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DETERMINATION OF BEZAFIBRATE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective and time-saving high-performance liquid chromatographic method to assess bezafibrate plasma and urine levels is described. Bezafibrate is extracted from plasma matrix using diethyl ether, after acidification with hydrochloric acid. The urine samples are directly analysed, after dilution with the mobile phase. The method is used to assess bezafibrate plasma and urine levels in man, after administration of therapeutic doses of bezafibrate. The results obtained are in agreement with previously published data.

INTRODUCTION

Bezafibrate, 2-{*p*-[2-(*p*-chlorobenzamido)ethyl] phenoxy}-2-methylpropionic acid (Fig. 1), is a lipid-lowering agent used in the treatment of patients with anomalies in lipid metabolism, characterized by very high cholesterol and triglyceride plasma levels.

A rapid, sensitive and selective method is needed to assess bezafibrate plasma and urine levels. Although a gas chromatographic method has already been reported in the literature [1], a high-performance liquid chromatographic (HPLC) procedure was developed. This is a time-saving method because there is no need for a derivatization step. The assay was applied to the measurement of bezafibrate in plasma and urine from human volunteers after oral administration of 400 mg of the drug.

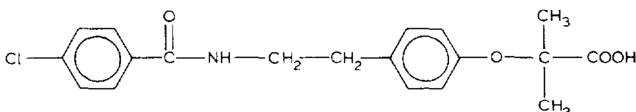

Fig. 1. Structural formula of bezafibrate.

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EXPERIMENTAL

Chemicals

Potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid, methanol for HPLC (Carlo Erba, Milan, Italy) and diethyl ether (Merck, Darmstadt, F.R.G.) were used. All reagents were of analytical grade. Bezafibrate and clofibric acid were supplied by Boehringer (Mannheim, F.R.G.).

Chromatography

An isocratic HPLC system was used, consisting of a Model 6000A pump and a variable-wavelength spectrophotometer Model 450 (Waters Assoc., Milford, MA, U.S.A.) focused at 230 nm. The samples were injected through an automatic sampler WISP 710B (Waters Assoc.). A Model SP-4270 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.) was used.

Chromatographic conditions

A reversed-phase LiChrospher RP-18 column (30 cm × 4.6 mm I.D.; 10 μm particle size) from Merck was used. The mobile phase was 0.01 *M* phosphate buffer (pH 3.5)—methanol (40:60). The chromatography was carried out at a flow rate of 1.0 ml/min at room temperature (ca. 25°C).

Solution preparation

A bezafibrate concentrated standard solution (1 mg/ml) was prepared by dissolving 10 mg of bezafibrate in 10 ml of methanol. The internal standard stock solution was prepared by dissolving clofibric acid and diluting with methanol to 1 μg/10 μl for the plasma assay.

Sample preparation

Plasma. Bezafibrate was extracted from 1 ml plasma samples in 1 μg of internal standard solution (1 μg/10 μl). After acidification with 10 μl of concentrated hydrochloric acid, the samples were extracted with 8 ml of diethyl ether. After centrifugation (1500 *g* for 10 min), the organic layer was dried under a nitrogen flow at room temperature. The residue was redissolved with 2 ml of the mobile phase, and quantitatively transferred into the vials of the automatic sampler. Aliquots of 100 μl were injected into the chromatograph.

Urine. Aliquots of 0.25 ml of the internal standard solution (1 mg/ml) were added to 1.0-ml urine samples and diluted with 1.75 ml of mobile phase. Following a brief centrifugation (1500 *g* for 10 min), the supernatant was transferred to a glass vial and capped. Volumes of 5 μl were injected into the chromatograph through an automatic sampler WISP 710B. The chromatographic assay was conducted as already described.

RESULTS

Fig. 2 shows typical chromatograms of plasma and urine samples. Interfering peaks do not appear on the chromatograms after the injection of samples corresponding to plasma and urine from untreated subjects. Complete resolution is observed between the peaks of bezafibrate and the internal standard.

