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Conventional and enantioselective determination of a new blood glucose-lowering agent in biological fluids using liquid–liquid extraction and high-performance liquid chromatography

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ABSTRACT

Two analytical methods are described for the determination of 2-(4-*tert.*-butylphenoxy)-7-(4-chlorophenyl)heptanoic acid sodium salt (I) in animal models (beagle dog and rat). Method 1 is conventional reversed-phase high-performance liquid chromatography on an octadecylsilane column with an eluent of acetonitrile–0.02 *M* potassium buffer (pH 3) (65:35, v/v). Method 2 is used for the enantioselective determination of I. This method uses a chiral column (Chiralcel OJ) with an eluent of *n*-hexane–2-propanol (95:5, v/v) containing 3 ml/l trifluoracetic acid. The analytical procedure has a recovery of more than 90%, within-run precision of less than 5.1%, and between-run precision of less than 4.3%.

INTRODUCTION

2-(4-*tert.*-Butylphenoxy)-7-(4-chlorophenyl)-heptanoic acid sodium salt (I, Fig. 1) has been developed as an antidiabetic agent, and may have potential to be a blood glucose-lowering agent for non-insulin-dependent diabetes mellitus (NIDDM). NIDDM is one of the most common metabolic diseases still lacking fully effective therapy and is characterized by subnormal tissue responsiveness to insulin, resulting in excessive hyperglycemia [1,2]. For pharmacokinetic studies in beagle dogs and rats, two methods to determine this compound in biological fluids were developed and compared: method 1 is used to determine racemic I, method 2 for the enantioselective determination of the (−)-enantiomer [(−)-I] and the (+)-enantiomer [(+)-I]. This separation of

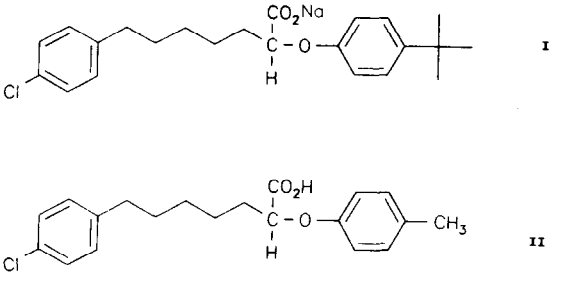

Fig. 1. Structures of I and II (the internal standard).

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the enantiomers is necessary, because animal models have shown pharmacological activity only for (−)-I. Therefore the active (−)-enantiomer was administered during animal experiments. In order to measure a possible *in vivo* racemization, the enantioselective method was applied. Both methods employ an internal standard, 2-(4-methylphenoxy)-7-(4-chlorophenyl)heptanoic acid (II, Fig. 1), liquid–liquid extraction, reversed-phase liquid chromatography (method 1) or chromatography with a chiral column (method 2), and UV detection. The chiral column (Chiralcel OJ) has been developed by Okamoto's group [3–5] and contains a stationary phase of cellulose tris-(4-methylbenzoate). Recently, several different cellulose derivatives have been successfully applied for the enantiomeric separation of drugs in biological fluids [6–9].

In this report we compare a conventional reversed-phase with a direct enantioselective separation of this new oral blood glucose-lowering agent.

EXPERIMENTAL

Reagents and buffer (methods 1 and 2)

The reference standards (I and II) were provided by Boehringer Mannheim (Mannheim, Germany). HPLC-grade acetonitrile, HPLC-grade hexane, potassium phosphate, boric acid and phosphoric acid were obtained from E. Merck (Darmstadt, Germany). Diethyl ether and glacial acetic acid were purchased from Riedel-de Haen (Seelze, Germany). Purified water was produced by a Milli-Q plus reagent water system (Millipore, Eschborn, Germany).

A Britton–Robinson buffer (0.04 *M*, pH 5) was prepared by weighing 2.47 g of boric acid in a 1000-ml flask, adding 2.28 ml of glacial acetic acid, 2.82 ml of phosphoric acid and *ca.* 800 ml of deionized water. The pH was adjusted to 5.0 with 10 *M* sodium hydroxide. The buffer was diluted to volume with deionized water.

