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Short communication

New high-performance liquid chromatography method for the determination of (*R*)-warfarin and (*S*)-warfarin using chiral separation on a glycopeptide-based stationary phase

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ABSTRACT

Warfarin is a well-known anticoagulant agent that occurs in two enantiomers, (*R*)-(+)-warfarin and (*S*)-(−)-warfarin. A new liquid chromatography method for the determination of both enantiomers was developed, validated and applied in *in vitro* studies with the aim of evaluating the accumulation of (*R*)-warfarin and (*S*)-warfarin in the hepatoma HepG2 cell line. OptiMEM cell cultivation medium samples and cellular lysates were purified using Waters Oasis® MAX extraction cartridges. The chiral separation of warfarin and the internal standard *p*-chlorowarfarin enantiomers was performed on an Astec Chirobiotic™ V2 column at a flow rate of 1.2 mL/min. The mobile phase was composed of 31% acetonitrile, 5% of methanol and 64% of ammonium acetate buffer (10 mmol/L, pH 4.1). The enantiomers were quantified using a fluorescence detector (λ_{excit} = 320 nm, λ_{emiss} = 415 nm). The limit of detection was found to be 0.121 μmol/L of (*S*)-warfarin and 0.109 μmol/L of (*R*)-warfarin. The range of applicability and linearity was estimated from 0.25 to 100 μmol/L. The precision ranged from 1.3% to 12.2% of the relative standard deviation, and the accuracy reached acceptable values from 95.5% to 108.4%. The new bioanalytical method confirmed the same accumulation of (*R*)-warfarin and (*S*)-warfarin in the hepatoma HepG2 cell line.

1. Introduction

Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin) is one of the most commonly used oral anticoagulants [1]. Given the presence of a centre of chirality at C9, the compound exists in two enantiomeric forms, (*R*)- and (*S*)-warfarin. The drug, however, is administered as a racemic mixture.

(*S*)-warfarin has been reported to have a faster metabolism and an anticoagulant potency that is 2–5 times higher than that of (*R*)-warfarin. The anticoagulant effect is mediated by the inhibition of vitamin K epoxide reductase complex 1 [2]. Phase I metabolism of warfarin is stereoselective [3].

The development of a suitable method for chiral separation is one of the necessary steps that enable further investigation between pharmacological properties and stereochemistry. Reported methods for the determination of warfarin enantiomers include liquid chromatography-tandem mass spectrometry [4,5] or liquid chromatography (LC) with UV [6,7] and fluorescence detection [8]. The chiral separation of warfarin enantiomers utilizes β-cyclodextrin analytical column [9,10] or a cellulose-derivative column [11].

In this paper, we describe the development and validation of a new LC method using chiral separation on a glycopeptide-based stationary phase. Macrocyclic antibiotics, such as vancomycin, have unique structural functions which provide multiple types of interactions, including strong ionic interactions, hydrophobic, hydrogen bonding, dipole stacking, π–π interactions, etc. [12]. The method has been applied to the evaluation of the accumulation of (*S*)-warfarin and (*R*)-warfarin in the hepatoma HepG2 cell line with the aim to correlate intracellular concentrations with the effects on regulation of several genes.

2. Experimental

2.1. Chemical and reagents

Enantiomerically pure (*R*)-(+)-warfarin and (*S*)-(−)-warfarin, racemic warfarin (purity min. 98%) and the internal standard p-chlorowarfarin (Coumachlor Pestanal®, Riedel de Haën, Germany), formic acid (puriss.p.a. for mass spectroscopy), ammonium acetate (puriss.p.a ACS), sodium acetate trihydrate (purum p.a.) and acetonitrile (Chromasolv for HPLC, gradient grade) were obtained from

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Sigma–Aldrich Inc. (St. Louis, MO, USA). Methanol (LiChrosolv, for liquid chromatography), orthophosphoric acid solution (85%, for analysis, ACS) and glacial acetic acid (for analysis, Lab grade ACS) were supplied by Merck (Darmstadt, Germany). A Milli-Q Plus system (Millipore, Bedford, USA) was used to purify water.

