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Short Communication

Quantitation of the new hypoglycaemic agent AG-EE 388 ZW in human plasma by automated high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A sensitive and selective high-performance liquid chromatographic assay has been developed for a new hypoglycaemic agent AG-EE 388 ZW (I) in human plasma. Plasma samples containing I were acidified with 0.2 *M* hydrochloric acid, directly injected into C_2 reversed-phase pre-columns, and cleaned up on-line. After pre-column switching, the substance was separated isocratically in 15 min on a C_{18} reversed-phase column. Quantitation was performed after amperometric detection by external standard calibration curves. The linearity of the assay was demonstrated over the therapeutic concentration range 5–200 ng/ml. The between-day coefficient of variation was 9.2% at 30 ng/ml. The limit of detection was 5 ng/ml in plasma. Determination of human plasma samples after intravenous and oral administration of 1 mg of ^{14}C-labelled I demonstrated the applicability of the assay for pharmacokinetic studies in humans.

INTRODUCTION

2-Ethoxy-N-[α-(2-methyl-1-propyl)-2-piperidinobenzyl]-4-carbamoylmethylbenzoic acid (AG-EE 388 ZW, I, Fig. 1) is a new hypoglycaemic agent. The high efficacy of this drug is reflected in the low therapeutic dose of *ca.* 2 mg, which requires a very sensitive assay for determination of its pharmacokinetics in humans. During the development of each new drug a large number of plasma samples have to be analysed to assess carefully the safety and efficacy. It was therefore mandatory to establish a selective and sensitive automated assay for determination of I in plasma. This paper describes a fully automated high-performance liquid chromatographic (HPLC) assay with electrochemical detection (ED) for the quantitation of I in human plasma.

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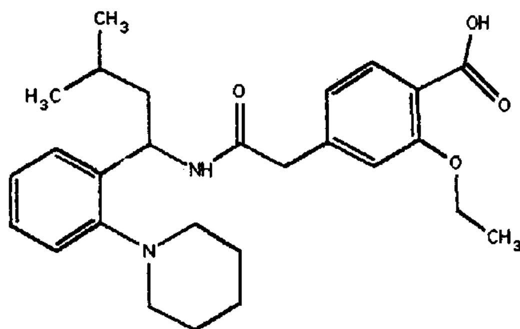


Fig. 1. Structure of AG-EE 388 (I).

EXPERIMENTAL

Preparation and sources of reagents

Potassium dihydrogenphosphate (p.a.), orthophosphoric acid (85%, w/v, p.a.), acetonitrile (LiChrosolv) and hydrochloric acid (37%, w/v) were obtained from E. Merck (Darmstadt, Germany). Lithium perchlorate was supplied by Aldrich Chemie (Steinheim, Germany). Dioxane and methanol were obtained from Promochem (Wesel, Germany) and Baker (Deventer, Netherlands), respectively. Compound I, with a purity of >99.9%, and ^{14}C-labelled I, with a specific activity of 0.4 MBq/mg and a radiochemical purity of >98%, were synthesized at Dr. Karl Thomae GmbH. Demineralized water was purified using Milli-Q system (Millipore, Bedford, MA, USA). Solvent A was methanol–acetonitrile–dioxane (68:24:8, v/v). Solvent B contained 3 g of potassium dihydrogenphosphate and 0.5 g of lithium perchlorate in 1 l of water, adjusted to pH 2.7 with orthophosphoric acid.

Preparation of standards and controls

A mass of 10 mg of I was dissolved in 100 ml of methanol, yielding a concentration of 100 μg/ml. This methanolic solution was diluted 1:500 with heparinized human plasma, yielding a plasma stock standard of 200 ng of drug per ml of plasma. Spiked plasma standards ranging from 5 to 200 ng/ml drug were prepared from the stock standard to define the linearity of the assay. Analogous spiked plasma samples with a concentration of 30 ng of drug per ml of plasma were prepared as controls for routine analysis. Standards and controls were stored in aliquots at −20°C until analysis.

Preparation of samples

Blood samples were collected from the antecubital forearm vein with heparinized monovettes from Sarstedt (Nümbrecht, Germany) and immediately centrifuged. Plasma supernatants were collected and stored at −20°C until HPLC analysis.

