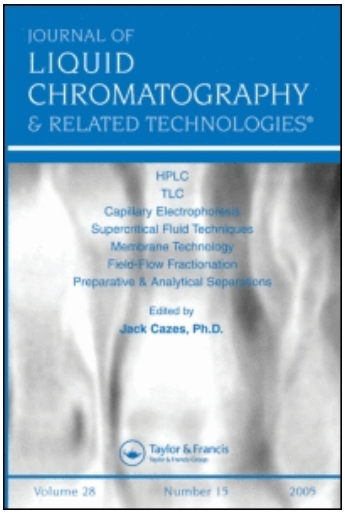


Journal of Liquid Chromatography & Related Technologies

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

A Novel Method for the Quantitation of Warfarin and its Metabolites in Plasma

Michael J. Fasco[a]; Mark J. Cashin[a]; Laurence S. Kaminsky[a]
[a] New York State Department of Health, Division of Laboratories and Research, Albany, New York

To cite this Article Fasco, Michael J. , Cashin, Mark J. and Kaminsky, Laurence S.(1979) 'A Novel Method for the Quantitation of Warfarin and its Metabolites in Plasma', Journal of Liquid Chromatography & Related Technologies, 2: 4, 565 — 575

To link to this Article: DOI: 10.1080/01483917908060085

URL: http://dx.doi.org/10.1080/01483917908060085

JOURNAL OF LIQUID CHROMATOGRAPHY, 2(4), 565-575 (1979)

A NOVEL METHOD FOR THE QUANTITATION OF WARFARIN AND ITS METABOLITES IN PLASMA

Michael J. Fasco , Mark J. Cashin and Laurence S. Kaminsky
Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

ABSTRACT

A procedure for the rapid, quantitative recovery of warfarin and its metabolites (diastereoisomeric alcohol, 4'-, 6-, 7-, 8-benzylic-hydroxywarfarin and dehydrowarfarin) from plasma with Sep-Pak C_{18} cartridges has been developed. A solution of warfarin and its metabolites in plasma was acidified with NH_4OAc buffer (pH 4.85), adsorbed on the Sep-Pak C_{18} resin, washed free of polar constituents and eluted with methanol. Dilution of the eluate with buffer followed by gradient high performance liquid chromatography permitted accurate quantitation of the desired compounds when detected at 313 nm. The recovery of warfarin and each metabolite was greater than 95% over an investigated range of 0.5-10.0 μg/ml of plasma and the limit of quantitation by the assay was 0.1 μg/ml of plasma. For more rapid quantitation of warfarin, without simultaneous analysis of metabolites, the chromatographic parameters were modified so that elution of warfarin occurred within 13 minutes, and the sensitivity of the assay increased to 0.03 μg of warfarin/ml of plasma. The quantitative recovery of warfarin and its metabolites coupled with the chromatographic versatility of the method make it ideally suited for either detailed pharmacokinetic studies or routine plasma analysis of warfarin.

INTRODUCTION

Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one) is clinically utilized as a vitamin K_1 antagonist in the treatment of a number of hematological disorders including

thrombophlebitis, pulmonary embolism and myocardial infarction. The drug is therapeutically administered as a racemate, but its R and S enantiomers have been resolved (1) and demonstrated to exhibit different anticoagulant potencies and to be differentially metabolized in both man and the rat (e.g., 2,3).

Because of its clinical importance, a variety of analytical methods utilizing spectrophotometric (4), fluorometric (5,6), thin-layer chromatographic (TLC) (7), gas-liquid chromatographic (GLC) (8) and high performance liquid chromatographic (HPLC) (9-11) techniques have been developed to measure warfarin levels in plasma. Each of these methods, however, suffers one or more disadvantages including: (a) low and sometimes variable recovery of warfarin and its metabolites; (b) inability to simultaneously quantitate warfarin and all of its known metabolites; (c) inability to quantitate warfarin without interference from its metabolites; (d) poor precision and sensitivity.

