Simultaneous GLC Determination of Clofibrate and Clofibric Acid in Human Plasma

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Abstract □ A simultaneous assay for the detection of clofibrate and its metabolite, clofibric acid [2-(*p*-chlorophenoxy)-2-methylpropionic acid], is described. This GLC method is rapid and does not require a derivatization step. It is sensitive to 1-μg/ml levels of either compound in biological samples and can be used to characterize the *in vivo* conversion of clofibrate ester to the free acid.

Keyphrases □ Clofibrate—simultaneous GLC assay with clofibric acid, human plasma □ Clofibric acid—simultaneous GLC assay with clofibrate, human plasma □ Hypolipemic agents—clofibrate, simultaneous GLC assay with clofibric acid, human plasma

Clofibrate, an oral hypolipemic agent, is administered traditionally as the intact ester and assayed as the free acid, clofibric acid [2-(*p*-chlorophenoxy)-2-methylpropionic acid]. In the past decade, considerable attention has been devoted to the development of assays for these compounds (1–12). These methods have been used to generate pharmacokinetic data for clofibric acid.

Clofibric acid assays include spectrophotometric (1), radiometric (2), GLC (3–8), and high-pressure liquid chromatographic (9) techniques, all having approximately the same sensitivity. Clofibrate can be assayed easily by both spectrophotometric and GLC (1) methods, but clofibric acid determinations usually are tedious and time consuming, involving numerous extraction and evaporation steps. A major obstacle in the development of a GLC method for the simultaneous determination of clofibrate and clofibric acid has been the lack of a liquid phase sufficiently polar to yield reproducible clofibric acid peaks without derivatization. A flash derivatization would be ideal for the simultaneous determination, but the heat required would degrade the parent ester.

This paper describes a simultaneous assay method for clofibrate and clofibric acid that is rapid and does not require a derivatization step. It is sensitive to 1-μg/ml levels of either compound in biological samples and can be used to characterize the conversion of clofibrate to clofibric acid.

EXPERIMENTAL

Reagents—Fresh reagent grade ether was used without prior distillation. Purified granular ammonium sulfate was used in the salting-out procedure, and a 50% (w/v) NaF solution was used for plasma esterase deactivation. Commercially available benzocaine was recrystallized and used as the internal standard.

Clofibric acid was prepared by alkaline hydrolysis of clofibrate[1]. Clofibrate, 10 g, was combined with 80 ml of 30% KOH and 40 ml of alcohol USP, and the solution was refluxed for 4 hr as described previously (10). The hydrolysate was cooled to room temperature, extracted with 20 ml of ether to remove any remaining clofibrate, and acidified with 5% H_2SO_4 until litmus paper showed it to be strongly acidic. The acidified solution was extracted with ether (3 × 15 ml), and the extracts were combined and evaporated to dryness. The resulting crystalline residue was recrystallized with hot methanol, yielding ~70%, mp 118–118.5°. Qualitative confirmation was obtained by mass spectrometry[2] and NMR spectroscopy[3].

Sample Preparation—Human plasma, 1 ml, spiked with clofibrate and clofibric acid was added to a 10-ml test tube containing 20 μl of sodium fluoride solution and 550 mg of ammonium sulfate. The mixture was agitated for 30 sec with a vortex mixer[4] and then centrifuged for 10 min at 3500 rpm. The supernate (0.5 ml) was transferred to a fresh test tube, and 100 μg of benzocaine was added. The resulting solution was extracted with 1 ml of ether by agitating for 3 min. The sample tubes were sealed[5] and then centrifuged at 3500 rpm for 30 min to separate the phases. After centrifugation, the ether layer was withdrawn, and 1 μl was injected into the gas chromatograph.

GLC Conditions—The gas chromatograph[6] was equipped with a hydrogen flame-ionization detector. The glass column (1 m × 2 mm i.d.) was packed with 100–120-mesh Gas Chrom Q coated with 10% Silar 10 C[7]. Nitrogen was used as the carrier gas at a 24-ml/min flow rate. The oxygen and hydrogen flow rates were 240 and 40 ml/min, respectively; the injection port, oven, and detector temperatures were 200, 220, and 250°, respectively.

Hydrolysis—A 250-ml three-necked flask was placed in a heating mantle supported on a magnetic stirrer. Two necks were sealed, and a thermometer was placed in the third neck. The temperature control was calibrated and maintained at 37°. Clofibrate, 5 mg, was added within the reaction vessel to a pH 10.5 buffer system consisting of 0.1 *N* Na_2CO_3 and 30 ml of 0.1 *N* $NaHCO_3$. Samples were withdrawn and assayed by the simultaneous method.

RESULTS

Clofibric Acid Identification—The free acid of clofibrate, clofibric acid, when subjected to chemical-ionization mass spectrometry, provided a M + H ion at *m/e* 216. This mass is consistent with the formula of clofibric acid and is supported further by abundant ions at *m/e* 169 (equivalent to the loss of –COOH from the protonated compound) and at *m/e* 128 (corresponding to *p*-C_6H_4ClOH). The structure was confirmed further by other small peaks corresponding to the expected mass fragments.

Further confirmation was provided by an independent NMR spectrum. NMR demonstrated the presence of an aromatic ring (δ 6.75–7.3), –COOH (δ 10.1), and $-C(CH_3)_2-$ (δ 1.55), consistent with the clofibric acid structure. Chloroform was the solvent.

Assay—Under the described GLC conditions, clofibrate and clofibric acid eluted with retention times of 1.8 and 2.5 min, respectively (Fig. 1). Blank plasma samples did not produce any interfering peaks with retention times similar to either clofibrate or clofibric acid.

