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Short Communication

Enantioselective assay of warfarin in blood plasma by liquid chromatography on Chiralcel OC

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ABSTRACT

A stereoselective method for the two enantiomers of warfarin has been developed. Warfarin is extracted into dichloromethane–hexane from blood plasma. After concentration by evaporation, the enantiomers are resolved by liquid chromatography on Chiralcel OC employing a non-polar mobile phase. By fluorometric detection, concentrations in plasma down to 80 nmol/l (25 ng/ml) can be determined with a coefficient of variation of less than 15%. At the 200 nmol/l level, a coefficient of variation of *ca.* 4% was found.

INTRODUCTION

Warfarin, a widely used anticoagulant drug, is administered in racemic form. Since many years it is well known that the pharmacological effect and the pharmacokinetic properties of warfarin are different for the *S*- and *R*- enantiomer [1]. Because of the narrow therapeutic index of the drug, an enantioselective assay may be requested especially in drug interaction studies [2].

Liquid chromatographic methods have been employed to separate the enantiomers of warfarin. Banfield and Rowland [3,4] formed diastereomeric ester derivatives by reaction with the chiral reagent N-carbobenzyloxy-L-proline. Recently a chiral bicyclic carboxylic acid [5] and menthylchloroformate [6] have been used to prepare diastereomers. In these applications separation was achieved on an achiral chromatographic column. Stereochemical resolution of underivatized warfarin was shown by Allenmark [7] using a stationary phase of immobilized bovine serum albumin. Chu and Wainer [8] used this chiral phase in a coupled-column system for a stereoselective assay of warfarin in plasma samples.

In the present study we have applied Chiralcel OC, a chiral stationary phase with cellulose triphenylcarbamate as selector on macroporous silica gel [9], for a simplified enantioselective method for warfarin in blood plasma. As organic mobile phase a mixture of 1-propanol and acetonitrile in hexane was used.

EXPERIMENTAL

Chemicals

Warfarin, as sodium salt, was supplied as reference substance and *p*-chlorowarfarin was obtained from Aldrich (Steinheim, Germany). Ace-

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tonitrile, dichloromethane, hexane, methanol and 1-propanol were of HPLC grade (Rathburn, Walkerburn, UK) and HCl, NaOH, NaH_2PO_4 and Na_2HPO_4 of analytical grade (E. Merck, Darmstadt, Germany). Water was supplied by a Milli-Q water purification system (Millipore, Molsheim, France).

Instrumentation

The liquid chromatograph comprised a Kontron 420 pump (Tegimenta, Milan, Italy), a Kontron 465 autosampler and a Jasco 820 FP fluorescence detector (Tokyo, Japan) operated at 315 nm (excitation) and 370 nm (emission) with a cell volume of 16 μl. The chromatograms were recorded on a VG Multichrom system (Fisons, Manchester, UK).

The analytical column was Chiralcel OC (10 μm, 250 mm × 4.6 mm I.D.) from Daicel Chemical Industries (Tokyo, Japan), which was used with a flow-rate of 0.5 ml/min at 40°C. The mobile phase contained acetonitrile–1-propanol–hexane (3:11:86). A Brownlee (Applied Biosystems, San José, CA, USA) CN guard column (7 μm, 15 mm × 3 mm I.D.) was used for the protection of the separation column.

Calibration solution and internal standard

A standard solution of racemic warfarin (65 μmol/l) used for calibration was made up in phosphate buffer pH 7.0 (I = 0.01). Racemic *p*-chlorowarfarin was dissolved in mobile phase and resolved into the two enantiomers after repeated injections onto the Chiralcel OC column. The fractions containing the *S*-enantiomer were collected, and the organic solvent was evaporated. The *R*-enantiomers were assumed to elute first with reference to the pharmacokinetic properties and chromatographic elution order of the warfarin enantiomers [3]. The retention times for the *R*- and *S*-isomers were 20 and 28 min, respectively. The retention times for the *R*- and *S*-isomers of the internal standard were 20 and 24 min, respectively, which means that the *R*-isomer overlapped with the *R*-form of warfarin. *S-p*-Chlorowarfarin (25 μmol/l) in methanol–phosphate buffer pH 7.0 (14:86) was used as internal standard solution.

