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Journal of Chromatography B, 826 (2005) 75–80
JOURNAL OF CHROMATOGRAPHY B
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A new high-performance liquid chromatographic method for determination of warfarin enantiomers

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Received 23 March 2005; accepted 17 August 2005
Available online 6 September 2005

Abstract

Warfarin is the most common agent used for control and prevention of venous as well as arterial thromboembolism. Although warfarin is administered as a racemic mixture of two stereoisomers (S and R), the S-form is mainly responsible for the anticoagulant effect. The anticoagulant effect of the drug is monitored by analysis of prothrombin complex (International Normalised Ratio,INR). In some cases, however, the measurements of plasma warfarin concentration are needed. Here, we present a new, rapid, sensitive and cost-effective HPLC-method for the determination of warfarin enantiomers in plasma. The chromatographic system consisted of Waters 616 gradient pump, Waters 996 photo diode array detector, Gilson 230 autoinjector and Pirkle (R,R) Whelk-O1 column (25 cm × 4.6 mm I.D., 5 μm). An isocratic mobile phase of methanol/acetonitrile/water (50/10/40, v/v) with 0.1% glacial acetic acid was used. The follow rate was 1 mL/min. Data analysis was carried out with Waters Millennium^{32}. The absorbance at 305 nm was measured with a total run-time of 15 min. Method linearity was studied by establishing regression data containing eight points over the range 0.08–10 μg/mL. In this range, warfarin showed to be linear (r^2 = 0.9997 for S-warfarin and r^2 = 0.9998 for R-warfarin). The limit of detection in plasma was 16 ng/mL for S-warfarin and 18 ng/mL for R-warfarin. Limit of quatitation was defined as 10 × LOD. The extraction recovery was approximately 80%. Also the relation between INR and warfarin concentration was investigated. As expected, there was a low correlation between these two variables (r = 0.23, y = 0.3044x + 0.9712). This method offers a rapid and cost-effective determination of warfarin enantiomers in human plasma.

Keywords: S- and R-warfarin; Oxybenzone; INR

1. Introduction

Vitamin K antagonists have been used as oral anticoagulants for more than 60 years. Even when new drugs are evolving the Vitamin K antagonists are still highly reliable [1]. There are few anti Vitamin K (AVK) drugs on the market; in the Nordic countries, warfarin has a market share exceeding 90%. AVK treatment is used for arterial as well as venous thromboembolism, primary as well as secondary prevention. The most common indications for treatment are arterial fibrillation, mechanical heart valves, deep venous thrombosis and pulmonary embolism. The therapeutic window of warfarin is very narrow. The treatment must be monitored by repeated analysis of prothrombin complex (International Normalised Ratio, INR) because there is a very large variation in dosing required for optimal INR. The difference of weekly dose between patients can be 20-fold. Most of this variation is due to liver metabolism of the drug, concomitant medication and variable ingestion of Vitamin K with food. In some situations, knowledge of the warfarin concentration is valuable for clinical decisions. For example, if after starting treatment, a therapeutic INR is not reached even though the prescribed daily dose is increased or if the INR value on a control visit is unexpectedly low (or high) despite previous stable values for a long time. In both these cases, patient compliance must be in doubt. Other causes for the unwanted INR value may, however, exist and a warfarin concentration can guide further action. Also, the warfarin S-enantiomer has a shorter half-life than the R-enantiomer [2]; thus, the relation gives a hint on time since last ingestion of drug. In rare cases, resistance towards Vitamin K antagonists is due to mutations in the Vitamin K epoxide reductase complex (VKORC1) [3,4]. If the warfarin concentration is high but therapeutic INR is not reached, a mutation in

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doi:10.1016/j.jchromb.2005.08.011

VKORC1 might be the cause. Furthermore, the authors have encountered patients with severe bleedings and high INR when warfarin had not been prescribed to the patient but the patient had nevertheless taken warfarin of fear for thromboembolism. Others have been intoxicated by accident or even intentionally. A warfarin concentration might thus be extremely valuable for both immediate patient care and forensic documentation.

