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Short communication

High-performance liquid chromatographic assay for the simultaneous determination of ethyl clofibrate and clofibric acid in plasma
Evaluation of plasma stability of ethyl clofibrate polylactic nanocapsules in human and rat plasmas

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Abstract

A rapid, sensitive, and selective HPLC assay was developed for the simultaneous determination of ethyl clofibrate and its major metabolite, clofibric acid, in plasma of humans and rats. The assay involves extraction of the compounds into chloroform–isoamyl alcohol (99:1, v/v) from plasma acidified with sulfuric acid. For human plasma, the overall recoveries of ethyl clofibrate and clofibric acid were 63 and 90%, respectively. The limits of detection of the assay for ethyl clofibrate and clofibric acid in human plasma were 1.1 and 1.5 μg/ml, respectively. Limits of quantitation of the assay for ethyl clofibrate and clofibric acid in human plasma were 3.6 and 4.9 μg/ml, respectively. The HPLC assay was used to monitor the plasma concentration–time profiles of ethyl clofibrate released from polylactic nanocapsules both in man and rat. The simultaneous determination of ethyl clofibrate and clofibric acid provided evidence that these colloidal systems are stable in human plasma whereas they are lysed in rat plasma.

1. Introduction

Ethyl clofibrate, also called ethyl chlorophenoxyisobutyrate, is a well-known antihyperlipidemic drug. Many chromatographic assays have been developed to determine clofibric acid (the ethyl clofibrate metabolite) concentration in plasma [1–5]. The HPLC method described here allows the simultaneous determination of the plasma concentrations of ethyl clofibrate and clofibric acid. The assay was used to evaluate the plasma stability of ethyl clofibrate polylactic nanocapsules in human and rat plasmas.

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2. Experimental

2.1. Reagents

Ethyl clofibrate was obtained from Pharmacie Centrale des Hôpitaux de Paris (Paris, France, lot no. 24118/1 to 5). 4-Dimethylaminobenzaldehyde (pDAB, reference standard; lot no. 437G239358) and acetonitrile (lot no. 14291) were supplied by Merck (Darmstadt, Germany). 2-Chlorophenoxy-2-methylpropionic acid was obtained from Sigma (Saint-Quentin-Fallavier, France; lot no. 106F3435). Acetic acid was RP Normapur from Prolabo (Paris, France; lot no. 89197). Isoamyl alcohol (lot no. 1485F100) and chloroform (lot no. 438601) were from Carlo Erba (Milan, Italy). Sulfuric acid was from UCB, Grauwmeer (Leuven, Belgium; lot no. 14601099). Newly outdated human plasma came from a blood bank whereas rat plasma was obtained from sacrifice of Whistar rats.

2.2. Instrumental parameters

A Perkin-Elmer 551S UV-Vis spectrophotometric detector (Perkin-Elmer, Bodenseewerk Ueberlingen, Germany) was used to determine the ultraviolet absorption spectra of ethyl clofibrate and clofibric acid in methanol from 200 to 300 nm. Spectra of ethyl clofibrate and clofibric acid exhibit maximum absorption peaks at 223 and 224 nm, respectively. The wavelength selected was 223 nm.

A Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), set at a flow-rate of 2.5 ml/min, was connected to a Lichrocart 125-4 Lichrospher 100 RP18 analytical column 12.5 cm × 4 mm I.D., 5 μm particle size (Merck). The mobile phase was acetonitrile–acetic acid–water (48:0.5:51.5, v/v/v). The eluate was monitored with a Shimadzu SPD-6A detector at 223 nm. This mobile phase was thoroughly degassed by ultrasound. All chromatograms were recorded on a Shimadzu C-R5A Chromatopac integrator at a chart speed of 5 mm/min.

2.3. Calibration of ethyl clofibrate and clofibric acid

The peak-area ratios of ethyl clofibrate (or clofibric acid) to the reference standard were plotted versus the ethyl clofibrate (or clofibric acid) concentration in the range 5–100 μg/ml. Calibration curves were prepared on each day of analysis to establish the linearity and reproducibility of the HPLC system.

