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Determination of bezafibrate, ciprofibrate and fenofibric acid in human plasma by high-performance liquid chromatography

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Abstract

A selective high-performance liquid chromatographic method to assess either bezafibrate, ciprofibrate or fenofibric acid plasma levels is described. Drugs are extracted with diethyl ether, after acidification with HCl. An isocratic acetonitrile–0.02 M H_3PO_4 (55:45) mobile phase, a C_{18} (5 μm) column and UV detection are used. The LOQ found was 0.25 μg/ml for the three fibrates. Intra- and inter-assay accuracy ranges were 90–107% and 82–111%; 96–115% and 94–107%; 94–114% and 94–126% for bezafibrate, ciprofibrate and fenofibric acid, respectively. Intra- and inter-assay precision (C.V.% ranges) were 1.72–3.06% and 2.66–7.67%; 1.88–4.64% and 0.62–2.99%; 1.26–4.69% and 3.56–7.17% for the three fibrates studied. Its sensitivity, accuracy and precision make it a useful tool for monitoring plasma levels of these drugs in a clinical setting and for research purposes.

Keywords: Fibrate monitoring; Bezafibrate; Ciprofibrate; Fenofibric acid

1. Introduction

Hypertriglyceridemia is commonly seen in patients with chronic renal failure [1] and fibrates (Fig. 1) are useful drugs to treat this lipid disorder [2]. However, except for gemfibrozil [3], renal excretion of fibrates is impaired in renal insufficiency, resulting in accumulation of active drug and an increased risk of adverse effects [4–6]. It has recently been suggested that dyslipidemic renal patients might be treated with these kinds of drugs only if facilities to monitor blood concentrations were available [4–6]. Several HPLC methods to assess separately bezafibrate, ciprofibrate or fenofibric acid plasma levels have been reported [7–9]. We have developed a sensitive and selective HPLC method suitable to determine either bezafibrate, ciprofibrate or fenofibric acid plasma concentrations. This method might be of value for clinical and research applications.

2. Experimental

2.1. Chemicals

Chromatographic grade acetonitrile (Omnisolv, MCB Manufacturing Chemist, Cincinnati, OH, USA) and p.a. grade H_3PO_4 and diethyl ether (Merck, Buenos Aires, Argentina) were used. Chromatographic grade water (Nanopure deionization system, Barnstead, IA, USA) was used. Bezafibrate, ciprofibrate and fenofibric acid pure substance were supplied

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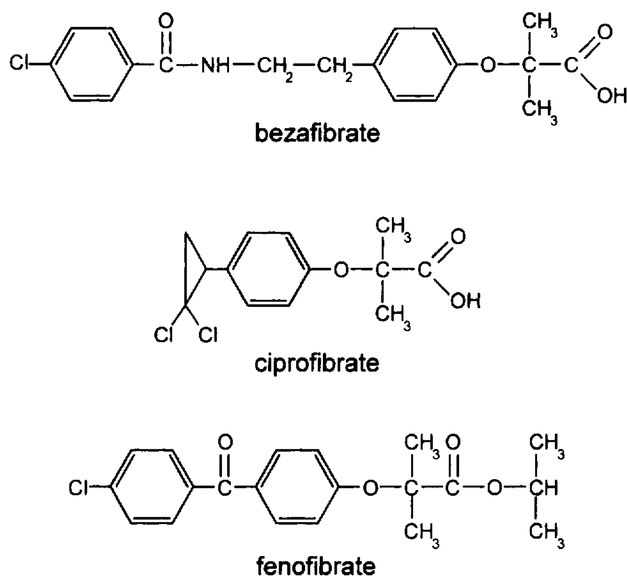

Fig. 1. Structures of bezafibrate, ciprofibrate and fenofibrate.

by ELEA S.A.C.I.F., Sanofi Wintrop and Ladco (Buenos Aires, Argentina), respectively.