The standard curves of peak height ratio *versus* concentration in the 10–200-μg/ml range were linear for both compounds. The least-squares estimate for the line fitting a plot of the mean of the peak height ratios obtained on different days *versus* the clofibric acid concentration ($n = 7$, $r = 0.999$) was $y = (6.17 \times 10^{-3})x + 0.024$. For clofibrate ($n = 9$, $r = 0.999$), the regression line was $y = (3.04 \times 10^{-2})x - 0.012$. The standard errors of the estimate were 1.53×10^{-2} and 1.02×10^{-1}, respectively. There was a systematic reduction in all peak height ratios over 1 month, but the change was not statistically significant.

While the assay as outlined has a lower sensitivity limit of ~10 μg/ml for both compounds, the sensitivity can be increased 10-fold to ~1 μg/ml by evaporating the solvent after extraction and redissolving the residue in 100 μl of ether.

[1] Provided by Ayerst Laboratories, New York, N.Y.

[2] Hewlett-Packard model 5930 A.
[3] Varian model 60.
[4] Vortex-Genie, Scientific Industries, Springfield, Mass.
[5] Parafilm, American Can Co., Neenah, Wis.
[6] Hewlett-Packard model 5710 A.
[7] Applied Science Laboratories, State College, Pa.

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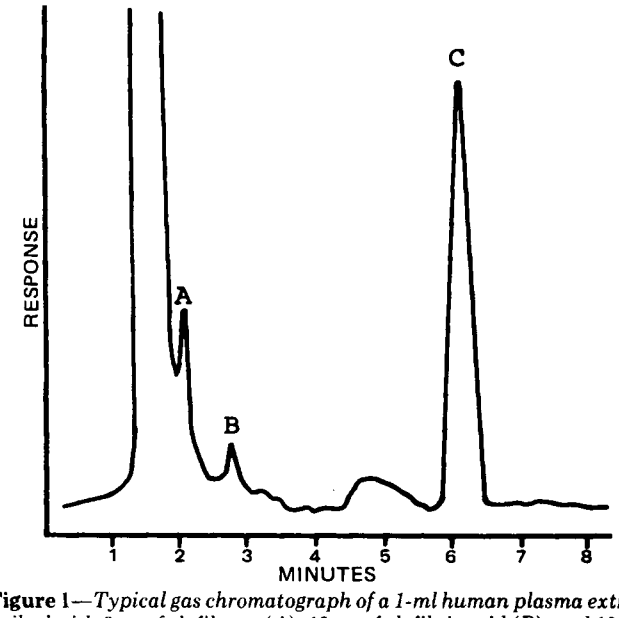

Figure 1—*Typical gas chromatograph of a 1-ml human plasma extract spiked with 6 μg of clofibrate (A), 10 μg of clofibric acid (B), and 100 μg of benzocaine (C).*

Recovery—To achieve maximum recovery of both the ester and the acid, a salting-out technique was utilized. The technique was based on a literature method (11) in which the efficacy of ammonium chloride as an agent for salting drugs out of plasma was examined. A later study (12) showed the effectiveness of ammonium sulfate for the same purpose. A standard mixture of 200 μg of clofibrate and 50 μg of benzocaine was used to study this technique for clofibrate recovery from aqueous solution and plasma. A peak height ratio of 12 was obtained from 1 ml of an aqueous solution of this mixture. For the plasma determination, varying amounts of ammonium sulfate were used to determine the amount needed to achieve the same peak height ratio. Amounts ranged from 300 (ratio of 9.45) to 900 (ratio of 6.65) mg/ml, with the maximum peak height ratio of 11.95 obtained using 550 mg of ammonium sulfate/ml of plasma.

Table I—Recovery of Clofibrate and Clofibric Acid after Alkaline Hydrolysis at 37° and pH 10.51 [a]

Minutes	Clofibrate, μmole/ml	Clofibric Acid, μmole/ml	Recovery, μmole/ml	Percent of Initial Concentration
10	0.190	0.019[b]	0.190	101
20	0.161	0.051	0.212	102
30	0.149	0.060	0.209	101
45	0.145	0.069	0.214	103
60	0.128	0.079	0.207	100
90	0.099	0.102	0.201	97
120	0.074	0.133	0.207	100

[a] Initial clofibrate concentration was 0.207 μmole/ml. Constant pH was maintained with a carbonate buffer. [b] This value was close to the assay sensitivity limit and could not be measured as accurately as other values.

Hydrolysis—The accuracy of the simultaneous assay was tested by following the disappearance of clofibrate and the appearance of clofibric acid during alkaline hydrolysis of the ester at pH 10.51 and 37°. There was excellent recovery at each period, indicating that the assay accurately measured the ester and the hydrolysis product simultaneously (Table I). Degradation of the ester followed pseudo-first-order kinetics over the 2-hr run, yielding a least-squares estimate for the degradation rate constant of 7.98×10^{-3} min^{-1}.

DISCUSSION

The described GLC method has several advantages over previously described assays. It is fast and specific. About 10 samples can be determined in 1 hr by one person. Its specificity is of particular interest because the disappearance of the parent ester and the appearance of the acid metabolite can be followed simultaneously, facilitating the determination of their respective pharmacokinetic parameters. The method does not require derivatization, which can introduce an additional variable.

The method sensitivity may be increased severalfold by evaporating the solvent and redissolving the residue in an appropriate amount of solvent. Column degradation by the free acid cannot be avoided. Under heavy use and with an overnight oven temperature of 200° or greater, column repacking may be necessary every 6–8 weeks.

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