Columns

The pre-columns (17 mm × 2.9 mm I.D.) were filled with Perisorb RP-2 (30–40 μm particle size) from E. Merck, the guard columns (17 mm × 4.6 mm I.D.) were filled with ODS-Hypersil (5 μm particle size), supplied by Shandon (Cheshire, UK). The analytical columns were two serial LiChroChart HPLC cartridges (each 125 mm × 4 mm I.D.) packed with LiChrospher 100 RP-18 (5 μm particle size) from E. Merck.

Instrumentation

A diagram of the HPLC system is shown in Fig. 2. The system consisted of an autosampler (Perkin-Elmer, ISS 100), a miniPump (LDC Milton Roy, Riviera Beach, FL, USA), a column-switching module (Gynkotek, Germering, Germany), a gradient system CM 4000 (LDC Milton Roy), an electrochemical detector 460 (Waters) and an integrator CI-10 B (LDC Milton Roy).

HPLC analysis

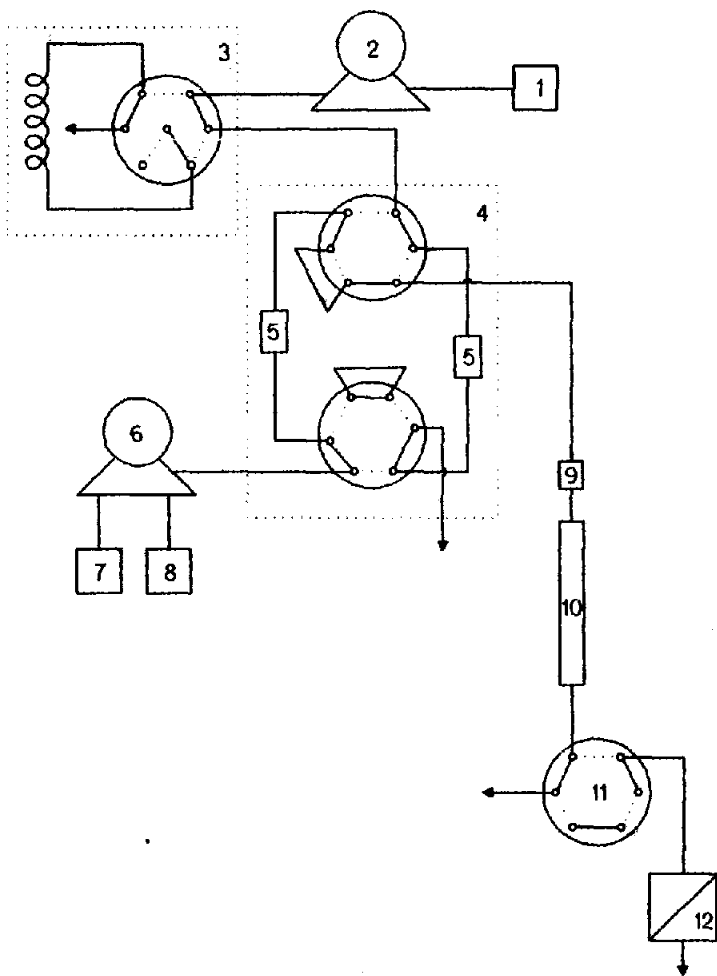
On the day of HPLC analysis the frozen samples were thawed at room temperature (*ca.* 20°C), centrifuged at 13 000 *g* for 2 min to remove residual clottings, and diluted 1:2 with 0.2 *M* hydrochloric acid. Then 160 μl of the prepared samples were injected into the pre-columns at intervals of 15 min. Each sample was cleaned-up for 3 min on the pre-columns with demineralized water at a flow-rate of 1 ml/min. Then the substance was back-flushed isocratically (solvent A–B = 76:24, v/v) on to the analytical column at a flow-rate of 1 ml/min and a pressure of *ca.* 10 MPa, followed by a washing step with solvent A. The detection was performed by an amperometric detector using a potential of 1.04 V. In order to prevent contamination of the electrochemical cell, the column outflow bypassed the amperometric cell between 0 and 7.5 min after sample injection (Fig. 2). Then the analytical column was switched to the amperometric cell during the time period 7.5–13.8 min. The drug appeared at 8.8–9.1 min. Quantitation of 1 was performed using the peak area and external standard calibration curves.

Recovery studies

^{14}C-Labelled 1 was dissolved in plasma at a concentration of 200 ng/ml according to the preparation of unlabelled standard. Ten injections were carried out as described above. The eluates of the pre-columns and the analytical column were collected separately, and the radioactivity of each was quantified.