Apparatus and HPLC conditions (methods 1 and 2)

HPLC analyses were performed using a Shimadzu LC-6A pump (Kyoto, Japan), a Perkin-Elmer ISS-100 autosampler (Überlingen, Germany), a Biotronik BT-8200 UV detector (Maintal, Germany) and a Hewlett-Packard HP 1000 computer system (Böblingen, Germany). Both compounds (I and II) were detected at 220 nm with a sensitivity range of 0.04 a.u.f.s.

Method 1. The column (125 mm × 4.6 mm I.D., Bischoff, Leonberg, Germany) was packed by a conventional slurry method with Nucleosil 120-5 C_{18}, 5 μm particle size (Macherey-Nagel, Düren, Germany). The mobile phase was acetonitrile–0.02 *M* potassium phosphate buffer (pH 3) (65:35, v/v). At a flow-rate of 1 ml/min, the retention times were 9 min for I and 19 min for II; the separation time was 22 min.

Method 2. The analytical chiral column (250 mm × 4.6 mm I.D.) and the precolumn (50 mm × 4.6 mm I.D.) (Chiralcel OJ column, Diacel Chemical Industries, Tokyo, Japan) contained a stationary phase of tris-(4-methylbenzoate)-modified cellulose coated on 10-μm silica gel particles. The mobile phase was *n*-hexane–2-propanol (95:5, v/v) containing 3.0 ml/l trifluoracetic acid. At a flow-rate of 0.9 ml/min and a separation time of 42 min, (−)-I was eluted after 14 min, (+)-I after 18 min and the (−)-enantiomer of II [(−)-II] after 37 min.

Preparation of standards

Method 1. A stock solution was prepared by dissolving 10.56 mg of racemic I in 10 ml of methanol to give a concentration of 1 mg/ml of the free acid. The internal standard solution was prepared by dissolving 5.00 mg of racemic II in 100 ml of methanol to yield a concentration of 50 μg/ml. Serum standards were prepared by aliquoting appropriate volumes of stock solution into control (drug-free) dog/rat serum to produce a calibration curve containing concentrations of 1, 5, 10, 20, 50 and 100 μg/ml racemic I.

Method 2. Stock solutions were prepared by dissolving separately 10.56 mg of (−)-I and (+)-I in 10 ml of methanol. The internal standard solution was prepared by dissolving 10.00 mg of (−)-II in 100 ml of methanol to give a concentration of 100 μg/ml. Serum standards

were prepared using the same procedure as in method I to produce two calibration curves containing 1, 5, 10, 20, 50 and 100 μg/ml of each enantiomer.

Sample preparation and quantification (methods 1 and 2)

Serum samples (0.05 ml) and serum standards were mixed with 1 ml of 0.04 *M* Britton–Robinson buffer (pH 5) and 0.05 ml of the internal standard. Diethyl ether (5 ml) was added, the tubes were shaken in a test-tube rack on a reciprocating shaker for 10 min at a frequency of 150/min and centrifuged for 3 min at 3000 *g*. After phase separation, the diethyl ether was evaporated by a stream of nitrogen for 30 min at 45°C. The residue was reconstituted with 250 μl of the mobile phase and transferred to a sample vial, and 150 μl were injected into the HPLC system.

Calibration graphs were prepared by assaying plasma (serum) samples to which known amounts of racemic I (method 1) or different concentrations of the enantiomers of I (method 2) had been added. Peak-height ratios of racemic I (method 1) or of each enantiomer (method 2), relative to the internal standard, were plotted *versus* the known concentrations.

For the experimental fit of the data points to the regression line, two modes of regression were used: linear and power-fit. The coefficients of correlation of the different regression equations were routinely calculated to evaluate the fit of the calibration data to regression line (> 0.99). The function with the best correlation was used for the calibration of the sample concentrations.