2.4. Sample preparation

Preparation of plasma standards

To 20 ml of an ethanol stock solution of ethyl clofibrate (2.14 g/l) in a 50-ml volumetric flask 2.340 ml of an aqueous stock solution of clofibric acid (0.25 g/l) were added. This mixture was diluted to 50 ml with human plasma to yield a working solution containing 100 μg/ml of clofibric acid and ethyl clofibrate. Aliquots of the working solution were transferred to separate volumetric flasks. The aliquots were diluted with human plasma to yield mixed standard solutions containing the reference standard and each compound (ethyl clofibrate and clofibric acid) in the following concentrations: 5, 20, 40, 60 and 100 μg/ml. Native plasma was used as blank. Aliquots of each standard were stored frozen.

A 500-μl aliquot of a stock solution of pDAB (reference standard) in acetonitrile (1.25 g/l) was diluted to 50 ml with acetonitrile to yield a working solution containing 12.5 μg/ml of pDAB.

Analysis of human and rat plasma

To 200 μl of plasma in a 15-ml centrifuge tube, were added 50 μl of sulfuric acid (0.25 mol/l), 150 μl of the working reference standard solution in acetonitrile (12.5 μg/ml) and 1 ml of extraction solution (chloroform–isoamyl alcohol, 99:1, v/v). The mixture was vortex-mixed for 30 s on a Vortex action mixer (Bioblock, Strasbourg, France). Along with the samples the six plasma standards were run. The samples were centrifuged at 3600 *g* for 5 min. The aqueous solution and protein slice were discarded. The

organic layer was evaporated to dryness under a stream of clean nitrogen. The residue was redissolved in 100 μl of the mobile phase and vortex-mixed for 30 s. After centrifugation for 5 min at 3600 g, a 20-μl aliquot was injected onto the HPLC column. Representative chromatograms of human and rat blank plasmas, and of human plasma containing 200 μg/ml of ethyl clofibrate and clofibric acid processed by the extraction procedure are shown in Fig. 1.

2.5. Application: stability of ethyl clofibrate polylactic nanocapsules in rat and human plasmas

Polylactic acid nanocapsules of ethyl clofibrate were prepared using an interfacial polymer-deposition solvent-displacement technique as described by Fessi et al. [6]. The drug concentration in the final suspension was 25.9 mg/ml. The particle-size distribution of the resultant nanocapsules (155 ± 27 nm) was measured by light scattering using a monochromatic laser diffusion counter (SuperNanosizer N4, Coultronics, Margency, France).

An identical volume of the nanocapsule suspension was added to 10 ml of human or rat plasma maintained at 37°C. At 1, 1.5, 2, 3, 4, 6, 8, 10 and 15 min, 200-μl samples were taken and assayed with the HPLC assay described above. The use of acetonitrile as solvent for the reference standard results in the immediate precipitation of the plasma proteins and thus stops all enzymatic reactions. It also releases the ethyl clofibrate from the polylactic nanocapsules.

3. Results and discussion

3.1. HPLC assay

Using the HPLC assay described above, blanks of human and rat plasma samples did not show peaks interfering (Fig. 1A) with the peaks of ethyl clofibrate, clofibric acid or 4-dimethylaminobenzaldehyde (reference standard). A representative chromatogram of a human plasma extract is given in Fig. 1B. The retention times of the reference standard, clofibric acid