Stability studies

Eight to ten controls were thawed daily and analysed over six weeks. Furthermore, plasma samples were incubated over 24 h at 30°C, and the electrochemical signals before and after this incubation were compared.



1 = Water
2 = Solvent Pump
3 = Autosampler
4 = Column Switching Module
5 = Precolumn
6 = Multiple Solvent Delivery System
7 = Solvent A
8 = Solvent B
9 = Guard Column
10 = Analytical Column
11 = Valve
12 = Detector
⟶ = Waste

Fig. 2. Diagram of the chromatographic system.

Statistics

The linearity of the concentration–signal data was proved using linear regression analysis of thirteen calibration curves. The detection limit was calculated according to Mücke [1] using the 99% confidence interval of the regression line. The precision and accuracy were determined by measurement of control samples

during routine analysis over twelve days (ten determinations per day). The precision was determined by calculating the within- and between-day coefficients of variations (C.V.) of the controls. The accuracy was determined by calculating the difference between the measured concentration and the true value of the spiked controls.

Human study

^{14}C-Labelled I (1 mg) administered intravenously or orally to four healthy volunteers, and the plasma concentration–time curves of I were determined using the described assay. The study protocol was approved by the Ethical Review Board, and the volunteers gave informed consent.

RESULTS

Recovery

A recovery of 98.8 ± 0.1% (mean ± S.D., n = 10) was determined at a drug concentration of 200 ng/ml.

Chromatograms

Typical chromatograms are shown in Fig. 3.

Stability of plasma samples

Compound I was stable in plasma at −20°C for at least six weeks, which was sufficient time for storage between blood sampling and analysis. It was also stable in plasma for over 24 h at 30°C, allowing automated analysis around the clock.

Assay linearity

The calibration curve of I was reproduced thirteen times. Each curve was linear in the range 5–200 ng/ml. The correlation coefficient of each calibration curve was greater than 0.99. The mean intercept and slope were −76 429 ± 13 555 and 24 775 ± 2632, respectively (mean ± S.D., n = 13).

Detection limit

Based on the 99% confidence intervals of the calibration curve a detection limit of 5 ng/ml was calculated.

Assay validation

The imprecision of the assay was characterized by a within-day C.V. of 8–11% and a between-day C.V. of 9.2% at a concentration of 30 ng/ml. The inaccuracy bias was 1%.