2.2. Stock solutions and preparation of calibration samples

The standards of (*R*)-warfarin (0.6667 mg/mL), (*S*)-warfarin (0.6667 mg/mL) and the internal standard p-chlorowarfarin (0.25 mg/mL) were dissolved in methanol, and the stock solutions were stored in the dark at −20 °C for at least three months. The methanolic solutions of the enantiomers were further diluted with methanol. The calibration samples were prepared daily using a blank OptiMEM medium (Gibco/Invitrogen) or blank samples of cellular lysates. The volume of 0.95 mL of a blank sample was spiked with a 0.05 mL aliquot of a diluted stock solution, which gave the concentrations of 0.125, 0.25, 0.5, 1.0 and 2.5 μmol/L (for the measurement of cellular lysate samples) or 2.5, 5, 25, 50 and 100 μmol/L (for the measurement of OptiMEM medium samples) of an enantiomer in the calibration sample. The stock solution of p-chlorowarfarin in methanol (0.25 mg/mL) was diluted daily in the OptiMEM medium to obtain the concentration of 1.2 μmol/L (in cellular lysate samples) or 12 μmol/L (in OptiMEM medium samples).

2.3. Sample preparation procedure

The volume of 0.06 mL of a calibration sample (quality control, medium or cellular lysate sample) was spiked with a blank sample (0.06 mL) and 0.1 mL of the internal standard solution. The samples were acidified with a 0.02 mL of orthophosphoric acid solution (21%). After this step, the samples were purified using Waters Oasis® MAX extraction cartridges (3 mL, 60 mg). The sorbent in the cartridges was equilibrated using 2 mL of methanol and 2 mL of water. The acidified samples (0.2 mL) were loaded onto the cartridges without vacuum. Proteins were removed using 2 mL of sodium acetate buffer (50 mmol/L, pH 7), and the cartridges were subsequently washed with 2 mL of methanol. Warfarin enantiomers and the internal standard were then eluted into micro reaction tubes (Supelco, Bellefonte, PA, USA) with 1 mL of 2% formic acid in methanol. The solvent was evaporated under a stream of nitrogen at 45 °C, and the residues were reconstituted in 0.1 mL of methanol prior to LC analysis. The volume of 0.03 mL (extract of cellular lysate samples) or 0.01 mL (extract of medium samples) was injected into the chromatographic system.

2.4. Apparatus and chromatographic condition

The liquid chromatographic system was composed of a 2695 Waters Separations Module (Waters Corp., Milford, MA, USA) equipped with a Peltier column-thermostat Jet-Stream (Thermotechnic Products), Waters 2475 fluorescence detector and Empower Software for data processing.

(*R*)-warfarin, (*S*)-warfarin and the internal standard p-chlorowarfarin were separated on an Astec Chirobiotic™ V2 column (Supelco, Bellefonte, PA, USA) 5 μm particle size (250 mm × 4.6 mm I.D.) at an isocratic flow rate of 1.2 mL/min. The mobile phase contained 31% of acetonitrile, 5% of methanol and 64% of ammonium acetate buffer (10 mmol/L, pH 4.1). The quantification of warfarin enantiomers was performed using fluorescence detection at excitation and emission wavelengths of 320 and 415 nm, respectively. The peak-area ratios of warfarin enantiomers and the internal standard enantiomers were used for data evaluation.