Several methods using high-performance liquid chromatography (HPLC) exist in the literature for the quantification of warfarin enantiomers in human plasma [5–16]. Many of these methods are suffering either from an inappropriate choice of internal standard or from laborious extraction procedures using solid phase extractions with or without liquid–liquid phase extractions. Naproxen is a common anti-pain drug and has been used as internal standard for warfarin [7,10]. This substance can, however, be detected in human plasma from individuals who have taken the drug making it unsuitable as internal standard [8]. Other reported methods [6,9,11,13] have employed solid phase extraction techniques in their sample processing. In those cases, C_{18}-based cartridges with time-demanding conditioning and washing measures have been used. Most of the methods in the literature used columns containing β-cyclodextrins, a widely used stationary phase common in chiral chemistry. We had, however, some difficulties with this phase and chose a Pirkle (R,R) Whelk-O1 column. The latter offered us better reproducibility and stability although this statement may not be valid in all cases. Viewed on the whole, the methods available in the literature could not meet our demands in terms of speed, accuracy, stability and cost-efficiency. In hospital laboratories where large volumes of patient samples are daily analysed, it is often required that a method preferably has a high analytical performance and as few steps as possible. In this paper, a single liquid–liquid phase extraction step with ethyl acetate as the organic component was sufficient to generate pure chromatograms free from interferences. The total run-time per sample in the HPLC was also relatively short (15 min). The limit of detection and the limit of quantitation were comparable to the other reported methods.

We present here a rapid and simple method for the detection of warfarin enantiomers in human plasma using Pirkle (R,R) Whelk-O1 chiral column and oxybenzone as internal standard. Oxybenzone has been used as internal standard for warfarin analysis with β-cyclodextrins [8]. In that specific study, there was some interference in the internal standard peak from early eluting components. The interference showed, however, to be much more disturbing for us when we used the β-cyclodextrins containing column as recommended by the method in the literature. The purpose of this study was therefore to set up a rapid and stable method free from interferences and sufficiently sensitive and reproducible.

2. Experimental

2.1. Chemicals and reagents

All organic solvents and water used in this work were HPLC-grade. Methanol and acetonitrile were purchased from VWR-international (Stockholm, Sweden). Racemic warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin, 98%), acetic acid, ethyl acetate and sulphuric acid (p.a. grade) were purchased from Sigma–Aldrich (Stockholm, Sweden). Oxybenzone (2-hydroxy-4-methoxybenzophenone, ≥98%) was available from Riedel-de Haën (Seelze, Germany).

2.2. Blood samples and plasma preparation

Whole blood was collected, into vacuum tubes containing EDTA, from patients undergoing warfarin treatment. The samples were centrifuged at $2500 \times g$ for 15 min to get cell free plasma. The tubes were kept at −20 °C unless they were analysed in the same day. Plasma for the Quality Controls (QCs) was prepared in the same way from healthy blood donors.

2.3. Standard preparation

Stem solution of warfarin was prepared at 1 mg/mL in methanol. Working solutions at the levels 5, 25 and 50 μg/mL were then prepared from the stem solution. Stem solution at 4 mg/mL and a working solution at 12 μg/mL of oxybenzone (internal standard) were also prepared in methanol. All warfarin and internal standard solutions were stable in the dark at −20 °C for at least 3 months. The solutions for the standard curve were freshly prepared before the analysis. For the standard curve, 900 μL plasma was spiked with 100 μL of warfarin working solution to make final standard concentrations of 0.5, 2.5 and 5 μg/mL. QCs were prepared in the same way at two levels.

2.4. Sample preparation and extraction

Fifty microliters of 12 μg/mL internal standard (Oxybenzone) was added in 1 mL standard, QC or patient plasma. The samples were acidified with 0.7 mL of 0.5 M sulphuric acid. After mixing with 5 mL ethyl acetate, the samples were rigorously vortexed in 1 min and centrifuged shortly to separate the different phases. The upper organic phase was transferred into fresh glass tubes which were pre-washed with 2% RBS 25 and deionised water. The supernatant was ultimately evaporated under nitrogen gas and reconstituted in 120 μL of the mobile phase (see below). Fifty microliters was injected into the HPLC.

