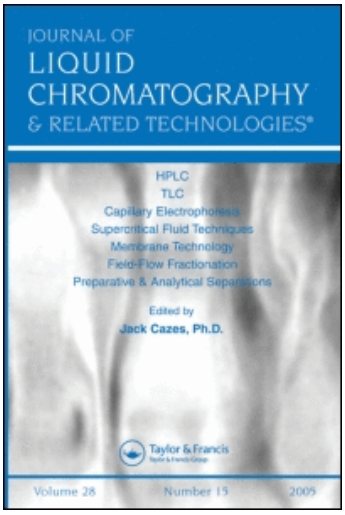

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of (R) and (S) Warfarin in Plasma by High Performance Liquid Chromatography Using Precolumn Derivatization

M. Aycard[a]; S. Letellier[b]; B. Maupas[b]; F. Guyon[b]

[a] Laboratoire Central de Biochimie et de Toxicologie de l'Hôtel Dieu de Paris 1, Place du Parvis-Notre-Dame, Paris Cedex 04, France [b] Laboratoire de Chimie Analytique UFR des Sciences Pharmaceutiques et Biologiques 4, Avenue de l'Observatoire, Paris Cedex 06, France

To cite this Article Aycard, M. , Letellier, S. , Maupas, B. and Guyon, F.(1992) 'Determination of (R) and (S) Warfarin in Plasma by High Performance Liquid Chromatography Using Precolumn Derivatization', Journal of Liquid Chromatography & Related Technologies, 15: 12, 2175 — 2182

To link to this Article: DOI: 10.1080/10826079208016333

URL: http://dx.doi.org/10.1080/10826079208016333

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(12), 2175-2182 (1992)

DETERMINATION OF (R) AND (S) WARFARIN IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING PRECOLUMN DERIVATIZATION

M. AYCARD[1], S. LETELLIER[2],
B. MAUPAS[2], AND F. GUYON[2]
[1]*Laboratoire Central de Biochimie et de Toxicologie de l'Hôtel Dieu de Paris 1*
Place du Parvis-Notre-Dame 75181 Paris Cedex 04, France
[2]*Laboratoire de Chimie Analytique*
UFR des Sciences Pharmaceutiques et Biologiques 4
Avenue de l'Observatoire 75270 Paris Cedex 06, France

ABSTRACT

The enantiomers of warfarin were determined in human plasma after derivatization of warfarin with (-)1-menthylchloroformate to form diastereomeric derivatives and separation by HPLC on a silica column.
The analytical method was employed to determine human plasma level of warfarin enantiomers after administration of racemate .

INTRODUCTION

Many of the drugs used in clinical practice are chiral. Most of time, these drugs are administrated as racemates (mixture of R and S enantiomers).
The great difference in pharmacological effect and pharmacokinetics between two enantiomeric forms of many drugs has emphasized the need for methods allowing enantioselective separation and determination in biological samples (1).
With the development of chromatographic techniques, in particular high performance liquid chromatography (HPLC), the latter has become the method of choice for chiral separation .

Today, chiral separations are mainly carried out by three HPLC methods :
-using chiral HPLC columns.
-using achiral HPLC columns with chiral mobil phase.
- by derivatization with optical reagents and separation on achiral columns.
A method for the determination of both warfarin enantiomers based on enantioselective immunoassay has been reported (2), but exhibited important cross reaction with inactive metabolites.

Proteins as serum albumin and α_1-acid glycoprotein (AGP) are usefull as stationnary phases in the HPLC resolution of enantiomeric compounds, with wide range of applications. Warfarin is resolved on these stationnary phases (3), but poor column efficiency may be a problem in case of small concentration determination.

The purpose of this study was to achieve the chromatographic separation of the warfarin enantiomers with an achiral chromatographic system via diastereoisomeric derivatization . The first step consisted of the quantification of the (+-) warfarin by HPLC, according to a method already adjusted (4) . The second step consisted of the determination of the ratio of the enantiomers with the achiral chromatographic system after a diastereoisomeric derivatization .

Warfarin (α 3-acetonylbenzyl-4 hydroxycoumarin) (schema 1) is a widely used oral anticoagulant drug . In humans, the hypothrombinemic effect of S-(-)warfarin is two to five times more potent than R- (+)warfarine (5, 6) .