Analytical procedure

The frozen plasma samples were thawed, mixed and centrifuged (5 min, 1500 *g*). A 1.00-ml volume of the sample was mixed with 100 μl of internal standard solution and 100 μl of 1 mol/l HCl and extracted with 5.00 ml of dichloromethane–hexane (1:5) by shaking for 20 min. After centrifugation for 5 min (1500 *g*) the aqueous phase was frozen and the organic phase decanted into a tapered centrifuge tube. After evaporation under nitrogen the residue was dissolved in 250 μl of mobile phase, of which 100 μl were injected with the autosampler onto the chromatographic column.

Reference samples for calibration were included in the daily series of analysis. A 100-μl volume of the standard solution, 50 μmol/l in phosphate buffer, was added to 1 ml of blank plasma in six to ten replicates which, together with one blank plasma sample, were processed in parallel with the authentic samples and with daily quality control samples.

Calculation

Peak-height ratios between *R*- and *S*-warfarin and the internal standard, *S-p*-chlorowarfarin, were calculated for each chromatogram by the chromatographic data system which used the mean value of the calibration samples to estimate plasma concentrations of the authentic samples. Full standard curves in the range 80–5000 nmol/l were determined at intervals of not more than a month during analysis periods.

RESULTS AND DISCUSSION

Extraction from plasma

We used a mixture of dichloromethane and hexane (1:5) to extract *R*- and *S*-warfarin and the internal standard *S-p*-chlorowarfarin from the acidified plasma sample. An extraction recovery of more than 90% was achieved for all three components. For these compounds diethyl ether may be a more powerful extractant and has been preferred by others (*e.g.* refs. 3–5). However, in those cases purification was necessary which was fulfilled by a preceding extraction from an alkali-

nized plasma sample [3,4] or by a subsequent passage of the extract through a silica column [5]. Precipitation of plasma proteins by acetonitrile and evaporation were combined with a coupled-column liquid chromatographic system [8].

Liquid chromatographic separation

We found the Chiralcel OC column quite useful for the resolution of the warfarin enantiomers as displayed in the application brochure from the producer Daicel. For this particular separation the introduction of acetonitrile in the 1-propanol–hexane mobile phase significantly improved the column efficiency, giving more narrow peaks. *S*-*p*-Chlorowarfarin was a suitable internal standard eluting between the *R*- and *S*-enantiomers of warfarin (Fig. 1) and was isolated from the racemate by semipreparative separation on the same kind of column.

The mobile phase acetonitrile–1-propanol–hexane (2:15:83) was chosen as a result of studies on blank plasma samples with added warfarin. However, when analysing authentic plasma samples there was interference with the latest-eluted peak, *S*-warfarin, in the chromatogram, this was assumed to be a metabolite of warfarin. We examined the separation selectivity in an authentic urine sample in which the interfering compound was easily localized and where no intact warfarin was present. By changing the composition of the mobile phase successively and comparing with added warfarin we found that with a mobile phase of acetonitrile–1-propanol–hexane in the proportions 3:11:86 the disturbing peak eluted well after *S*-warfarin. A chromatogram from a blank plasma sample and an authentic plasma sample is shown in Fig. 2A and B, respectively.

The *R*- and *S*-enantiomers were not available as pure reference substances. Their elution order was judged from the fact that at steady state the plasma concentration of the *S*-isomer is lower due to its more rapid clearance [1,4,8]. The last-eluted peak for both warfarin and *p*-chlorowarfarin was then assumed to be the *S*-enantiomer. An