2.2. Chromatography

Isocratic HPLC was performed using an automatic sample injector Model Sil-10A, a single plunger reciprocating pump Model LC-10 AS, a variable-wavelength UV detector Model SPD-10A focused at at 232 nm for bezafifrate and ciprofibrate and at 287 nm for fenofibric acid, and a computing integrator CR-4A, all from Shimadzu Corporation, (Tokyo, Japan).

2.3. Chromatographic conditions

A guard column Shimpack G-ODS (10×4 mm, 5 μm particle size) and a reversed-phase Shim Pack CLC-ODS RP-C18 (25 cm×4.6 mm, 5 μm particle size) from Shimadzu were used. The mobile phase was a mixture of acetonitrile and 0.02 M H_3PO_4 (55:45). Chromatography was performed at room temperature and using the following flow program:

Time (min)	Flow (ml/min)
0	2.0
6	2.0
8	2.5
17	Stop

After pumping for 6 min, in order to washout the column, the flow was increased to 2.5 ml/min and held at that rate till the end of each run (17 min). Before restart, the column was restabilized for 3 min. Therefore the total lack time before injecting a new sample was 20 min.

2.4. Sample preparation

Stock solutions of bezafibrate, ciprofibrate and fenofibric acid were prepared by dissolving 1 mg of drug in 1 ml of acetonitrile. Standard curves were prepared by supplementing 1 ml of control pool human plasma with 50 μl of suitable dilutions (in mobile phase) of stock solutions. Internal standards were prepared from stock solutions diluted with mobile phase to 100 μg/ml and 50 μl were included in each plasma sample (0.5 μg/ml final concentration). Bezafibrate was used as internal standard to assess ciprofibrate or fenofibric acid plasma levels. Ciprofibrate was used as internal standard when bezafibrate plasma levels were investigated.

Plasma samples (1 ml) were placed in 15-ml glass tubes and acidified with 1 ml of 1 M HCl. After vortex-mixing, the samples were extracted twice with diethyl ether (5 ml) by slow end-over-end rotation for 20 min. Centrifugation was performed at 1200 g for 15 min at 4°C and the organic phase was transferred to a clean tube. The ether phases were

combined and evaporated to dryness under a nitrogen flow at room temperature. The residue was dissolved in mobile phase (1 ml) by vortex-mixing, filtered through a 0.22-μm Nylon cartridge MSI and a 10-μl aliquot was injected into the chromatographic system.

After plotting the tested drug/internal standard area ratios (*TD/IS*) vs. standard concentrations, the data were fit using a least squares regresion. The values corresponding to unknown samples were obtained by interpolation.

3. Method validation

3.1. Recovery

A recovery test was carried out by comparing peak areas obtained from plasma samples added with suitable amounts of stock solution with the corresponding to non extracted standards: %recovery= [peak area (extracted sample)/(peak area (stock sample)]×100. Recovery tests for bezafibrate, ciprofibrate and fenofibric acid were made in quadruplicate at the following concentrations: 1, 5, 10 and 25 μg/ml.

3.2. Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) were defined by the statistical method according to Anderson [10]. Briefly, 10 blank (negative) plasma samples were tested and the mean blank value and the S.D. were calculated. The LOD was the mean blank value plus 3 S.D.. The LOQ was the mean blank value plus 10 S.D.. These LOQ values were reanalysed after performing accuracy and reproducibility tests.

3.3. Accuracy and precision

The accuracy and precision were investigated both intra- and inter-assay. For intra-assay, replicate analyses (n=5) at five different drug concentrations (0.5, 2.5, 5, 10 and 25) were performed. For inter-assay, quadruplicated analyses at four drug concentrations (1, 5, 10 and 25 μg/ml) were employed. The real found value of each fibrate was expressed as mean±standard deviation. Accuracy was defined as the range of percent differences between the mean±2 S.D. back-calculated concentrations and real standard values. Intra- and inter-assay reproducibility was expressed as the percent coefficient of variation of found concentrations: C.V.%=(S.D./ mean)×100.