Plasma level of (+-) warfarin of treated patients is in the range 1-3 $mg.l^{-1}$.

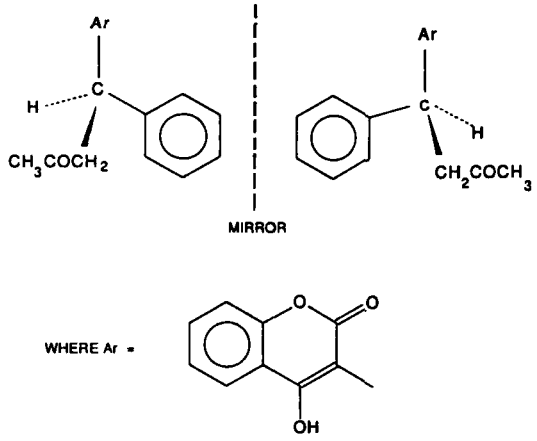

Figure 1 : Chemical structure of warfarin enantiomers

EXPERIMENTAL

Reagents and Standards

Heptan, ethylacetate, dichloromethane, dichloro 1-2 ethane (HPLC grade) are Fluka reagents . All the other reagents were of analytical grade .
(-)1-menthylchloroformate , (+-)warfarin and (+-) 8 chlorowarfarin were obtained from Aldrich . Stock solutions of warfarin and 8 chlorowarfarin (500 $mg.l^{-1}$) were prepared in ethanol.

Apparatus

The IR spectra of KBr pellets were recorded using a Perking Elmer 399 spectrophotometer .
UV-VISIBLE absorption spectra data were obtained using a VARIAN Superscan 3 spectrometer .
^1H NMR spectra were recorded on a Bruker A.M. 500 instrument with tetramethylsilane as an internal standard . Chemical shifts are reported in p.p.m. . The following abreviations are used in describing the spectra : s = singulet, d = doublet, q = quadruplet, m = multiplet, br = unresolved broad signal .
Mass spectra were recorded using desorption-chemical ionization (DCI:NH_3) on a R1010C Nermag (France) mass spectrometer .

Chromatographic conditions

The HPLC system consisted of a Varian 5000 solvent delivery system, equiped with a Rheodyne model 7125 injection valve provided with a 50 µl sample loop, a LC6A (Schimadzu, Japan) UV detector coupled with integrated data analyser Chromatopac C-R 3A (Schimadzu). The detector was set at 310 nm with a sensitivity of 0.05 a.u.f.s.

Racemic warfarin

The separation was performed at ambient temperature, using a 5 µm Spherisorb ODS 2 column (15 cm x 0,46 cm I.D.) The mobile phase consisted of acetonitrile-methanol-acetate buffer (0.05 M, pH = 4), 11:44:45 (v/v). The flow rate used was 1.5 $ml.min^{-1}$.

Diastereomeric warfarin derivatives

The separation was performed at ambient temperature using a 5 µm Nucleosil silica column (Macheray-Nagel) (15 cm x 0,46 cm i.d., specific area 200 m^2g^{-1}) .
The mobile phase consisted of a binary mixture heptane-ethylacetate 93:7 (v/v) . The flow rate used was 1 $ml.min^{-1}$.
The retention time reprocductibility require water concentration stability, the water concentration after chromatographic system equilibrium was measured at 51 ppm .

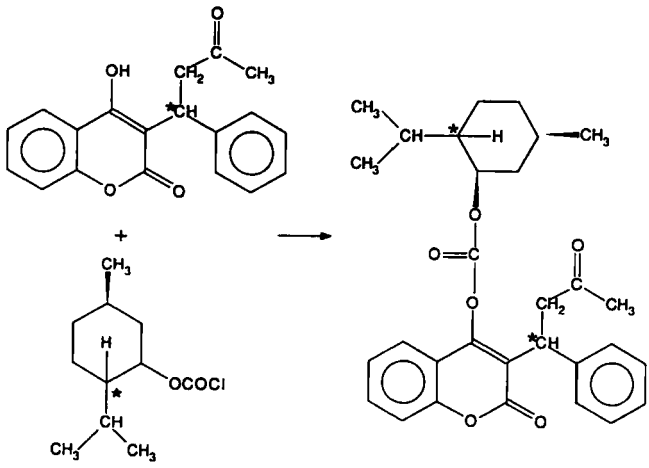

Figure 2 : Derivatization of (+-) warfarin with (-)1-menthylchloroformate

Before used, both mobile phases are filtred under vacuum, with a glass fiber Wathman GF/F 0.5 µm porosity filter .