2.5. Method validation

Validation characteristics of the new bioanalytical method were determined according to the FDA guideline [13]. The equation LOD = 3.3σ/S was used for the determination of the limit of detection (LOD), where the standard deviation of the background (σ) was found using six blank cellular lysate samples, and S was assessed as the slope of the calibration curve. The intra-day and inter-day precision (determined as the relative standard deviation, RSD), the intra-day and inter-day accuracy (assessed as the recovery) were tested using quality control samples (QC). The precision should not exceed 15% of the RSD and the accuracy should be in the range of 85–115%, except for the lower limit of quantification (LLOQ). The regression equation of the calibration curve ($y = kx + q$) was calculated by the least-squares procedure. The peak-area ratios of warfarin enantiomers and the corresponding *p*-chlorowarfarin enantiomers represent the values of y. The lower limit of quantification was determined as the lowest concentration in the calibration curve which was measured with a precision of 20% and accuracy of ±20%.

The recovery of the solid-phase extraction procedure for the warfarin enantiomers and the internal standard enatiomers in the

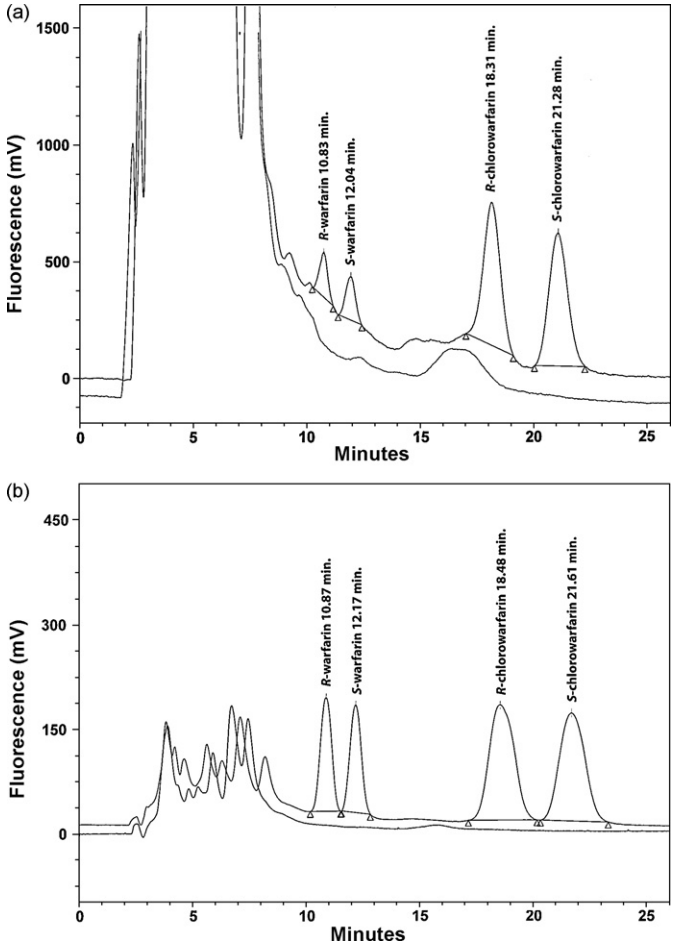

Fig. 1. (a) The chromatograms of extract from blank sample of cellular lysate (lower chromatogram) and blank sample spiked with the standards of enantiomers (upper chromatogram). The peaks of (*R*)-warfarin and (*S*)-warfarin correspond to concentration 0.47 and 0.46 μmol/L, respectively. The concentration of *p*-chlorowarfarin enatiomers was 1.20 μmol/L in the sample. (b) The chromatograms of extract from blank sample of OptiMEM medium (lower chromatogram) and blank sample spiked with the standards of enantiomers (upper chromatogram). The peaks of (*R*)-warfarin and (*S*)-warfarin correspond to a concentration of 12.20 and 12.27 μmol/L, respectively. The concentration of *p*-chlorowarfarin enatiomers was 12 μmol/L in the sample.

Table 1
Intra-batch and inter-batch precision and accuracy of the HPLC method for the determination of (*R*)-warfarin and (*S*)-warfarin in the cellular lysates* and the OptiMEM medium samples.