4. Results

Typical chromatograms of fibrates are shown in Fig. 2(1)–(5). There was a complete resolution between peaks corresponding to the tested drug and the corresponding internal standard. No interfering peaks appeared, as seen either in blank plasma or in samples obtained after administration of each drug to healthy volunteers. The mean±S.D. retention times were 3.1±0.02, 4.9±0.07 and 5.5±0.02 min for bezafibrate, ciprofibrate and fenofibric acid, respectively. The separation factors (α) were 1.78 (for bezafibrate and ciprofibrate assay) and 2.04 (for fenofibric acid assay), giving this method a suitable selection capacity.

4.1. Sample stability

Samples obtained from plasma spiked with the three fibrates were stable for at least a week, stored at 4°C. Studies carried out with frozen plasma showed that up to six months, the samples were stable either at −20 or −70°C.

4.2. Plasma calibration curves

The calibration curves corresponding to each fibrate were linear (r =0.99, regression on means, by least squares analysis, of four samples at each concentration) from 0.25 to 50 μg/ml plasma. The corresponding mathematical expressions for bezafibrate (a), ciprofibrate (b) and fenofibric acid (c) are the following:

a: $y = 0.9391 + 3.0318x$

b: $y = 0.5858 + 1.6224x$

c: $y = 0.6565 + 2.3288x$

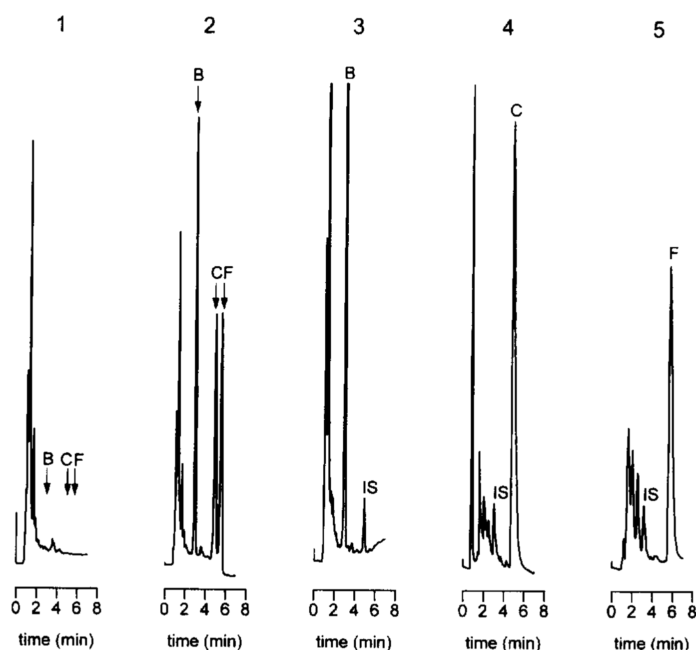

Fig. 2. Representative liquid chromatograms (see Section 2 for experimental conditions) of pool plasma samples containing 0 μg/ml (1) or spiked with 5 μg/ml each of bezafibrate (B), ciprofibrate (C) or fenofibric acid (F) (2). Extracted samples from healthy volunteers who took bezafibrate (400 mg, sustained release, p.o.), ciprofibrate (100 mg, p.o.) and fenofibrate (350 mg, p.o.) are also included: 5.0 μg/ml of B (3), 16.8 μg/ml of C (4) and 4.4 μg/ml of F (5).

4.3. Recovery

The mean and range values were ≥75% (75.3–89.6), ≥88% (88.6–101.9) and ≥79% (79.2–89.8) for bezafibrate, ciprofibrate and fenofibric acid, respectively (Table 1). Recovery of each drug did not depend on the initial amount in the analyzed samples.

4.4. Sensitivity

The calculated values of LOD were 0.10, 0.07 and 0.09 μg/ml (mean + 3 S.D.) for bezafibrate, ciprofibrate and fenofibric acid, respectively. Likewise, the found values of LOQ were 0.14, 0.14 and 0.15 μg/ml (mean + 10 S.D.). However, since the accuracy and reproducibility test for these LOQ values

Table 1
Fibrates recovery

Concentration (μg/ml)	Recovery (%)		
	Bezafibrate	Ciprofibrate	Fenofibric acid
1	75.32	89.42	89.76
5	83.53	101.92	80.12
10	89.63	88.63	87.18
25	83.64	88.54	79.21

Fibrate recoveries were evaluated by performing replicate analyses ($n=4$) of four different concentrations of bezafibrate, ciprofibrate or fenofibric acid.

