. Regarding different LC methods previously reported [10,15,16], the procedure developed involves a fully automated sample preparation combined to LC analysis. Moreover, the sensitivity of this method is slightly higher since the determined LOQ is around 0.1 μg/ml. Recoveries of fenofibric acid are also close to 100%. The method was validated to meet the requirements of the pharmacokinetic investigation of this compound. The procedure developed has been successfully applied to the determination of FEFA plasma levels for investigating the bioequivalence of a new oral formulation of fenofibrate (Lidose™) and has been proved rugged.

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