	(*R*)-warfarin			(*S*)-warfarin		
	Concentration found (mean ± SD; μmol/L)	Precision (RSD; %)	Accuracy (%)	Concentration found (mean ± SD; μmol/L)	Precision (RSD; %)	Accuracy (%)
Concentration spiked (μmol/L) (*n* = 8) intra-batch						
0.25*	0.250 ± 0.015	6.0	100.0	0.254 ± 0.024	9.6	101.7
0.50*	0.497 ± 0.038	7.6	99.3	0.487 ± 0.059	12.1	97.4
1.0*	1.074 ± 0.070	6.5	107.4	1.018 ± 0.089	8.7	101.8
2.5*	2.533 ± 0.055	2.2	101.3	2.574 ± 0.039	1.5	103.0
5.0	5.127 ± 0.129	2.5	102.3	5.000 ± 0.100	2.0	100.0
25.0	25.034 ± 0.353	1.4	100.1	24.84 ± 0.476	1.9	99.4
50.0	49.371 ± 2.275	4.6	98.7	49.809 ± 1.567	3.1	99.6
100.0	100.545 ± 1.304	1.3	100.6	99.683 ± 1.321	1.3	99.7
Concentration spiked (μmol/L) (*n* = 6) inter-batch						
0.25*	0.257 ± 0.031	12.2	102.7	0.248 ± 0.016	6.6	99.3
0.50*	0.505 ± 0.023	4.5	101.0	0.477 ± 0.019	4.0	95.5
1.0*	1.084 ± 0.060	5.5	108.4	0.992 ± 0.110	11.1	99.2
2.5*	2.546 ± 0.049	1.9	101.8	2.386 ± 0.092	3.8	95.5
5.0	5.060 ± 0.102	2.0	101.2	4.970 ± 0.120	2.4	99.3
25.0	24.860 ± 0.920	3.7	99.4	24.96 ± 0.870	3.5	99.9
50.0	49.767 ± 2.500	5.0	99.5	50.588 ± 1.848	3.7	101.2
100.0	99.190 ± 3.346	3.4	99.2	101.674 ± 3.445	3.4	101.7

RSD, relative standard deviation.

OptiMEM medium was tested in triplicate at four concentration levels of the QC samples (5, 25, 50 and 100 μmol/L). The mean extraction recovery was determined by comparing the peak area before and after extraction using the dilution of the compounds in methanol.

The selectivity was assessed by comparing the chromatograms from the blank OptiMEM medium (blank samples of the cellular lysates) and drug-free samples spiked by the enantiomers under study. The stability of warfarin enantiomers in the QC samples was tested at five concentration levels after storage at −70 °C for 11 weeks. The QC samples were then thawed and analyzed in triplicate. To prove stability, the mean recovery at each concentration level had to be within the limit of 85–115% of the spiked concentration.

2.6. Application of the method to in vitro experiments—accumulation experiments in the hepatoma HepG2 cell line

The human Caucasian hepatocyte carcinoma (HepG2) cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). The cells were used within 20 passages after delivery and maintained in the antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate and 1% non-essential amino acids (Sigma–Aldrich, St. Louis, MO). The HepG2 cells (1×10^6) were seeded into 6-well plates 24 h before accumulation experiments. After 24 h in the culture, the medium of the HepG2 cells was aspirated, and the cells were incubated in 600 μL of the prewarmed serum-free OptiMEM medium (Gibco/Invitrogen) with 30 or 50 μM of (*R*)-, (*S*)- or racemic (*E*)-warfarin for 24 h in a humidified incubator. The stock solutions of (*S*)- and (*R*)-warfarin and the racemate were prepared in a sterile dimethyl sulfoxide (DMSO, final concentrations: 10, 30 and 50 mM; maximum DMSO concentration in the media was 0.1%). Accordingly, the control cultures were incubated with 0.1% DMSO. The accumulation of warfarin was terminated by the removal of the medium, and the cells were washed twice with ice-cold Mg^{2+}- and Ca^{2+}-free phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mL). The cells were then lysed with the Cell lysis buffer (25 mM Tris-phosphate, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton® X-100, pH 7.8, Promega, Madison, WI, USA), and the cellular lysate samples were frozen and stored at −70 °C. At least three

Table 2
Validation characteristics of the HPLC method for the determination of (*R*)-warfarin and (*S*)-warfarin.