Table 2
LOQ accuracy and reproducibility

Fibrate	Added fibrate (μg/ml)	Found fibrate (μg/ml)	Accuracy (%)	C.V. (%)
Bezafibrate	0.25	0.269±0.011	99–116	3.9
Ciprofibrate	0.25	0.233±0.012	83–103	5.3
Fenofibric acid	0.25	0.271±0.018	93–123	6.9

The accuracy and reproducibility of the fibrate LOQ measurements were evaluated by performing replicate analyses ($n=4$) at this concentration for bezafibrate, ciprofibrate or fenofibric acid.

were not satisfactory, 0.25 μg/ml was found as the nearest value to fulfill the LOQ requirements for the three fibrates (see Table 2).

4.5. Accuracy and reproducibility

The accuracy and precision obtained for intra- and inter-assay studies are shown in Table 3 and Table 4. The values obtained for the intra-assay and inter-assay accuracy were between 91% and 116% for bezafibrate, ciprofibrate and fenofibric acid. The C.V. values found for the reproducibility test for the three fibrates were below 5 and 8% for intra- and inter-assay, respectively.

Table 3
Intra-assay accuracy and reproducibility

Added (μg/ml)	Found (μg/ml)	Accuracy (%)	C.V. (%)
Bezafibrate			
0.5	0.42±0.01	80–89	2.54
2.5	2.49±0.04	96–103	1.72
5.0	5.06±0.15	95–107	2.93
10.0	9.72±0.22	93–102	2.32
25.0	24.73±0.76	93–105	3.06
Ciprofibrate			
0.5	0.50±0.01	98–105	1.74
2.5	2.64±0.10	97–115	4.15
5.0	5.03±0.10	97–104	1.88
10.0	10.61±0.49	96–116	4.64
25.0	25.00±0.53	96–104	2.10
Fenofibric acid			
0.5	0.52±0.02	94–114	1.42
2.5	2.60±0.01	103–105	1.26
5.0	5.42±0.08	105–112	1.64
10.0	10.16±0.15	99–104	1.43
25.0	26.13±1.22	95–114	4.69

The accuracy and intra-assay reproducibility of fibrate measurement were evaluated by performing replicate analyses ($n=5$) of five different concentrations of bezafibrate, ciprofibrate or fenofibric acid.

Table 4
Inter-assay accuracy and reproducibility

Added (μg/ml)	Found (μg/ml)	Accuracy (%)	C.V. (%)
Bezafibrate			
1.0	0.98±0.04	90–106	4.14
5.0	5.12±0.21	94–111	4.00
10.0	10.17±0.28	96–107	2.66
25.0	24.73±0.76	82–111	7.67
Ciprofibrate			
1.0	1.00±0.02	96–104	1.88
5.0	5.04±0.15	95–107	2.99
10.0	9.90±0.06	98–100	0.62
25.0	24.96±0.72	94–106	2.90
Fenofibric acid			
1.0	1.09±0.06	97–121	5.51
5.0	5.52±0.40	94–126	7.17
10.0	9.86±0.37	91–106	3.76
25.0	25.78±0.92	96–110	3.56

The accuracy and inter-assay reproducibility of fibrate measurement were evaluated by performing replicate analyses ($n=4$) of four different concentrations of bezafibrate, ciprofibrate or fenofibric acid.

5. Conclusion

The HPLC method described here provides sensitive, accurate, reproducible and relatively fast determinations of bezafibrate, ciprofibrate or fenofibric acid plasma levels through a single procedure. We consider that these features make it not only a useful tool for the management of dyslipidemia in unique populations but also a suitable instrument for research purposes.

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