2.5. Chromatography

The chromatographic system consisted of Waters 616 pump, Waters 996 photo diode array detector (Waters corp., Milford, MA, USA), 230 XL auto sampler (Gilson Inc., Middleton, WI, USA), chiral column of type Pirkle (R,R) Whelk-O1 column (250 mm × 4.6 mm, 5 μm, Regis Technologies, Morton Grove, IL, USA) and Hypurity C4 guard cartridge (10 mm × 4 mm, 5 μm, Thermo Hypersil-Keystone, Bellefonte, PA, USA). An isocratic mobile phase of methanol/acetonitrile/water (50/10/40, v/v)) with 0.1% glacial acetic acid was used. The follow rate was 1 mL/min. Before analysis, the column was equilibrated with the mobile phase for at least 2 h. Data analysis and valley-to-valley integration was performed with Millennium^{32} (Waters corp., Milford, MA, USA). Linear calibration curves were generated

by setting the concentration of the standards on the *x*-axis and the response ratios of standards/internal standard times the concentration of the internal standard on the *y*-axis. To fit the curve to the calibration data, least-squares fit was used ($y = A + Bx$). The absorbance at 305 nm was measured with a total run-time of 15 min. The diode array detector had the advantage that we could extract different wavelengths from the absorbance spectrum (220–400 nm). In our conditions, wavelength 305 nm showed to be our best choice. We found an interfering peak with the same retention profile as S-warfarin. This peak was identified to be a contaminant originating from the borosilicate glass tubes used in the experiment. We therefore washed all glassware thoroughly with 2.5% RBS 25 followed by deionised water after machine washing.

2.6. Method validation

The linearity of the method was investigated by establishing an initial standard curve containing eight points over the range 0.08–10 μg/mL. Plasma was spiked with known amounts of racemic warfarin in triplicates. The relationship between response and concentration was then examined by least-square method. When the linearity of the method was established, a simpler calibration curve containing three points and covering the expected range of the patient samples was set up (Section 2.3, standard preparation). The latter was then used for analysis of patient samples. The limit of detection (LOD) was defined as the concentration corresponding to a signal to noise ratio of 3:1. The limit of quantitation was defined as 10 × LOD. Recovery was calculated from plasma spiked with warfarin at three levels (0.5 μg/mL, 2.5 and 5.0 μg/mL) ($n = 6$ at each level). After extraction, the response values (peak area) of these samples were compared with unextracted (direct injected) warfarin of the same levels. The coextraction of the internal standard with warfarin was investigated by measuring the area ratios of the internal standard and warfarin after injections of extracted samples each containing 0.5 μg/mL warfarin and 1.2 μg/mL oxybenzone ($n = 6$). Intra-day assay variations were calculated from a set of six pairs of QCs (2.5 and 5.0 μg/mL) originating from the same batch and run in the same day. Inter-day variations were evaluated by running six QC pairs over a period of 6 days.

2.7. Comparing the measured concentrations of warfarin and the International Normalised Ratio

The measured S-warfarin concentrations were plotted against the International Normalised Ratio (INR) of Prothrombin Time (PT) to investigate if there was a correlation between these two variables. The ratios of S- and R-enantiomers of warfarin (S/R) were also calculated to study the clearance profiles of the enantiomers.

3. Results and discussion

3.1. Stationary phase

Warfarin has previously been analysed with Pirkle (R,R) Whelk-O1 columns using both normal and reversed mobile phases [5,6]. We have tested both of these phases and found that the reversed phase, compared to the normal phase mode, gave less interference from the plasma matrix. We have also tested columns containing β-cyclodextrins, a stationary phase which has frequently been employed for warfarin [7,8]. Pirkle (R,R) Whelk-O1 column worked, however, better in our conditions.