Derivatization

Carbonates derived from 1-menthylchloroformate and racemic alcohols and amines have been used in preparative resolutions by cristallization .

The hydroxyl group of the (+-)warfarine may react with (-)1-menthylchloroformate to yield a mixture diastereoisomeric carbonates in 94 % yield (figure 2) .

The general procedure for the preparation of carbonates of (+-) warfarin has been previously reported (7) .

A solution of 10 mmoles of (-)1-menthylchloroformate (M.W. 218.73) in 10 ml of dichloromethane is added to 10 mmoles of (+-) warfarine (M.W. 308.32) dissolved in 15 ml of dichloromethane .

After 1.5 ml of triethylamine are added, the reaction mixture is stirred at 25°C for 0.5 hour . The crystalline precipitate of the amine salt is removed by filtration . The filtrate is washed first with hydrochloric acid solution (1 mol.l-1) then with water several times, dried and evaporated (yeld 94 %) .

Sample preparation

Racemic warfarin in plasma

The internal standard solution (25 mg.l^{-1}), 0.05 ml is added to 1 ml of patient's plasma (or spiked blank plasma) followed by 0.1 ml of 1 M hydrochloric acid. The acidified sample is then extracted with 5 ml of dichloromethane by stirring for 10 minutes. After separation of the phases by centrifugation the organic layer is

transfered into an evaporation tube and then evaporate at 40°C under a stream of nitrogen. The residue is redissolved in 0.1 ml of mobile phase, of which 0.05 ml is injected into the liquid chromatograph. A three points standard curve (0.5, 1, 3 $mg.l^{-1}$) prepared in plasma was analysed with each set of samples.

Enantiomers of warfarin in plasma

Same extraction procedure is used than for racemic determination but without internal standard, organic layer is evapored in a 10 ml glass test tube with a teflon cap, 0.1 mmol of 1-menthylchloroformate in dichloromethane and 20µl of triethylamin are added . The resulting mixture was left at 30°C during 20 minutes. After centrifugation the supernatant is washed by 3 ml of 1 M hydrochloric acid and then evaporated .The residue is dissolved in 0.1 ml of mobile phase, of which 0.05 ml is injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Studies of derivatization product

The mass spectrum (DCI-NH3) of the derivatization product has a peak at m/z 508 ($M\text{-}NH^{4+}$), a pseudomolecular ion peak at m/z 490 (MH^{+}) with abundant fragment peaks at m/z 308, 265 .

The NMR spectrum shows twin peaks due to the protons closed to the asymetrics carbons, especially at 2.1 ppm : proton of the methyl group of the CH_3COCH_2- brougth by the warfarin, at 4.6 ppm : proton of the -CHOCO brought by the (-)1-menthylchloroformate, at 4.7 ppm : proton of the asymetric carbon brought by the warfarin .

The IR spectrum shows the caracteristic vibration $\nu_{C=O}$ at 1730 cm^{-1} ; the caracteristic vibration $\nu_{C=O}$ of the carbonate at 1240 cm^{-1} .

The UV spectrum shows a maximum wave lenght at 310 nm (in methanol) with a molecular extinction coefficient of 6200 $mol^{-1}.l.cm^{-1}$.

Many chiral derivatizing agents are commercially available and the resulting diastereomeric products can be resolved on standard achiral stationary phase .

It is very important for indirect resolution of enantiomers by HPLC that the chiral resolving agent has optical purity and no racemization occured in the process of derivatization as well as chromatography .

The chiral 1-menthylchloroformate is usually considered to be a kind of the most usefull resolving reagent .

Chromatography

Chromatographic analysis of racemic warfarin

Under the describerd chromatographic conditions, separation of warfarin and internal standard was performed within 15 minutes. No endogenous interference was noticed because the detection was carried out at 310 nm.