Our original HPLC procedure for the analysis of warfarin and its metabolites in plasma (12) did not suffer the disadvantages of other published methods, but did produce an increase in column pressure due to incomplete removal of protein and lipid components resulting in shortened column life. We have consequently developed a method for the quantitative recovery of warfarin and its metabolites from plasma and have employed the technique in the analysis of over one hundred samples without any abnormal increase in column pressure.

MATERIALS AND METHODS

Apparatus

A Waters Associates Model 204 Liquid Chromatograph was equipped with an additional Model 6000 A pump, Model 660 Solvent Programmer, WISP 710 autoinjector, and Spectra-Physics 4000 recording integrator. Warfarin and its metabolites were detected at 313 nm. The analytical column was a Waters Associates μBondapak C_{18}. Solvents were deionized, double distilled water and HPLC grade acetonitrile (Fisher Scientific).

Compounds

Racemic warfarin was purchased from Calbiochem and the R and S enantiomers resolved by the method of West et al. (1) to optical purities of 100% and 101%, respectively, based on published rotation values. 4'-, 6-, 7-, and 8-Hydroxywarfarin were synthesized as previously described (12). The R,R; R,S, and S,S; S,R warfarin alcohols were prepared by the method of Trager et al. (13). The compound tentatively identified by Pohl et al. (14) as benzylic hydroxywarfarin was prepared by the in vitro rat hepatic microsomal metabolism of R warfarin using pregnenolone-16α-carbonitrile induced microsomes and cumene hydroperoxide as a source of activated oxygen (15). The isolation of benzylic hydroxywarfarin was performed by preparative HPLC in a manner analogous to that described for dehydrowarfarin (15). The extinction coefficient of warfarin at 309 nm in 0.1 M NaOH (11,540 M^{-1} cm^{-1}) was assumed to be equivalent to that of benzylic-hydroxywarfarin, since these two compounds have qualitatively the same uv spectrum in both acid and base solution. Dehydrowarfarin was synthesized by the reaction of warfarin with freshly prepared cuprous chloride in pyridine (15).

Sep-Pak C_{18} Chromatography

Stock solutions of warfarin and each metabolite were prepared in acetonitrile at a concentration of 2.0 mg/ml. An aliquot (0.1 ml) of each was pooled and the mixture diluted to a final volume of 2.0 ml with 0.02 M NaOH. Variable volumes of this solution were added to 1.5 ml of plasma and equilibrated at room temperature for 30 min before extraction.

A Sep-Pak C_{18} cartridge (Waters Associates) was attached to a graduated 5.0 ml glass syringe and washed successively with approximately 4.0 ml each of methanol, water, methanol and 1.5% HOAc brought to pH 4.85 with conc. NH_4OH (Buffer A). The 1.5 ml of spiked plasma was diluted with an equal volume of Buffer A and the mixture passed through the Sep-Pak C_{18} cartridge at a flow rate of 4-5 ml/min. The resin was washed with 4.0 ml of Buffer A and dried by forced passage of air from the air space between the

buffer and syringe plunger head. The syringe was filled to the 4.0 ml mark with MeOH, the first 0.5 ml of eluate was discarded, and warfarin and its metabolites collected in the next 1.5 ml fraction. The latter was then diluted with 1.5 ml of Buffer A and an aliquot (50-300 µl) analyzed for warfarin and its metabolites.

Coagulation Tests

One-stage prothrombin assays were performed with a Fibrometer. Fibroplastin (Becton, Dickinson and Co.) was used to restore coagulant activity to plasma samples.

HPLC Chromatography

Method 1

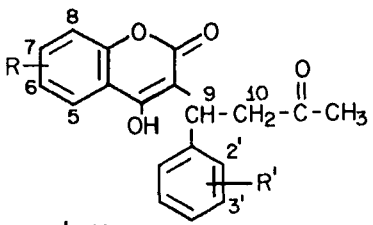
Warfarin and its metabolites were separated by a modification of our originally published procedure (12,16). Buffer B was prepared from Buffer A by dilution with an equal volume of acetonitrile. The initial condition was 20% Buffer B and the final condition 62% Buffer B. The flow rate was 2.0 ml/min throughout and the nonlinear gradient number 7 was run for 10 min. The initial buffer condition was retained for approximately 2 min, and during the next 8 min the Buffer B concentration increased in a near linear manner until the final condition was reached. Warfarin and its metabolites were identified by their retention times and quantitated using the integrator previously calibrated in the external standard mode. Retention times of the various compounds did not significantly vary during analysis of multiple samples over 20-24 hr of continuous chromatography.