3.2. Chromatography and data analysis

The separation and retention pattern of warfarin and the internal standard is shown in a typical chromatogram containing spiked plasma with values near the limit of quantitation (Fig. 1). The separation was complete with a baseline resolution for all analysed peaks (S- and R-warfarin, oxybenzone). The S and R enantiomers were identified according to Henne et al. [6]. All peaks of interest were eluted before 10 min, but further 5 min were required for all late eluting peaks. No interference from endogenous substances or warfarin metabolites was observed. Retention times of the analytes were 4.8 and 5.5 min for S- and R-warfarin, respectively, followed by the internal standard at 6.2 min. A typical patient sample is also shown (in Fig. 2). The

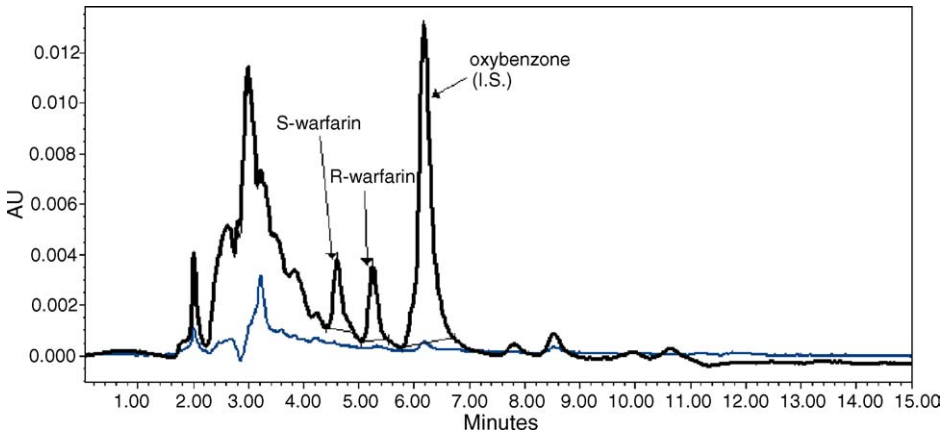

Fig. 1. A spiked chromatogram near the limit of quantitation (thick curve) and a blank (thin curve) on the same scale. The calculated concentrations were 220 ng/mL for S-warfarin and 205 ng/mL for R-warfarin. Retention times were 4.8 and 5.5 min for S- and R-warfarin, respectively, followed by the internal standard at 6.2 min.

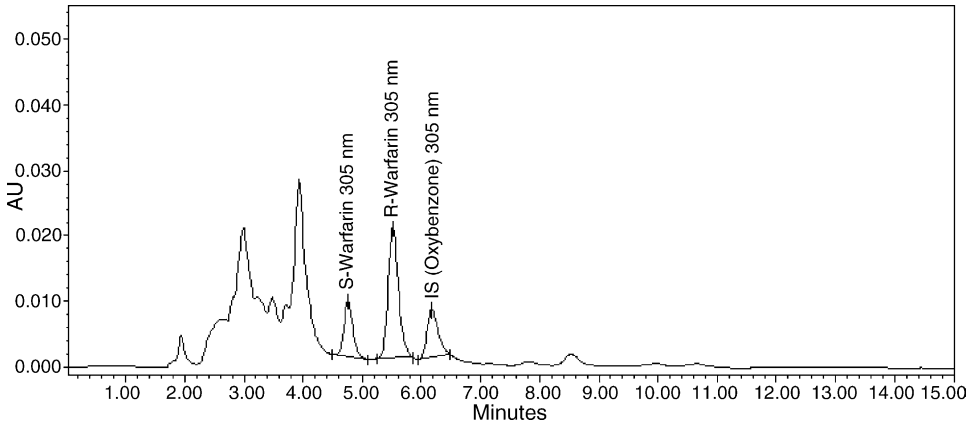

Fig. 2. A typical patient chromatogram. Warfarin values of this sample were 1.1 μg/mL for S-warfarin and 2.4 μg/mL for R-warfarin. The mean ratio of S/R warfarin in the studied population was around 0.6.