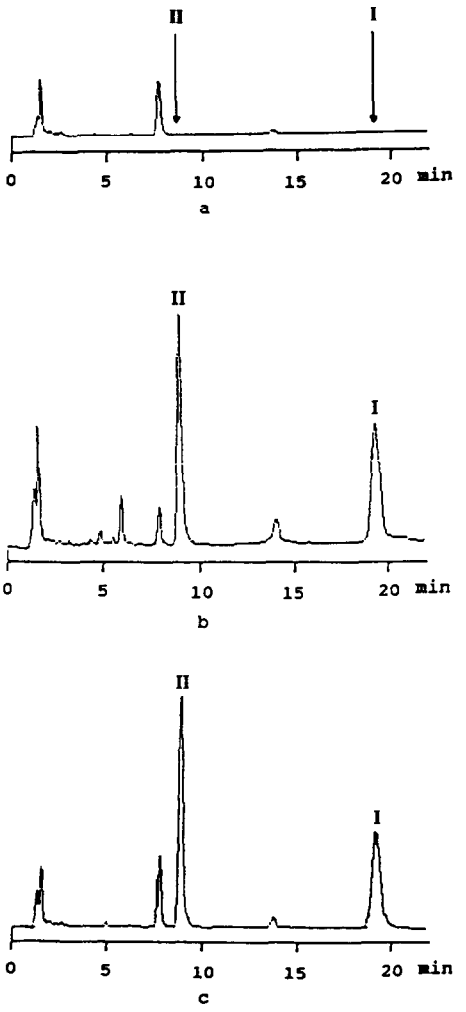

Fig. 2. Chromatograms of (a) an extract of a 50-μl sample of drug-free dog serum, (b) an extract of a 50-μl sample of dog serum spiked with 50.08 μg/ml I and 50.90 μg/ml II (internal standard) and (c) an extract of a 50 μl-sample of dog serum taken 24 h after a single oral dose of 7.5 mg/kg (−)-I; the concentration is found to be 40.5 μg/ml.

RESULTS

Selectivity

Method 1. Drug-free dog serum (control) shows no significant peaks at the retention times of I or of the internal standard when analysed by this method (Fig. 2a). Fig. 2b shows the chromatogram of an extract of 50 μl of dog serum spiked with 50.08 μg/ml I and 50.90 μg/ml internal standard. Fig. 2c shows the chromatogram of an extract of a 50-μl serum sample, taken from a beagle dog 24 h after an oral dose of 7.5 mg/kg (−)-I.

Method 2. Drug-free rat serum (control) shows no significant peaks at the retention times of (−)-I, (+)-I or of the internal standard when analysed by this method (Fig. 3a). Fig. 3b shows the chromatogram of an extract of 50 μl of rat serum spiked with 50.21 μg/ml (−)-I, 46.48 μg/ml (+)-I and 98.79 μg/ml (−)-II (internal stan-