Method 2

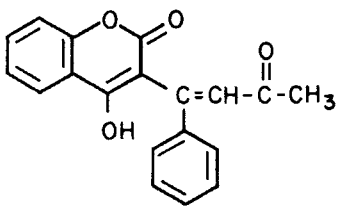
The more rapid analysis of warfarin which does not separate its metabolites from one another was performed at an initial condition of 20% Buffer B and a final condition of 90% Buffer B. The flow rate was maintained at 2.0 ml/min and the gradient was the same as that of Method 1, except the run time was 5 min. A column reequilibration time of 7 min was used in both methods.

RESULTS AND DISCUSSION

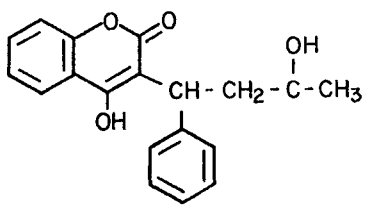
The structures of warfarin and its various metabolites are illustrated in Figure 1. The recoveries of each compound obtained from duplicate experiments are presented in Table 1. Due to a lack of sufficient quantities of 8- and benzylic-hydroxywarfarin, recovery studies of these warfarin metabolites were performed at 2.0 µg/ml plasma only, and their recoveries were equivalent to those of the other warfarin metabolites.



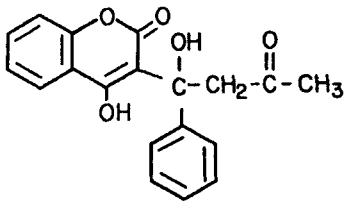
R = R' = H
R = 6-OH, R' = H
R = 7-OH, R' = H
R = 8-OH, R' = H
R = H, R' = 4'-OH



Dehydrowarfarin



Warfarin alcohols -
R,R-R,S ; S,S-S,R diastereoisomers



Benzylic-OH
warfarin

FIGURE 1

Warfarin and metabolite structures.

TABLE 1

Recovery of Warfarin and Its Metabolites from Rat Plasma

Concentration added to plasma (μg/ml)	Warfarin or Metabolite[1] Recovered (% theoretical)						
	Warfarin	Dehydro-warfarin	S,S;R,S Alcohols[2]	R,R;S,R Alcohols[2]	4'-OH	6-OH	7-OH
0.5	100; 100	102; 106	112; 108	92; 100	96; 104	102; 98	104; 104
1.0	97; 99	93; 95	96; 96	94; 96	87; 89	96; 97	95; 95
2.0	105; 105	100; 100	100; 100	105; 105	100; 100	105; 105	105; 105
5.0	94; 94	92; 92	96; 96	104; 104	92; 94	98; 96	94; 94
10.0	95; 94	91; 92	96; 102	100; 102	96; 98	98; 98	97; 97
Average %[3]	98.3	96.3	100.2	100.2	95.6	99.3	99.0
± SD	± 4.3	± 5.3	± 5.7	± 4.7	± 5.3	± 3.4	± 4.9

Values represent single analysis of duplicate experiments.

[1]Benzylic- and 8-hydroxywarfarin at a concentration of 2.0 μg/ml of plasma were 98% recovered.

[2]The concentration of each pair of diastereoisomers was one-half that cited.

[3]Averages were taken after an analysis of variance showed that recoveries were independent of added concentrations.

A typical HPLC elution pattern of warfarin and its metabolites in rat plasma following Sep-Pak C_{18} chromatography is illustrated in Figure 2. There were trace amounts of endogenous contaminants which eluted in the region of dehydrowarfarin and the warfarin alcohol diastereoisomers, but they did not significantly interfere with the accurate integration of these compounds. These contaminants were absent in human plasma.