Selectivity and pharmacokinetics of I in humans

After intravenous or oral administration of 1 mg of ^{14}C-labelled I to humans

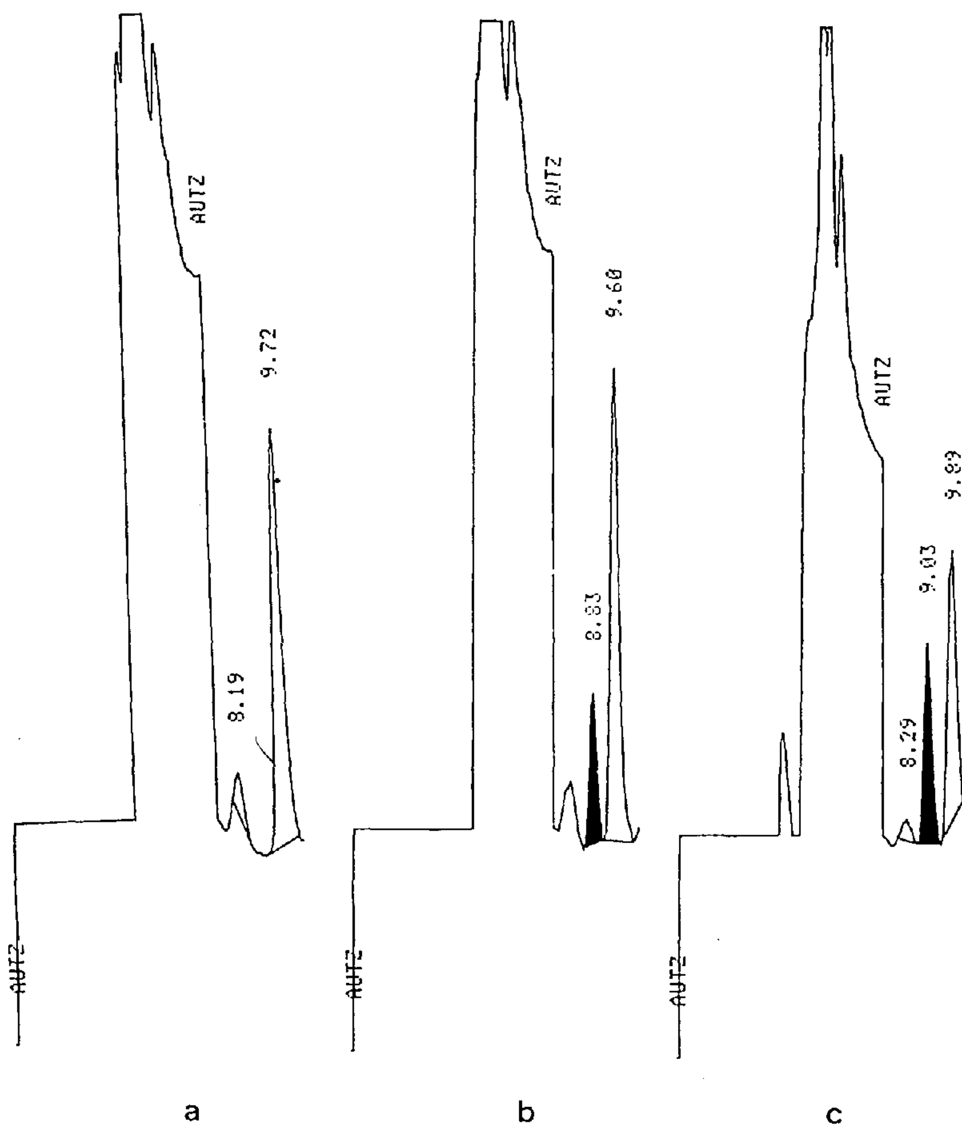


Fig. 3. Chromatograms of (a) a blank plasma sample, (b) a spiked standard of 30 ng/ml, and (c) an *in vivo* sample taken 1 h after oral administration of 1 mg of ^{14}C-labelled I.

ca. 80% of the total radioactivity in plasma represented the parent drug, as determined by the described assay. This was confirmed by the pattern of radioactivity in plasma using an HPLC gradient system, indicating the selectivity of the assay. After intravenous administration a mean residence time (MRT) of 3.0 ± 1.7 h, a terminal half-life ($t_{1/2}$) of 2.5 ± 0.9 h, a total plasma clearance (*Cl*) of 143 ± 53 ml/min and a volume of distribution at steady state (V_{ss}) of 20 ± 6 l

were calculated for the parent drug, using non-compartmental pharmacokinetic analysis (mean ± S.D.). After oral administration the maximum plasma level of the parent drug (C_{max}) was 33 ± 6 ng/ml which was reached after 0.7 ± 0.2 h (mean ± S.D.). The absolute bioavailability (*f*) of I, calculated on the basis of the area under the curve ratios after oral and intravenous administration, was 78 ± 26% (mean ± S.D.).

DISCUSSION

An automated assay methodology for the determination of I in human plasma was required. As I can be adequately separated from other plasma constituents by liquid chromatography on a reversed phase, and amperometric detection proved to be the most sensitive detection technique, an automated HPLC–ED assay applying the column-switching technique was developed [2]. During the development of the assay, problems arose in connection with the on-column extraction of I, owing to its amphoteric properties. In addition, external standard calibration was difficult to perform because of the rapid contamination of the amperometric cell by plasma constituents. Almost complete extraction of I from directly injected plasma at the pre-columns was achieved by acidifying the plasma. Blinding of the amperometric cell could be prevented by using an appropriate pre-column phase, reducing it to a minimum, and controlling the column outflow so that only the peak volume of interest passed through the cell.

CONCLUSION

A sensitive and selective HPLC–ED assay for the determination of the hypoglycaemic agent I in human plasma has been developed. The method has been automated and validated, and can be applied to the routine analysis of therapeutic plasma levels of this drug. However, I is a racemate (Fig. 1) and the described non-stereoselective method cannot differentiate between the enantiomers of this drug, of which the (+)-form is active. It is well known that the pharmacokinetics and pharmacodynamics of enantiomers can be markedly different [3–5]. This should be considered in interpreting the plasma concentration–time curves of I obtained with the described assay.

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