Table 1
Extraction recoveries of warfarin in human plasma

Level (μg/mL)	Mean recovery (S-warfarin) (%)	R.S.D.[a]	Mean recovery (R-warfarin) (%)	R.S.D.[a]
0.5 (n = 6)	78.6	5.2	79.3	4.9
2.5 (n = 6)	80.2	4.5	80.9	4.4
5.0 (n = 6)	82.1	4.1	79.7	4.0

[a] Relative standard deviation.

lower values of S-warfarin compared to R-warfarin is explained by the rapid clearance of S-warfarin in patients. We investigated the composition of the mobile phase and found that the proportion of acetonitrile was critical. Without acetonitrile, oxybenzone coeluted with R-warfarin. On the other hand, higher concentrations of acetonitrile (>10%, v/v) gave interference from early eluting components.

3.3. Validation

S- and R-warfarin were linear over the range 0.08–10 μg/mL. Regression data contained eight points with r^2 = 0.9997 for S-warfarin and r^2 = 0.9998 for R-warfarin. The limit of detection in human plasma was 16 ng/mL for S-warfarin and 18 ng/mL for R-warfarin. Limit of quantitation defined as 10 × LOD was calculated to 160 ng/mL for S-warfarin and 180 ng/mL for R-warfarin. The entire population analysed at our laboratory had values well above the calculated LOQ. The extraction recoveries are shown in Table 1. Warfarin was well recovered to approximately 80%. We investigated the coextraction of warfarin and the internal standard, and found that they behaved similarly in the extraction procedure (Table 2). The signal ratio of the internal standard and warfarin was constant (mean = 2.7 and R.S.D. = 3% for both S- and R-warfarin). Intra-day and inter-day assay precision was calculated from the QCs (Tables 3 and 4).

3.4. Examining the relationship between warfarin concentration and INR, and between S- and R-warfarin

The correlation between warfarin concentration and the INR was studied. INR-calibration of PT was performed by a method developed at our laboratory [17]. Values of INR from

Table 2
Investigation of the coextraction of the internal standard (IS) and warfarin enantiomers

Sample no.	Peak area (S-warfarin)	Peak area (IS)	Ratio (IS/S-warfarin)	Peak area (R-warfarin)	Peak area (IS)	Ratio (IS/R-warfarin)
1	363086	1026882	2.83	364522	1026882	2.82
2	357067	973381	2.73	344021	973381	2.83
3	342318	905953	2.65	331648	905953	2.73
4	316792	835072	2.64	309805	835072	2.70
5	341781	883604	2.59	336941	883604	2.62
6	341429	901855	2.64	335131	901855	2.69
Mean	343745	921124	2.68	337011	921124	2.73
S.D.[a]	16058	68308	0.09	17768	68308	0.08
R.S.D.[b]	4.67	7.42	3.24	5.27	7.42	2.92

Concentration for each sample was 0.5 μg/mL (n = 6).
[a] Standard deviation.
[b] Relative standard deviation.