	(*R*)-warfarin	(*S*)-warfarin
Concentration of enantiomers (μmol/L)	Mean recovery of extraction procedure (%, *n* = 3)	Mean recovery of extraction procedure (%, *n* = 3)
5	91.4	93.7
25	97.3	92.6
50	91.9	90.1
100	90.7	91.9
Linearity of calibration curve (six curves)	The range of concentration 0.125–2.5 μmol/L	The range of concentration 0.125–2.5 μmol/L
Regression equation		
Slope (±SD)	0.876 ± 0.028	0.896 ± 0.028
Intercept (±SD)	0.003 ± 0.023	−0.014 ± 0.022
Correlation coefficient	0.998	0.999
Mean standard error (±SD)	0.041 ± 0.031	0.031 ± 0.037
Linearity of calibration curve (six curves)	The range of concentration 2.5–100 μmol/L	The range of concentration 2.5–100 μmol/L
Regression equation		
Slope (±SD)	0.874 ± 0.022	0.918 ± 0.018
Intercept (±SD)	0.015 ± 0.406	−0.388 ± 0.354
Correlation coefficient	0.999	0.999
Mean standard error (±SD)	0.651 ± 0.326	0.721 ± 0.517

reproducible experiments were performed for each compound in triplicate per concentration.

3. Results and discussion

3.1. HPLC with fluorescence detection

At first the enantioselective separation of (*R*)-warfarin and (*S*)-warfarin was performed on the column with the underivatized β-cyclodextrin bonded to a 5 μm spherical silica gel, and the separation of both enantiomers was evaluated in the polar organic mode. These conditions with some modifications have been successfully applied in a previously published study [4]. Interference from endogenous compounds and a bad resolution of the peaks required a chiral separation in the reversed-phase mode using a different stationary phase. The native cyclodextrins have been described to be inefficient at separating furocoumarin enantiomers in the reversed-phase mode [14], and this finding was in agreement with our experiments. The macrocyclic glycopeptides have shown selectivity for coumarin-based compounds [15,16], but any study employing a vancomycin-based column for the separation of warfarin enantiomers in biological samples has not been reported yet. The thermodynamic study evaluated from an experimental point of view the effect of temperature changes with four macrocyclic glycopeptide chiral stationary phase has been described [17]. The mentioned study was useful for the method development. After optimization of the mobile phase composition, the resolution of warfarin and p-chlorowarfarin enatiomers was acceptable on an Astec Chirobiotic™ V2 column with no interference from the sample matrix. The volume of the sample was only 0.06 mL, and low concentrations of the enantiomers were expected in the samples of the cellular lysates. Therefore, a sensitive method with a sample preparation procedure based on the solid-phase extraction (SPE) was developed. Sample purification by SPE has been demonstrated [18,19]. In this work, Waters Oasis® MAX extraction cartridges containing a poly(divinylbenzene-co-*N*-vinylpyrrolidone) copolymer with strong anion exchange quaternary amine groups on the surface were employed. These reversed-phase and anion-exchange functionalities provide high selectivity for acidic and neutral compounds such as warfarin derivatives. The chromatograms of the sample extracts illustrate the retention times of (*R*)-warfarin, (*S*)-warfarin and enantiomers of the internal standard (Fig. 1a and b).

3.2. The validation parameters

The validation experiments confirmed the suitability of the new bioanalytical method for *in vitro* studies. The limit of detection was found to be 0.109 μmol/L of (*R*)-warfarin and 0.121 μmol/L of (*S*)-warfarin. The applicability of the method ranged from 0.25 (LLOQ) to 100 μmol/L (upper limit of quantification). Precision and accuracy do not cross the allowed limits (see Table 1).

