Journal of Chromatography, 378 (1986) 254—260
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3081

Note

Reversed-phase high-performance liquid chromatographic method for the determination of warfarin from biological fluids in the low nanogram range

J.M. STEYN* and H.M. VAN DER MERWE

Department of Pharmacology, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300 (South Africa)

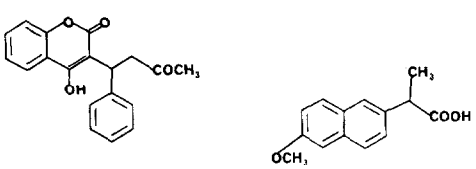
and

M.J. DE KOCK

Department of Microbiology, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300 (South Africa)

(First received October 7th, 1985; revised manuscript received January 2nd, 1986)

Warfarin, 3-α-phenyl-β-acetylethyl-4-hydroxycoumarin (Fig. 1), a drug that finds extensive use as an anticoagulant in man, is largely bound to plasma proteins, only about 0.5% being unbound [1]. This fact renders it particularly susceptible to interaction with other drugs, like some of the non-steroidal anti-inflammatory agents that may compete with it for protein binding sites. Such a competition may lead to an increase in the protein unbound concentration of the drug with a corresponding increase of anticoagulant effect, often with serious adverse effects on the patient. It is therefore important to be able



Warfarin Naproxen

Fig. 1. Structures of warfarin (3-α-phenyl-β-acetylethyl-4-hydroxycoumarin) and the internal standard, naproxen [(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid].

0378-4347/86/$03.50

to determine the interaction potential of any drug that may be administered concomitantly with warfarin.

Since the unbound concentration of warfarin for the therapeutic range of 1–4 μg/ml is in the order of 10—20 ng/ml, a very sensitive, accurate and precise method that would be able to detect an increase in the unbound fraction of warfarin is needed. This becomes especially important if possible interactions were to be studied in the in vivo situation in patients being maintained on warfarin therapy and if radioactively labelled warfarin cannot be administered.

Several high-performance liquid chromatographic (HPLC) methods exist for the determination of the total amount of warfarin in the therapeutic concentration range [2–12] in plasma or serum. None of these, however, is sensitive enough to allow the determination of warfarin in the concentration range that is expected for the unbound fraction in plasma or serum. The procedure described by Lee et al. [13], whereby warfarin fluorescence is enhanced by post-column acid/base manipulation, offers the possibility for the determination of the drug in the expected concentration range. Although the authors justifiably claim a very low detection limit (0.18 ng), no statistical data as to the accuracy and precision of the method when applied to the analysis of warfarin from ultrafiltrate or ultracentrifugate in the low nanogram range are offered (the two media mentioned being applicable to the study of protein-unbound warfarin).

This paper describes a reversed-phase HPLC method for the determination of warfarin using a variation of the acid/base manipulation technique described by Lee et al. [13]. This method is easy to perform, sensitive, accurate and precise and is suitable for the determination of warfarin in the low nanogram range from serum or plasma ultrafiltrate or ultracentrifugate as well as for the determination of total warfarin from plasma or serum. The method described is therefore ideally suited to the study of possible interactions between warfarin and any concomitantly administered drugs without recourse to the use of radioactively labelled warfarin. This is a distinct advantage since the use of radioactively labelled warfarin of which the radiochemical purity is not known may lead to erroneous conclusions [14, 15].

EXPERIMENTAL

Apparatus

Chromatography was carried out on a μBondapak C_{18} Radial Pak® reversed-phase column (10 μm) held in an RCM 100 radial compression unit (Waters Assoc., Milford, MA, U.S.A.). A Perkin-Elmer Series 3B liquid chromatography pump (Norwalk, CT, U.S.A.) was used to pump the mobile phase at 1.5 ml/min, while a Gow-Mac Model 080-19 pump (Gow Mac, Bridgewater, NJ, U.S.A.) was used to pump the reagent at 0.5 ml/min. The sample was introduced into the system via a Valco injection valve fitted with a 150-μl injection loop. Detection was by means of a Perkin-Elmer Model 650/105 fluorescence detector operated at an excitation wavelength of 320 nm and an emission wavelength of 390 nm. Slit width and sensitivity settings were adjusted according to the concentration range under investigation.

Peaks were recorded on a Unicorder Model U-125 recorder (Nippon Denshi

Kagaku, Japan). A Beckman Model L8M ultracentrifuge with a Type 75Ti rotor was used for the preparation of plasma and serum ultracentrifugate in polyallomer centrifuge tubes (Beckman, Palo Alto, CA, U.S.A.).

Materials

Warfarin (sodium salt) was obtained from Allen & Hanbury (Germiston, South Africa) while the internal standard, naproxen, (+)-6-methoxy-α-methyl-2-naphthaleneacetic acid (Fig. 1), was obtained from Syntex Labs. (Palo Alto, CA, U.S.A.). Ammonium dihydrogen phosphate, hydrochloric acid, triethanolamine and acetonitrile (analytical-reagent grade) were obtained from E. Merck (Johannesburg, South Africa). Anaesthetic diethyl ether was distilled before use. Water was double-distilled and filtered before use.