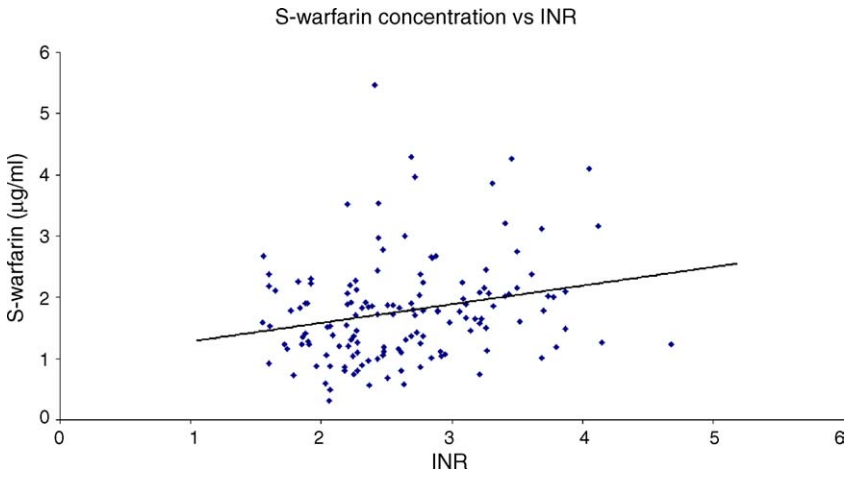

Fig. 3. A plot showing the correlation between the International Normalised Ratio (INR) of prothrombin complex and warfarin concentration. The correlation was low ($r = 0.23$, $y = 0.3044x + 0.9712$).

Table 3
Intra-day precision of the method used for warfarin ($n = 6$)

Nominal concentration in plasma (μg/mL)	Concentration found in plasma (μg/mL) (mean ± S.D.)	R.S.D.[a] (%)	Bias[b] (%)
S-warfarin: 2.5	2.45 ± 0.11	4.49	−2
R-warfarin: 2.5	2.46 ± 0.10	4.06	−1.6
S-warfarin: 5.0	4.92 ± 0.21	4.27	−1.6
R-warfarin: 5.0	4.95 ± 0.22	4.44	−1

[a] Relative standard deviation.
[b] Bias was calculated as: (concentration found in plasma − expected concentration) × 100/expected concentration.

patients undergoing warfarin treatment ($n = 141$) were plotted against warfarin concentrations (Fig. 3). The correlation between INR and warfarin concentration was low ($r = 0.23$, $y = 0.3044x + 0.9712$). This is in line with previous observations [9] and can be explained by interindividual differences caused by differing drug metabolisms, Vitamin K status, age and other factors. The relation between S- and R-warfarin in patients was also studied by plotting the values of S-warfarin on the y-axis and the values of R-warfarin on x-axis (diagram not shown). The following information was obtained from the diagram: $r = 0.67$, $y = 0.3966x + 0.5939$ where the intercept (≈0.6) corresponds to the mean ratio of S- and R-forms in patient plasma.

Table 4
Inter-day precision of the method used for warfarin ($n = 6$)

Nominal concentration in plasma (μg/mL)	Concentration found in plasma (μg/mL) (mean ± S.D.)	R.S.D.[a] (%)	Bias[b] (%)
S-warfarin: 2.5	2.49 ± 0.17	6.8	−0.4
R-warfarin: 2.5	2.51 ± 0.19	7.5	0.4
S-warfarin: 5.0	4.92 ± 0.27	5.5	−1.6
R-warfarin: 5.0	4.98 ± 0.27	5.4	−0.4

[a] Relative standard deviation.
[b] Bias was calculated as: (concentration found in plasma − expected concentration) × 100/expected concentration.

4. Conclusion

The aim of this study was to develop a rapid and cost-effective method for the analysis of both enantiomers of warfarin free from interferences and sufficiently sensitive and reproducible. With covalent bonding of the stationary phase, giving longer column durability and excellent coextraction of the internal standard, this method offers a reproducible and stable detection of warfarin enantiomers in plasma. With optimised mobile phase and extraction procedure, together with Pirkle (R,R) Whelk-O1 column, we solved many problems with matrix interference. At the same time, no laborious solid phase extractions or derivatisations are necessary. This method is well suited for a daily routine analysis of remitted patient samples in medical laboratories.

Acknowledgements

We are grateful to Kerstin Gustafsson for skilful technical assistance. The authors also want to thank doctors Sören Hanssen, Ricci Gotthard, Christer Kihlström, Leif Engquist, Torbjörn Wallén and their staffs and patients at the anticoagulation clinics at the hospitals in Eksjö, Linköping, Motala, Värnamo and Västervik. The study was supported by the Medical Research Council of Southeast Sweden.

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