The linearity of the method in two concentration ranges and the regression equations are shown in Table 2.

Mean recoveries of the solid-phase extraction procedure (SPE) for the warfarin enantiomers in the medium were observed between 90.1–97.3% (see Table 2). The extraction recoveries (the mean ± SD, $n = 12$) of the internal standard enantiomers (*R*)-chlorowarfarin and (*S*)-chlorowarfarin (the concentration of 12 μmol/L) were found to be 91.9 (±7.8)% and 88.2(±7.7)%, respectively. HPLC selectivity experiments confirmed no interference in the area of the retention times of the analytes. The stability of the warfarin enantiomers in the QC samples was evaluated in the concentration range 2.5–100 μmol/L. After storage at −70 °C for 11 weeks, the mean concentration of (*R*)-warfarin and (*S*)-warfarin was within the range of 91.6–108.4% of the spiked concentration.

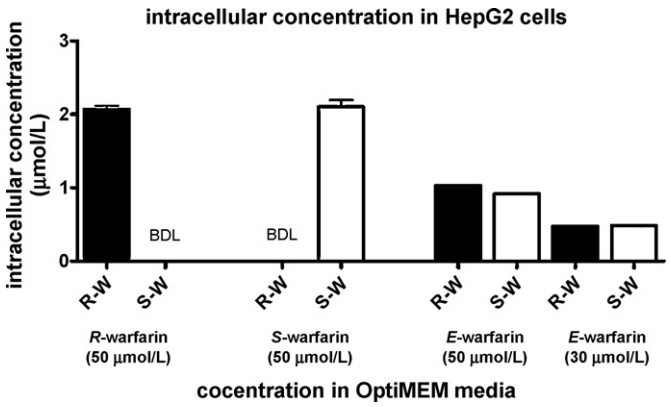

Fig. 2. The concentration of (*R*)- and (*S*)-warfarin in total cellular lysate of hepatoma HepG2 cells. HepG2 cells were exposed to 30 or 50 μM of *R*-, *S*- or racemate warfarin (in 600 μL of serum-free OptiMEM medium) for 24 h. Cells were than washed with ice-cold PBS and cell lysis buffer (100 μL) was added. 60 μL of lysate was subjected to HPLC analysis. Means and standard deviations of (*R*)- and (*S*)-warfarin concentration from at least three independent cellular lysate samples are presented. BDL-below detection limit.

3.3. Results of in vitro studies

The validated method was applied in a pilot project that evaluated the accumulation of (*R*)- and (*S*)-warfarin enantiomers in the hepatoma HepG2 cell line. For this purpose, HepG2 cells were cultivated with (*R*)-, (*S*)-warfarin as well as with the racemate, and the concentration was determined in the medium and in the total cellular lysate samples.

When the HepG2 cells were exposed to either *R*- or *S*-enantiomer, no chiral inversion of an enantiomer to the opposite isomer induced by the cell line occurred. Importantly, the same accumulation of both enantiomers was detected when the racemate was added into the medium (Fig. 2). This finding suggests that both enantiomers have an equal ability to enter the hepatoma cell line. Thus, in the subsequent ongoing experiments with warfarin enantiomers, the stereospecific intracellular effects of the stereoisomer will be discriminated.

4. Concluding remarks

A new bioanalytical method that demonstrated the suitability of macrocyclic glycopeptide-based chiral stationary phase for the separation of warfarin enantiomers has been developed and validated. The validation experiments showed that the analytical method is characterized by acceptable accuracy, precision and sensitivity. The HPLC method was successfully applied in *in vitro* studies, and enabled us to quantify (*R*)- and (*S*)-warfarin not only in the Opti-MEM medium but also in the cellular lysates with a mere 0.06 mL of a sample. *In vitro* experiments confirmed the same extent of accumulation of (*R*)- and (*S*)-warfarin enantiomers in the hepatoma HepG2 cell line.

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