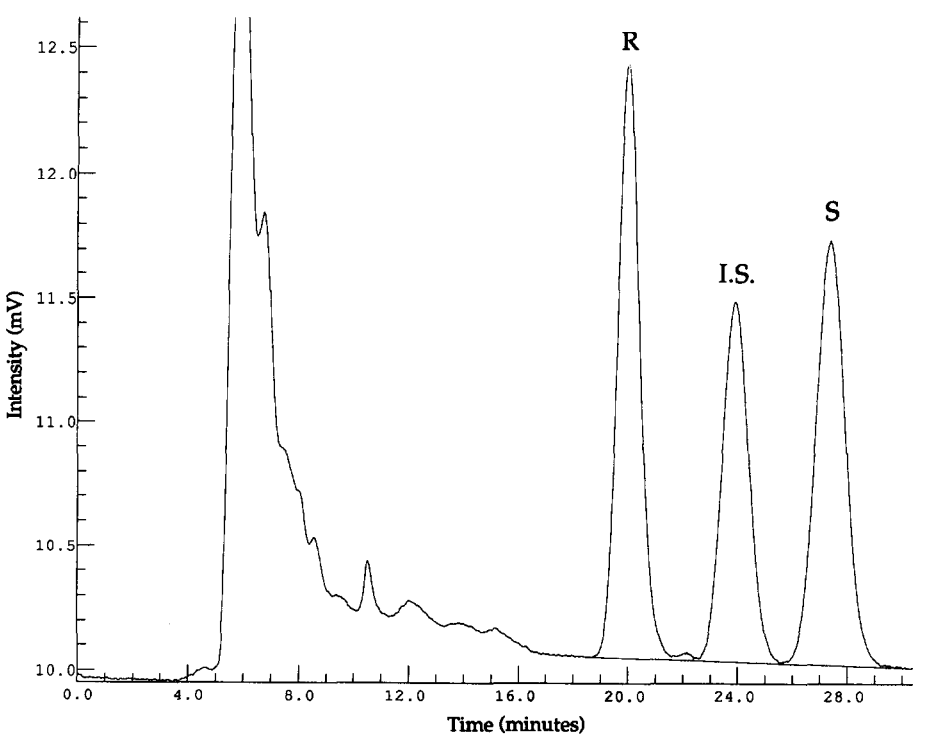

Fig. 1. Chromatogram of an extract from a plasma standard containing 3300 nmol/l each of *R*- and *S*-warfarin and 2500 nmol/l *S*-chlorowarfarin (internal standard).