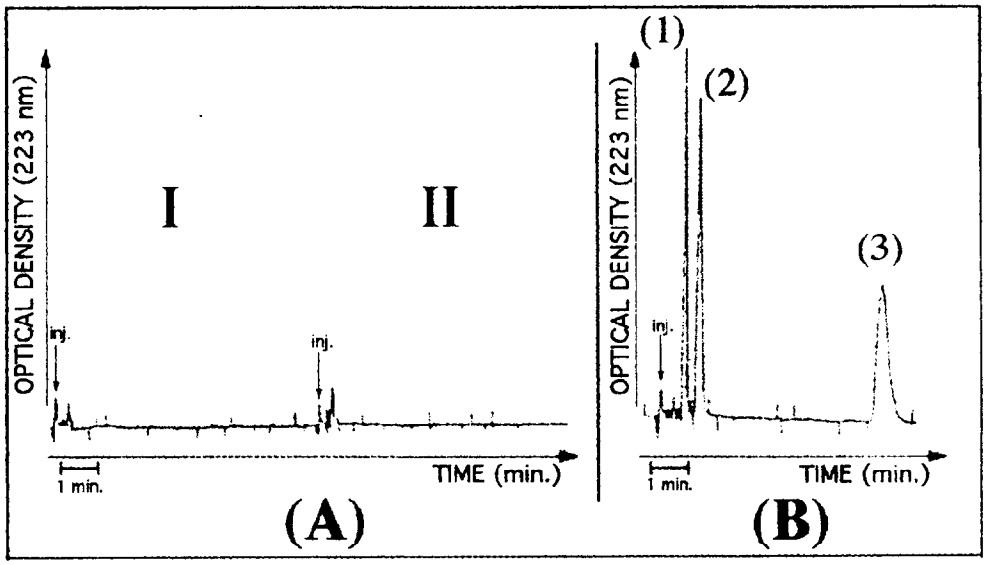

Fig. 1. Chromatograms of (A) human (I) and rat (II) blank plasmas, and (B) human plasma containing 200 μg/ml of ethyl clofibrate and clofibric acid processed by the extraction procedure. Peaks: 1 = 4-dimethylaminobenzaldehyde, 2 = clofibric acid, 3 = ethyl clofibrate.

and ethyl clofibrate were 1, 1.6 and 7 min, respectively.

The response of the assay was linear for ethyl clofibrate and clofibric acid plasma concentrations from 0 to 100 μg/ml (clofibric acid: slope: 0.0197 ± 0.0001, intercept: 0.0001 ± 0.0179, r^2 = 0.9993; ethyl clofibrate: slope: 0.0038 ± 0.0001, intercept: −0.0004 ± 0.0218, r^2 = 0.9765). Inter- and within-day coefficients of variation were determined using 60 μg/ml concentrations of the analytes. Results are the mean of five determinations. Inter-day coefficients of variation for clofibric acid and ethyl clofibrate were 2.2 and 5.9% respectively, whereas the within-day coefficients of variation were 5.7 and 7.8%, respectively. Limits of detection and quantitation were calculated from the standard deviation of the intercept and average of the slope from five calibration curves according to Caporal-Gautier et al. [7]. The limits of detection for ethyl clofibrate and clofibric acid in human plasma were 1.1 and 1.5 μg/ml, respectively. The limits of quantitation for ethyl clofibrate and clofibric acid in human plasma were 3.6 and 4.9 μg/ml, respectively.

Several chromatographic assays were developed for the determination of clofibric acid (ethyl clofibrate metabolite) concentration in plasma [1–4]. However, only a few HPLC assays have been described for the simultaneous determination of ethyl clofibrate and clofibric acid in plasma [5]. The reference standard used by Garrett and Gardner [5] was morphine sulfate, a compound structurally totally different from both analytes. We first selected the mobile phase described by Bjornsson et al. [2] (acetonitrile–acetic acid–water, 42:0.5:57.5, v/v). This mobile phase was not buffered and suppressed clofibric acid ionization. 4-Chloro-2-methylphenoxy acetic acid (CMPA) was chosen as the reference standard. With the mobile phase flow-rate set at 1.6 ml/min, the retention times of clofibric acid and ethyl clofibrate were 4 and 19 min, respectively. The retention time of the reference standard (CMPA) was 6 min. Due to its long retention time the ethyl clofibrate peak obtained was broad. To reduce the retention time of this compound, it was necessary to increase the acetonitrile concentration in the mobile phase up to 48% and increase the flow-rate to 2.5 ml/min. Since under these new conditions the clofibric acid and CMPA peaks merged, a new reference standard was selected. 4-Dimethylaminobenzaldehyde appeared more convenient to use.