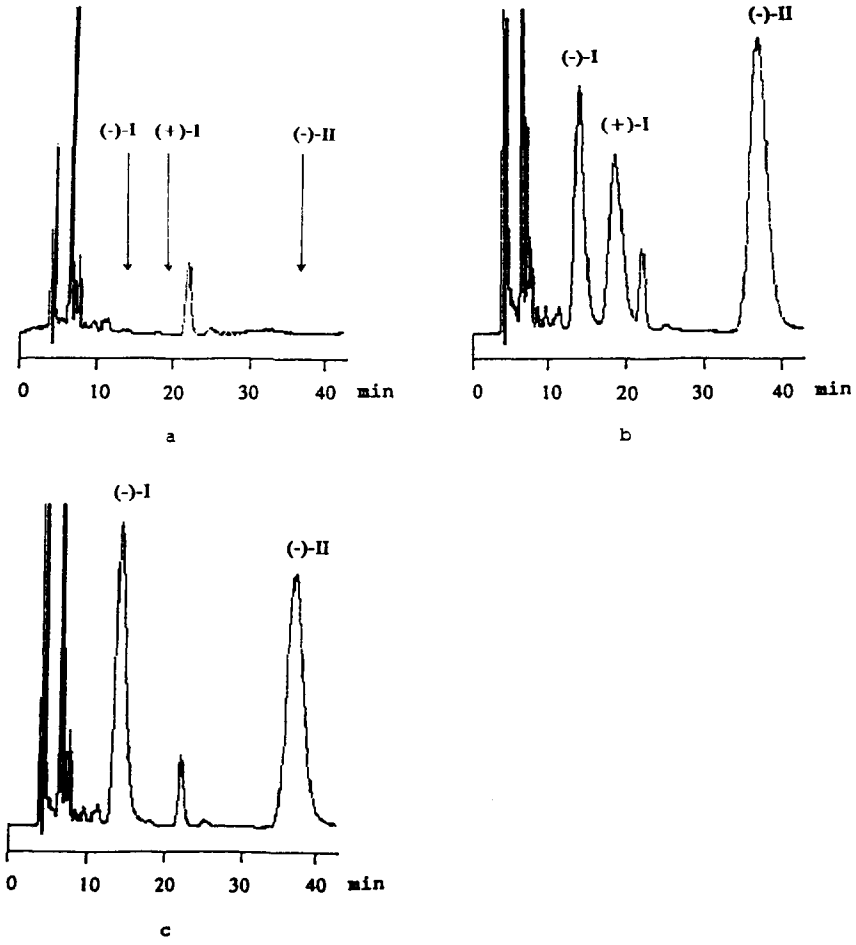

Fig. 3. Chromatograms of (a) an extract of a 50-μl sample of drug-free rat serum, (b) an extract of a 50-μl sample of rat serum spiked with 50.21 μg/ml (−)-I, 46.48 μg/ml (+)-I and 98.79 μg/ml of (−)-II (internal standard) and (c) an extract of a 50-μl sample of rat serum taken 8 h after the eighth administration of an oral dose of 25 mg/kg (−)-I; the concentration is found to be 70.30 μg/ml.

dard). Fig. 3c shows the chromatogram of an extract of a 50-μl serum sample, taken from a rat 8 h after the eight administration of a therapeutic oral dose of 25 mg/kg (−)-I.

Recovery study

The extraction recoveries were determined by method 1 at concentrations of 100 μg/ml of I and 50 μg/ml for II. Samples were extracted in ten replicates. The results were compared with the peak heights obtained from reference standards containing the corresponding concentrations of I and II. The recoveries for I and II ranged from 85.8 to 98.2% (mean ± S.D., 90.4 ± 4.3%) and 79.5 to 91.1% (mean ± S.D., 84.8 ± 3.9%), respectively.

Method validation (methods 1 and 2)

Sensitivity. The sensitivity of the methods was evaluated by analysing serum samples at the presumed minimum quantifiable level in six replicates. The limit of quantitation (R.S.D. < 10%) was 200 ng/ml for method 1 and 750 ng/ml for method 2. The detection limit, based on a signal-to-noise ratio of 3:1, was 100 ng/ml for method 1 and 500 ng/ml for method 2.

Accuracy and precision. To determine the between-run and the within-run precision and accu-

TABLE I

BETWEEN-RUN PRECISION AND ACCURACY DATA

Theoretical concentration of racemic I (μg/ml)	Method	Concentration found (mean ± S.D., n = 5) (μg/ml)	R.S.D. (%)
10	1	9.85 ± 0.24 rac. I	2.44
50	1	49.17 ± 1.60 rac. I	3.25
20	2	9.77 ± 0.37 (−)-I	3.79
		9.87 ± 0.42 (+)-I	4.26
100	2	50.32 ± 1.90 (−)-I	3.78
		50.48 ± 1.34 (+)-I	2.66

racy, four plasma pools containing 10 and 50 μg/ml (method 1) as well as 20 and 100 μg/ml (method 2) racemic I were prepared. On five different days, six samples from each pool were analysed, giving a total of sixty samples for each method. The within-run precision (R.S.D.) ranged from 0.8 to 5.1% (mean 2.0%) for method 1 and from 1.1 to 4.5% (mean 2.7%) for method 2. The between-run accuracy and precision (Table I) are indicated by the mean and S.D. of the five within-run calculations.

Stability. The stability of racemic I and (−)-I in serum samples maintained at 22°C (room temperature) for 24 h, at 4°C for one week and at −20°C for four weeks was evaluated. No significant decrease in concentrations was detected.

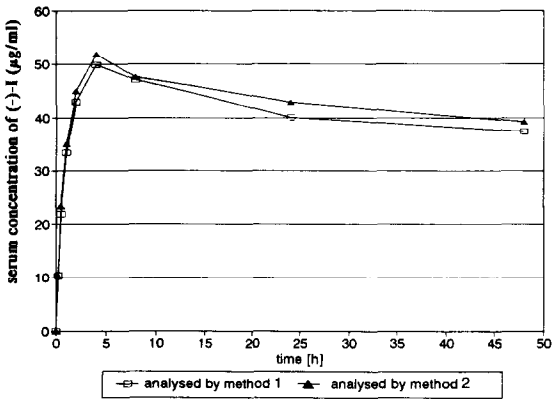

Fig. 4. Median concentration–time curves (n = 4) obtained in dogs receiving a 7.5 mg/kg single oral dose of (−)-I.