Elution profiles of a standard solution of warfarin and its metabolites and of a rat plasma sample drawn 16 hr after oral administration of sodium warfarin (10 mg/kg), which were obtained

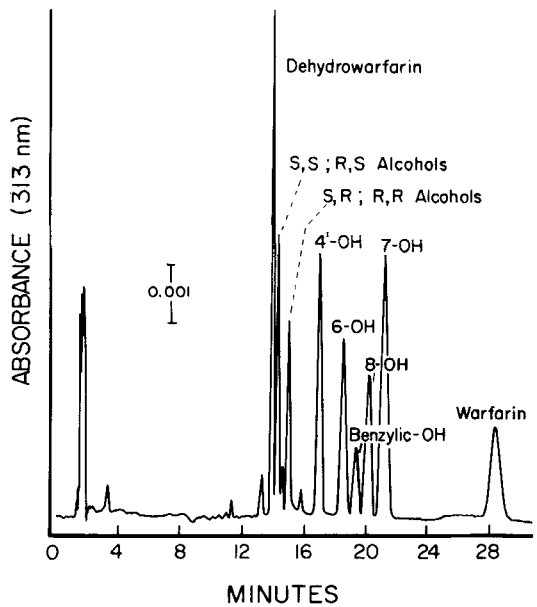


FIGURE 2

Warfarin and metabolite standards extracted from rat plasma by Sep-Pak C_{18} chromatography and separated by Method 1. The concentrations of dehydrowarfarin, 4'-, 6-, 7-hydroxywarfarin and warfarin were 2.0 µg/ml of plasma. The alcohol diastereoisomers, benzylic- and 8-hydroxywarfarin were 1.0, 0.5, and 0.9 µg/ml of plasma, respectively. The injection volume was 0.1 ml, the absorbance 0.12 AUFS, and the chart speed 0.5 cm/min.

by the more rapid chromatographic Method 2, are shown in Figure 3. Under these chromatographic conditions many of the warfarin metabolites are poorly resolved from one another and cannot be accurately quantitated. Warfarin, however, was completely separated from all its metabolites and eluted as a sharp peak. The concentration of warfarin in the citrated plasma sample was 1.8 µg/ml and represents a 0.1 ml injection of the Sep-Pak C_{18} eluate plus buffer dilution. The lower limit of quantitation of warfarin by this method is 0.03 µg/ml of plasma.

The precision of the assay for warfarin concentration in human plasma utilizing Sep-Pak C_{18} extraction and HPLC Method 2 was demonstrated by triplicate analysis of a pool of human plasma

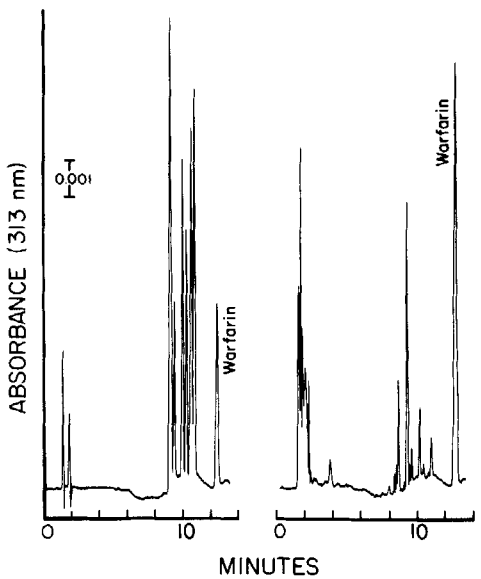


FIGURE 3

Left. Warfarin (0.05 µg) and metabolite standards chromatographed by Method 2. Right. Sep-Pak C_{18} extracted plasma of rat which had received R-warfarin. The concentration of R-warfarin was 1.5 µg/ml of plasma. The injection volume was 0.1 ml, the absorbance 0.12 AUFS, and the chart speed 0.5 cm/min.