To obtain the best extraction ratio for the two drugs studied (clofibric acid, ethyl clofibrate) from human plasma, several solvents were tested. An increase in the extraction ratio of clofibric acid was obtained when chloroform was used instead of toluene or dichloromethane. When sulfuric acid concentrations higher than 6–7% (v/v) in plasma were used, the extraction ratio of clofibric acid did not show any further increase. To increase the extraction ratio of ethyl clofibrate, it was necessary to add a more polar compound to the chloroform. The impact of various concentrations of isoamyl alcohol on the extraction ratio of clofibric acid and ethyl clofibrate was evaluated. The results are presented Fig. 2. Using a 1% (v/v) concentration of isoamyl alcohol in chloroform, the overall recoveries of clofibric acid and ethyl clofibrate from human plasma were maximal, 90% and 63%, respectively. In rat plasma, the recoveries of the reference standard and clofibric acid were the same as in human plasma, whereas ethyl clofibrate was rapidly transformed to clofibric acid when added to rat plasma.

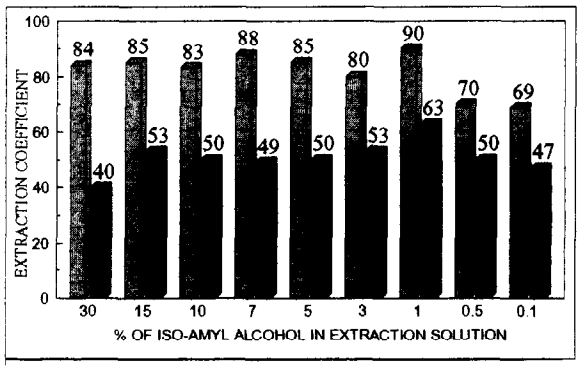

Fig. 2. Influence of iso-amyl alcohol on the extraction coefficients of (shaded) clofibric acid, and (black) ethyl clofibrate.

3.2. Application: stability of ethyl clofibrate polylactic nanocapsules in rat and human plasmas

Since the HPLC assay described above allowed the simultaneous determination of ethyl clofibrate and clofibric acid in human and rat plasmas, it was used to determine the stability of ethyl clofibrate polylactic nanocapsules in human and rat plasmas. Given that ethyl clofibrate in rat plasma is almost instantly metabolized to clofibric acid, the results suggest that nanocapsules are stable in human plasma (Fig. 3I) whereas they are destroyed in rat plasma (Fig. 3II). Therefore, rat can not be used as a model system to test the possibility to target ethyl clofibrate polylactic acid nanocapsules to the liver. Nevertheless, the idea of using this system in human is still valid since polylactic acid nanocapsules show good stability in human plasma.

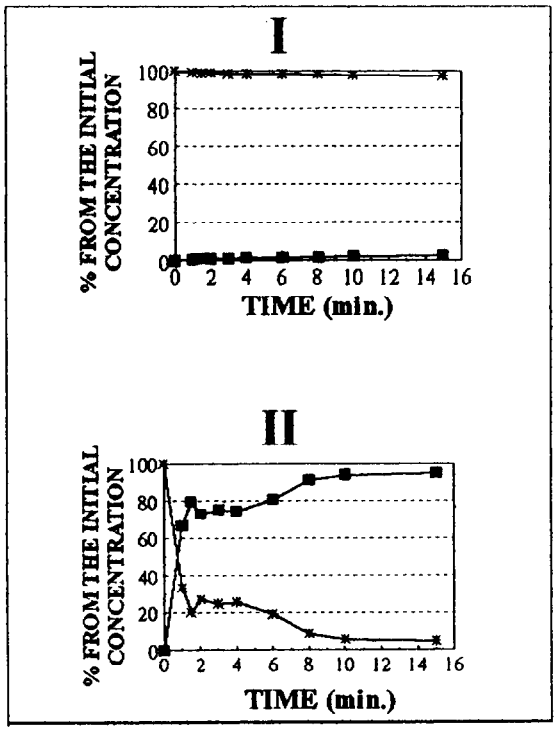

Fig. 3. Stability of ethyl clofibrate polylactic acid nanocapsules in human (I) and rat plasmas (II). (■) Ethyl clofibrate, (★) clofibric acid.

4. Conclusions

An easy, rapid and accurate analytical method has been developed to determine plasma concentrations of ethyl clofibrate and clofibric acid. The HPLC assay was used to monitor the plasma concentration–time profiles of ethyl clofibrate polylactic nanocapsules in both species (human and rat). The results showed that rat can not be used as a model system because of the instability of the nanocapsules in its plasma. Other species should be tested.

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