Stock solutions

An accurately weighed-out portion of warfarin (sodium salt) was dissolved in the appropriate volume of distilled water and diluted to obtain a final solution containing 1 ng/μl warfarin free acid. This solution was used for the preparation of standards for the lower concentration range (1- 25 ng/ml), while a solution containing 500 ng of free warfarin per 100 μl was prepared for the preparation of standards in the higher concentration range (0.5- 5.0 μg/ml). The internal standard solutions for the two concentration ranges were prepared by dissolving an accurately weighed portion of naproxen in the minimum amount of 0.1 *M* sodium hydroxide followed by appropriate dilution to obtain solutions containing 25 ng per 10 μl for use in the lower concentration range and 250 ng per 10 μl for use in the higher concentration range.

All these solutions were subdivided in 2-ml aliquots contained in 5-ml amber glass ampoules that were flame-sealed and stored in the dark at - 20° C until used.

Mobile phase

The mobile phase consisted of acetonitrile- 0.1 *M* ammonium dihydrogen phosphate (63:37) and was filtered before use.

Ultracentrifugate

Drug-free serum was centrifuged at 45 000 rpm at 15° C (203 000 *g* average) for 20 h in 11.5-ml polyallomer tubes. The top 3 ml of the supernatant was used for the preparation of standard and control solutions.

Extraction and assay procedure

Low concentrations. To 1 ml ultracentrifugate, unknowns, controls or standards in 10-ml glass-stoppered centrifuge tubes were added 10 μl (25 ng) of internal standard solution, followed by 250 μl of 4 *M* hydrochloric acid. Extraction was done with 5 ml distilled diethyl ether by rotating for 15 min on a rotating shaker revolving at 30 rpm. After centrifuging at 700 *g* for 5 min, the diethyl ether was transferred to 5-ml disposable glass ampoules and evaporated to dryness at 40° C under a stream of high-purity nitrogen. The residues were dissolved in 100 μl mobile phase and the whole volume was injected onto the column.

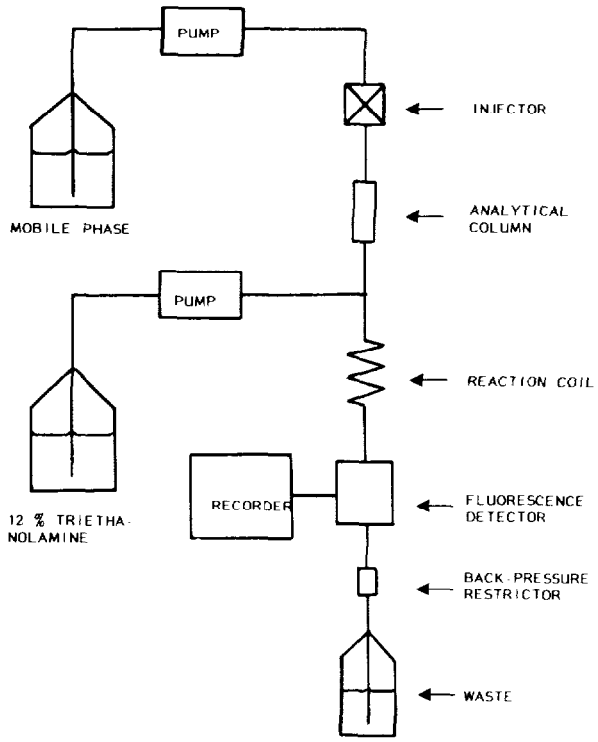


Fig. 2. Flow diagram for the post-column acid/base manipulation of warfarin.

Therapeutic concentrations. Total warfarin in plasma or serum was determined by adding 10 μl internal standard solution (250 ng naproxen) and 50 μl of 4 *M* hydrochloric acid to 100 μl serum or plasma (unknowns, standards or controls) and treating it as described above. Of the final extract 30 μl were injected onto the column.

Post-column acid/base manipulation. Fluorescence enhancement of warfarin was achieved by treating the column effluent with a 12% solution of triethanolamine (Fig. 2).

RESULTS AND DISCUSSION

Extraction efficiency

Drug-free ultracentrifugate (1 ml) was spiked with 50 ng warfarin, extracted and treated as described and injected onto the column. The peak height obtained was compared with the peak height obtained when 50 ng of warfarin were injected without extraction. Extraction efficiency was determined as 70%. This figure reflects the efficiency one would expect during a normal operational situation. The true extraction efficiency would probably be better since it is practically impossible to transfer all the diethyl ether after extraction and since it is likewise impossible to inject all of the redissolved extract without recourse to rinsing procedures — an action that may lead to unacceptable peak-broadening as a result of the large injection volume. The use of dichloromethane was found to result in a slightly better extraction efficiency (75%),

but diethyl ether was preferred owing to the practial consideration of it being easier to transfer after extraction than is dichloromethane.