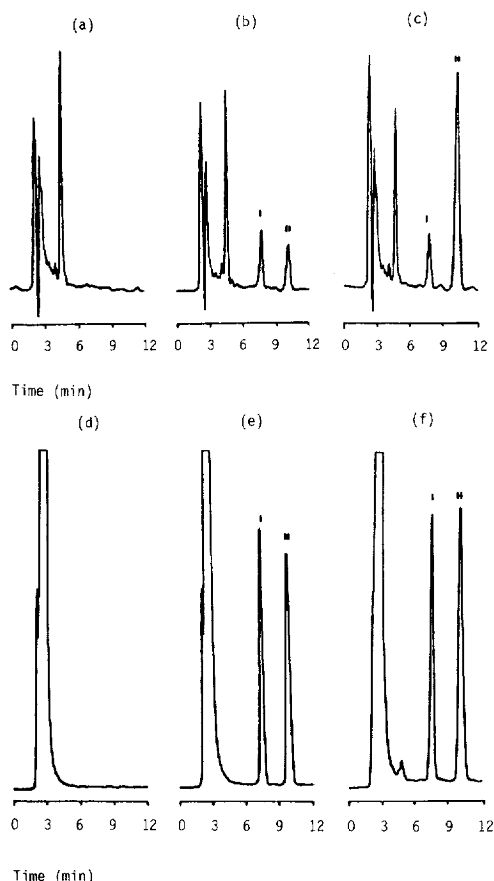

Fig. 2. Chromatograms of human plasma extracts and human urines. Human plasma extracts: (a) specimen from untreated subject; (b) normal plasma, spiked with bezafibrate (II, 1 μg/ml) and clofibric acid (I, 1 μg/ml); (c) specimen from a subject treated with bezafibrate, spiked with clofibric acid (see text). Clofibric acid 1 μg/ml, bezafibrate found 4.608 μg/ml. Human urines: (d) specimen from untreated subject; (e) normal urine, spiked with bezafibrate (II, 500 μg/ml) and clofibric acid (I, 250 μg/ml); (f) specimen from a subject treated with bezafibrate, spiked with clofibric acid (see text). Clofibric acid 250 μg/ml, bezafibrate found 569 μg/ml.

Sample stability

Samples obtained from plasma and urine as described above were stable for at least a week, stored at room temperature.

Plasma calibration curve

The calibration curve was obtained by assaying samples of plasma and urine in an appropriate amount of bezafibrate and internal standard stock solution (internal standard: 1 μg/ml for each sample; bezafibrate: 0.25, 0.50, 1.00, 2.50, 5.00 and 10.00 μg/ml). Table I shows the ratios between the peak areas of bezafibrate and the internal standard plotted against bezafibrate concentrations. The parameters for the regression curve are also reported.

TABLE I

BEZAFIBRATE CALIBRATION CURVE FOR THE PLASMA ASSAY

The internal standard concentration was 1 μg/ml. The regression curve, corresponding to the experimental results, was constructed by weighted linear regression fitting [4]. The mathematical expression of the curve is $y = 0.0397 + 0.9542x$.

Number of analyses	Concentration of added bezafibrate (μg/ml)	Mean ratio bezafibrate area/ internal standard area	Coefficient of variation* (%)
4	0.25	0.258	4.7
6	0.50	0.609	5.8
6	1.0	0.965	4.5
6	2.5	2.353	3.4
4	5.0	4.785	3.2
5	10.0	9.175	2.5

*Coefficient of variation = $\frac{\text{S.D.}}{\text{Mean}} \times 100$.

TABLE II

BEZAFIBRATE CALIBRATION CURVE FOR THE URINE ASSAY

The internal standard concentration was 250 μg/ml. The regression curve, corresponding to the experimental results, was constructed by linear regression fitting. The mathematical expression of the curve is $y = 0.0302 + 0.0024x$. A correlation coefficient (r) of 0.9996 was obtained. n=5.

Concentration of added bezafibrate (μg/ml)	Mean area ratio bezafibrate/internal standard	Coefficient of variation (%)
50	0.118	3.4
100	0.208	2.0
250	0.521	2.1
500	1.172	1.5
1000	2.356	1.9

Urine calibration curve

The calibration curve for urine (Table II) was obtained similarly to that for plasma. Since urine levels of the drug were higher than plasma levels, 250 μg/ml internal standard and 50 μg/ml, 100 μg/ml, 250 μg/ml, 500 μg/ml and 1 mg/ml bezafibrate were used. The detector response was found to be linear in all the ranges investigated.

Sensitivity

The minimum bezafibrate concentration detectable by this method was 50 ng/ml for plasma and 200 ng/ml for urine.