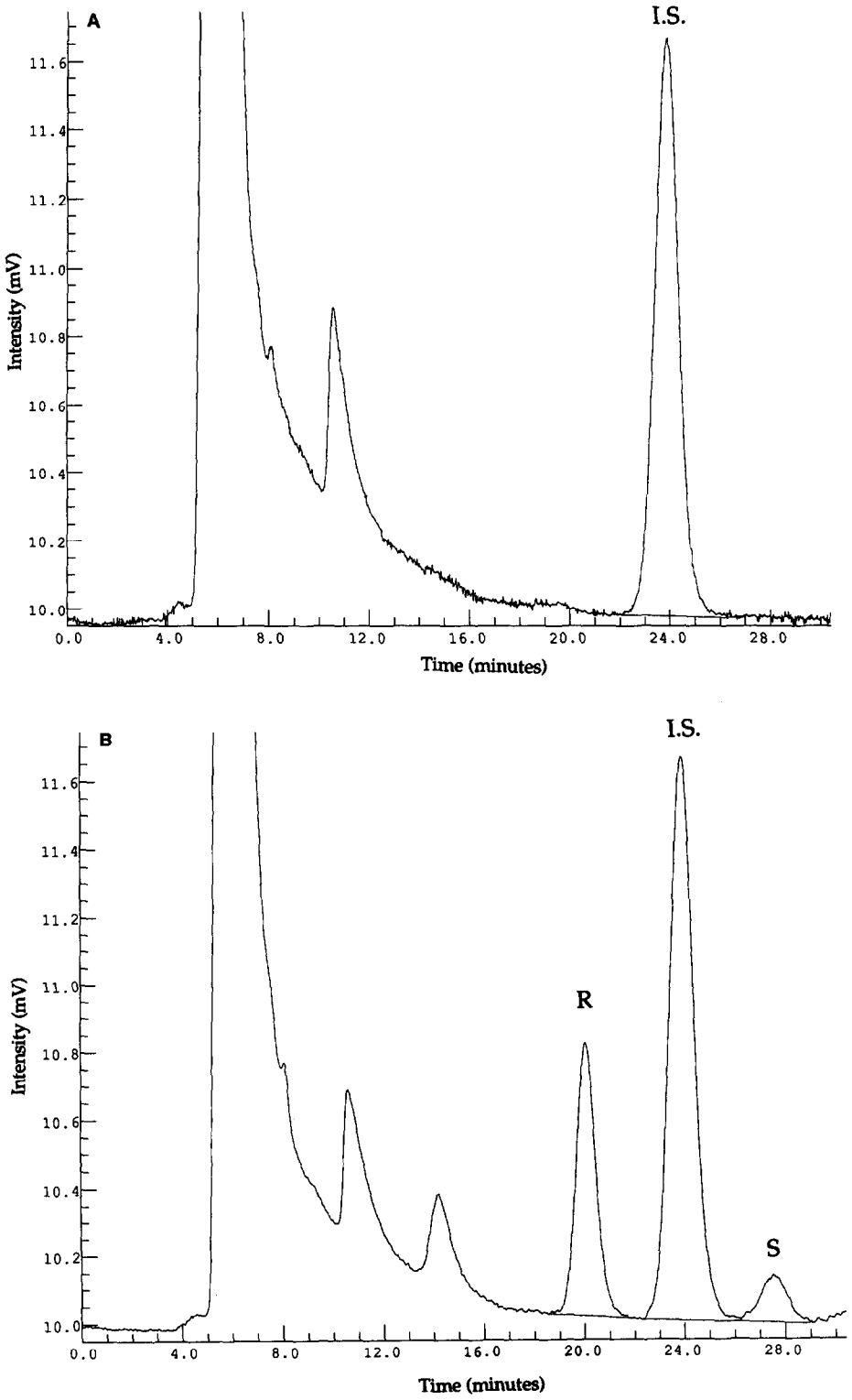

Fig. 2. Chromatograms of extracts from authentic plasma samples (A) before and (B) 120 h after a single dose of 25 mg of *R,S*-warfarin. The latter sample contained 950 nmol/l *R*-warfarin and 240 nmol/l *S*-warfarin.

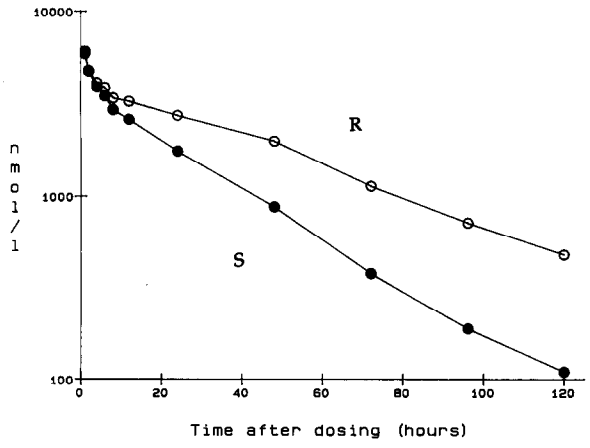

Fig. 3. Plasma concentration–time curve of *R*- and *S*-warfarin after a single oral dose of 25 mg of warfarin to a patient.

example from a plasma curve, obtained after a single dose of 25 mg of warfarin to a patient, is given in Fig. 3.

The stability of the chromatographic system and the durability of the Chiralcel column were excellent. The chiral column could be used for more than a thousand plasma samples without significantly loss of separation efficiency.

Quantitative evaluation

The absolute recovery of the warfarin enantiomers from plasma samples was estimated to be more than 90% when compared with a directly injected solution in mobile phase. The intra-assay precision (n = 10) was 4.4, 3.6 and 9.5% for the 2800, 700 and 80 nmol/l concentration level of the *R*-isomer, respectively. The corresponding figures for the *S*-isomer were 2.6, 4.0 and 13%, respectively. Consequently, 80 nmol/l was taken as the limit of quantitation which was quite sufficient even for pharmacokinetic studies. Peak heights of the *R*- and *S*-enantiomers relative to the internal standard were measured in the chromatogram. Single-point calibration in the daily analysis was employed and was regularly supplemented with full standard curves which were linear from 80 to 5000 nmol/l. The inter-assay precision (n = 28) was determined from quality control samples during fourteen working days. The relative standard deviation was 2.4 and 2.7% for the *R*- and *S*-isomer, respectively, at 1600 nmol/l of each isomer. The accuracy was 98.8 and 99.7% of the initial concentration.

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