TABLE 2

Patient	Sex	Age (years)	One-stage prothrombin time (sec)	Warfarin[3] concentration (μg/ml citrated plasma)
Control[1]	---	---	14.0	---
A[2]	F	52	18.9	0.42
A[2]	F	52	19.6	0.34
B[2]	M	64	17.6	0.45
B[2]	M	64	17.2	0.52
C	M	58	18.7	0.53
D	F	48	21.6	0.53
E	F	68	22.9	1.21

[1]Control was pooled human plasma.

[2]Same patient analyzed at different times.

[3]Plasma samples were obtained 1-3 hours after injestion of a maintenance dose of warfarin.

prepared from four patients on warfarin therapy. The one-stage prothrombin time of the pooled plasma was 19.4 sec and the warfarin concentration was 0.57 μg ± 0.02/ml citrated plasma. Plasma levels and one-stage prothrombin times were also determined on five patients on chronic warfarin therapy (Table 2). These data demonstrate the individual anticoagulant responses to varying warfarin plasma levels and suggest that more detailed investigations be performed at the clinical level.

It is becoming increasingly apparent that routine, or at least occasional, therapeutic monitoring of plasma levels of chronically administered drugs is necessary to ensure maximal drug activity with minimal side effects. Moreover, detailed plasma pharmacokinetic studies are required to determine concentrations of the parent compound and of pharmacologically active metabolites and the influence of other drugs on these concentrations. This is particularly true of warfarin whose anticoagulant potency is extremely sensitive to the presence of a variety of xenobiotic compounds. The influence of certain of these xenobiotics on rates of R and S warfarin clearance and concentration of plasma metabolites in rats is currently under investigation.

The Sep-Pak C_{18} extraction of warfarin and its metabolites from plasma is rapid, efficient and inexpensive. These features coupled with the widespread use of HPLC in clinical laboratories make it possible to readily analyze warfarin plasma levels in addition to its anticoagulant effect.

ACKNOWLEDGMENTS

The authors wish to thank Dr. David Brown, Albany Medical Center Hospital, Albany, New York, for providing the human plasma samples used in this investigation. This work was supported by NIH Research Grant HL 19772, awarded by the National Heart, Lung and Blood Institute, PHS/DHEW.

REFERENCES

1. West, B.D., Preis, S., Schroeder, C.H. and Link, K.P., J. Am. Chem. Soc., 83, 2676, 1961.
2. O'Reilly, R.A., Clin. Pharmacol. Ther., 16, 348, 1974.
3. Yacobi, A. and Levy, G., J. Pharmacokin. Biopharm., 2, 239, 1974.
4. O'Reilly, R.A., Aggeler, P.M., Hoag, M.S. and Leong, L.L., Thromb. Diath. Haemorrh., 8, 82, 1962.
5. Corn, M. and Berberich, R., Clin. Med., 13, 126, 1967.
6. Nagashima, R. and Levy, G., J. Pharmaceu. Sci., 58, 845, 1969.
7. Lewis, R.J., Ilnicki, L.P. and Carlstrom, M., Biochem. Med., 4, 376, 1970.
8. Midha, K.K., McGilveray, I.J. and Cooper, J.K., J. Pharmaceu. Sci., 63, 1725, 1974.
9. Vesell, E.S. and Shively, C.A., Science, 184, 466, 1974.
10. O'Reilly, R.A. and Motley, C.H., Fed. Proc., 35, 756, 1976.
11. Wong, L.T., Gnanaprakasam, S. and Thomas, B.H., J. Chromatogr., 135, 149, 1977.
12. Fasco, M.J., Piper, L.J. and Kaminsky, L.S., J. Chromatogr., 131, 365, 1977.
13. Trager, W.F., Lewis, R.J. and Garland, W.A., J. Med. Chem., 13, 1196, 1970.
14. Pohl, L.R., Garland, W.A., Nelson, S.D. and Trager, W.F., Biomed. Mass Spect., 2, 23, 1975.
15. Fasco, M.J., Piper, L.J. and Kaminsky, L.S., J. Med. Chem., 21, 1054, 1978.
16. Fasco, M.J., Vatsis, K.P., Kaminsky, L.S. and Coon, M.J., J. Biol. Chem., 253, 7813, 1978.