Recovery

The extraction recovery of bezafibrate was determined by a modified extraction procedure in which the internal standard was added after the extraction was completed. The extraction recovery check from human plasma was made in duplicate at six different concentrations: 0.25, 0.50, 1.00, 2.50, 5.00 and 10.00 μg/ml. Bezafibrate was quantitatively extracted (98.3%) from biological fluids. Bezafibrate recovery did not depend on its initial amount in the analysed specimens.

Accuracy and precision

The accuracy of this method was checked by taking five analyses of each of the five bezafibrate concentration levels (0.3, 1.0, 2.5, 5.0 and 10.0 μg/ml) through the entire method. The same procedure was carried out for the urine samples. In this case, the concentrations used were 100, 250 and 500 μg/ml.

Tables III and IV give the results.

Bezafibrate human plasma and urine levels

Doses of 400 mg of bezafibrate (two Bezalip® tablets) were given to four volunteers. Serial samples of blood and urine were collected during the 48 h period after administration. The samples were frozen after collection and stored at −20°C up to the time of the analysis. Plasma and urine levels were then evaluated, and Tables V and VI give the data.

TABLE III

ACCURACY AND PRECISION OF BEZAFIBRATE ANALYSIS IN HUMAN PLASMA

$n = 5$.

Concentration of added bezafibrate (μg/ml)	Concentration of found bezafibrate (μg/ml)	Standard error	Coefficient of variation (%)
0.30	0.331	0.011	7.4
1.00	0.976	0.023	5.3
2.50	2.533	0.054	4.8
5.00	4.902	0.109	5.0
10.00	10.218	0.208	4.5

TABLE IV

ACCURACY AND PRECISION OF BEZAFIBRATE ANALYSIS IN HUMAN URINE

$n = 5$.

Concentration of added bezafibrate (μg/ml)	Concentration of found bezafibrate (μg/ml)	Standard error	Coefficient of variation (%)
100	103.8	1.46	3.1
250	256.8	2.08	1.8
500	498.0	4.63	2.1

TABLE V

BEZAFIBRATE PLASMA LEVELS FOLLOWING A SINGLE ORAL DOSE OF 400 mg (TWO BEZALIP® TABLETS)

Values are given in µg/ml.

Subject No.	Time of collection (h)								
	0	1	2	3	4	5	8	12	24
1	0	1.973	12.668	11.990	9.285	4.853	1.511	0.314	0
2	0	3.636	12.289	10.297	8.101	3.478	2.013	0.231	0
3	0	11.368	8.046	4.434	3.202	1.492	0.657	0.302	0
4	0	13.180	8.680	5.280	3.450	1.550	0.690	0.210	0
$\bar{X}$	0	7.539	10.421	8.000	6.009	2.843	1.218	0.264	0
S.E.	–	2.779	1.198	1.855	1.569	0.813	0.331	0.026	–

TABLE VI

BEZAFIBRATE URINARY EXCRETION FOLLOWING A SINGLE ORAL DOSE OF 400 mg (TWO BEZALIP® TABLETS)

Values are given in mg.

Subject No.	Intervals of collection (h)						
	0–2	2–4	4–6	6–8	8–10	10–12	12–24	24–48
1	12.17	7.94	53.85	39.40	12.46	6.64	7.23	0.03
2	–	75.50	100.02	21.36	20.21	14.15	4.09	0.34
3	6.93	90.09	75.00	12.21	2.16	1.02	3.00	0.55
4	112.57	86.60	29.41	10.65	8.35	4.56	7.32	0.85
$\bar{X}$	43.890	64.28	64.57	20.90	10.79	6.59	5.41	0.44
S.E.	34.37	19.16	15.05	6.60	3.78	2.77	1.10	0.17

The maximum concentration of bezafibrate in plasma (10.421 µg/ml) was reached within 2 h; the unchanged drug elimination was relatively fast and the mean half-life was 1.86 h. About 50% of the bezafibrate dose (400 mg) was recovered in the urine within 48 h.

CONCLUSION

Our results agree with previously published data [2, 3] and confirm the possibility of using this analytical method to evaluate bezafibrate plasma and urine levels after administration of therapeutic doses of the drug. Its characteristics of sensitivity, accuracy and precision are an advantage in its use. The high speed of analysis enables the examination of seventy urine samples or forty blood samples within 24 h.

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