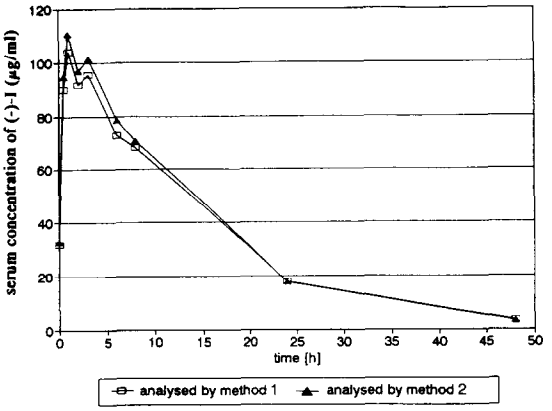

Fig. 5. Median concentration–time curves (n = 5) obtained in rats treated for eight days with 25 mg/kg (−)-I per day.

Application

Both methods have been successfully applied to the determination of I (conventional HPLC and enantioselective) during pharmacokinetic studies in beagle dogs and rats.

Four beagle dogs (two male, two female) were given a single oral dose of 7.5 mg/kg (−)-I. Blood samples (1 ml) were collected between 0 (predose) and 48.0 h after dosing.

Fifteen male Lewis rats were treated with a therapeutic oral dose of 25 mg/kg (−)-I for eight days. Blood samples were taken after the eighth administration in a volume of 0.8 ml between 0 and 48.0 h. Only three blood samples were taken from each rat within these 48 h. According to the procedure used, a total of five different blood samples for each of nine time-points was taken.

Blood samples of both studies were analysed by both methods. Concentration–time curves are shown in Figs. 4 and 5. In both species (dog and rat) the applied (−)-I was stable. With the described enantioselective method, no *in vivo* racemization was detectable (by method 2 all determined concentrations for (+)-I were below the detection limit).

A good correlation was observed was observed between concentrations of racemic I (measured with method 1) and (−)-I (measured with method 2) (Fig. 6). The correlation between the two methods fits the regression equation $y = 1.069x - 0.913$ (r = 0.992).

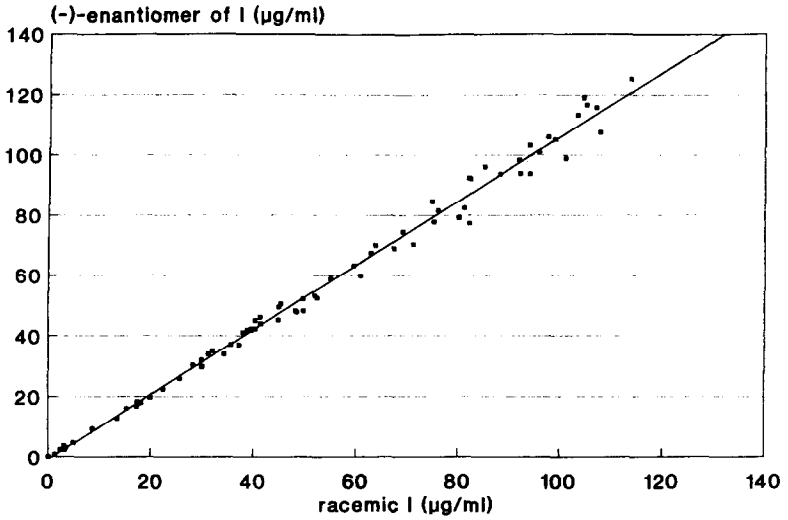

Fig. 6. Correlation between plasma concentrations of I determined by conventional HPLC and by the enantioselective method.

CONCLUSION

This paper reports two HPLC methods with UV detection for the determination of a new blood glucose-lowering agent in plasma (serum) samples. Diethyl ether was found to be an efficient solvent for single-step extraction without any interference from biological fluid constituents. The methods have been compared, validated and successfully employed for the analysis of serum samples obtained from beagle dogs and rats in a pharmacokinetic study. Applying the enantioselective determination, no *in vivo* stereoinversion from the administered pharmacological active enantiomer to its inactive antipode was observed in either of the two examined species.

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