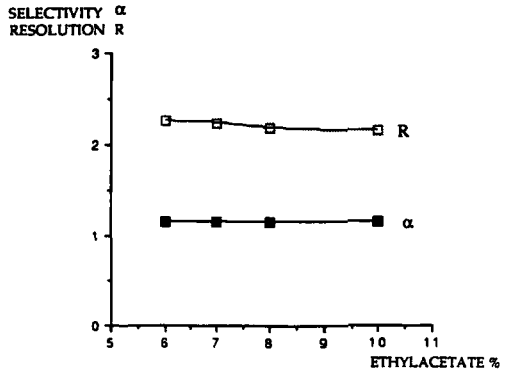

Fig. 3a

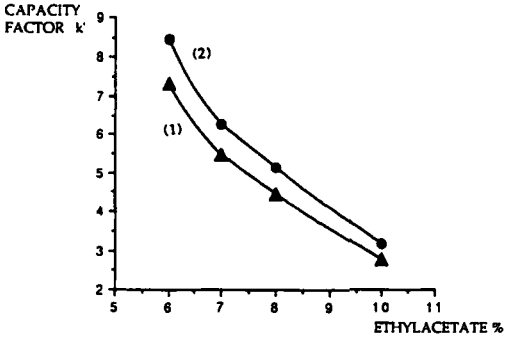

Fig. 3b

Figure 3. Variation of capacity factor k', selectivity α, resolution Rs, for (S) : curve (1) and (R) : curve (2) derivatized warfarin as function of percent ethylacetate in heptan.

A linear response to the UV detector was obtained for up to 10 mg/ml with a correlation coefficient found to be $R > 0.99$.

The limit of determination was defined as the concentration giving a signal-to-noise ratio of 3 and was found to be 15 $\mu g.l^{-1}$.

The intra-day variability (2.5 %) was determined by performing replicate analysis (n = 10) of spikeed plasma samples containing 1 mg/l . The inter-day variability (3.6 %) was determined by assaying two quality control samples each day of analysis, and calculating the relative standard deviation for the whole batch of control samples (n = 10) .

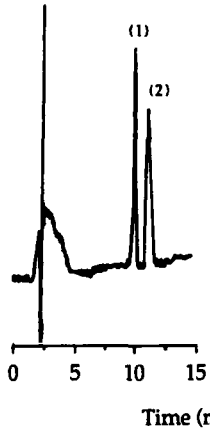

Figure 4. Resolution of the diastereomeric carbonate derivatives of warfarin extracted from human plasma (peaks 1 : (S) and 2 : (R))
column : length : 15 cm; I.D. : 0.46 cm
stationary phase : 5 µm, Nucleosil silica column
mobile phase : heptan-ethylacetate 93:7 (v/v)
flow rate : 1 ml.min^{-1} detection : UV at 310 nm

Chromatographic separation of derivatized warfarin

To optimize the separation, we have studied the influence of the variation of the percentage of ethylacetate in the mobile phase (heptane-ethylacetate v/v) .
Figure 3a shows variation of capacity factors k' of each diastereoisomer as function of ethylacetate percentage. Figure 3b shows variation of selectivity $\alpha = k'_2/k'_1$ and resolution Rs versus ethylacetate percentage in mobile phase.
The best results were obtained using 7% ethylacetate in heptan.
Figure 4 shows chromatographic separation of diastereomeric derivatives of (+-) warfarin.

In conclusion we have developed a sensitive HPLC assay for determination of each enantiomer of warfarin in plasma, using classical stationary phases.
The method has good selectivity and sufficient sensitivity for therapeutic drug monitoring according to plasma level of treated patients. The method allows pharmacokinetic studies of (+) and (-) warfarin after administration of racemic drug.

REFERENCES

1. E.J. Ariens, Drug Intell. Clin. Pharm., 21 : 827- 829 (1987).

2. C.E. Cook , N.H. Ballentine, T.B. Seltzman, C.R. Tallent, J. Pharmacol. Exp. Therap., 210 : 391-398 (1979).

3. S. Allenmark, J. Liq. Chromatogr., 9 : 425 - 442 (1986).

4. B. Maupas, C. Fenech, F. Guyon Analusis , 17 : 468 - 472 (1989).

5. S.Toon, K.J. Hopkins, F.M. Garstang, L. Aarons, A. Sedman, M. Rowland, Clin. Pharmacol.Ther., 42 : 33 - 41 (1987).

6. P. Hoglung, O. Paulsen, S.Bogentoft, Therapeutic Drug Monitoring , 11 : 667 - 673 (1989).

7. G.L. Jeyaraj, W.R. Porter, J. Chromatogr., 315 : 378 - 383 (1984).