Quantitation

A four-point calibration graph spanning the expected concentration range (1—25 ng/ml for protein unbound warfarin and 0.5—5 μg/ml for total warfarin) was constructed for each analysis run. Quantitation was done by means of the internal standard method utilizing peak heights. The calibration graphs for both concentration ranges conform to the equation for a straight line with a correlation coefficient better than 0.99 for both concentration ranges ($y = -0.073 + 10.251x$ with $r^2 = 0.9998$ for the concentration range 0—25 ng/ml and $y = 0.012 + 1.805x$ and $r^2 = 0.9985$ for the concentration range 0.5—5 μg/ml. The calibration graphs were tested for linearity and found to be linear up to at least 100 ng/ml and 10 μg/ml for the two respective concentration ranges.

Performance of the method

To evaluate the performance of the method, control solutions of warfarin were made up in drug-free serum ultracentrifugate for the lower concentration range and in drug-free serum for the higher concentration range and analysed on several occasions. The results for the two concentration ranges are presented in Tables I and II, respectively.

The method performs well with regard to both accuracy and precision, as is evident from the coefficient of variation of only 11.3% for the 2 ng/ml level

TABLE I

ACCURACY AND PRECISION OF THE METHOD AT LOW CONCENTRATIONS

Control No.	Concentration expected (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	*n*	Coefficient of variation (%)
Q1	0.65	0.61 ± 0.161	4	26.6
Q2	1.00	1.02 ± 0.165	6	16.2
Q3	1.99	1.86 ± 0.21	6	11.3
Q4	4.13	4.06 ± 0.39	6	9.6
Q5	8.25	7.69 ± 0.23	6	3.0
Q6	17.81	17.04 ± 0.45	6	2.6

TABLE II

ACCURACY AND PRECISION OF THE METHOD AT THERAPEUTIC CONCENTRATIONS

Control No.	Concentration expected (μg/ml)	Concentration found (mean ± S.D., $n = 6$) (μg/ml)	Coefficient of variation (%)
Q7	5.59	5.52 ± 0.156	2.8
Q8	3.38	3.29 ± 0.12	3.7
Q9	1.41	1.35 ± 0.027	2.0
Q10	0.57	0.55 ± 0.016	2.8

and 16.2% for the 1 ng/ml level and the close correlation between the concentration expected and found.

Detection limit

The detection limit for this method, defined as 2S.D. obtained at zero concentration from the intercept of a straight line plot of standard deviation of the mean versus plasma concentration of replicate spiked samples in the concentration range approaching the expected limit of detection, was found to be 0.3 ng/ml [Table I (controls Q1, Q2 and Q3) and Fig. 3].

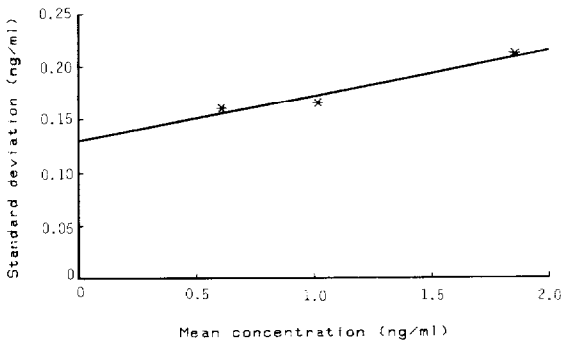


Fig. 3. Determination of the detection limit for warfarin assay. $y = 0.129 + 0.042x$; $r^2 = 0.9450$.

Post-column reaction

A flow diagram of the post-column acid/base manupilation procedure is represented in Fig. 2. In the original publication of Lee et al. [13] triethylamine was used as the reagent. Since this chemical is very unpleasant to work with owing to its unpleasant smell, it was replaced with a 12% solution of triethanolamine. It was determined that the pH of the column effluent rose from 5 before reaction to 8.5 after reaction. The reaction coil was also shortened from 3 m to approx. 1 m without any serious loss in the sensitivity of the method.

Selectivity

Chromatograms representing (a) serum ultracentrifugate blank, (b) serum ultracentrifugate containing 1 ng/ml warfarin and 25 ng/ml internal standard, (c) ultracentrifugate of a patient's serum containing 3 μg/ml warfarin, (d) serum blank and (e) serum from patient mentioned in c are displayed in Fig. 4, showing clearly that the signal-to-noise ratio in the 1 ng/ml range is more than adequate and that plasma components do not interfere with the method. Since the warfarin metabolites are considerably more polar than the parent compound one would not expect interference from these compounds. This was found to be the case in an investigation done by Bjornsson et al. [5].

A number of other acidic and neutral drugs, namely paracetamol, phenobarbitone, carbamazepine, phenytoin, furosemide, spironolactone, hydrochlorothiazide and salicylic acid, were tested for interference. Although salicylic acid displays some fluorescence under the conditions used, it separates

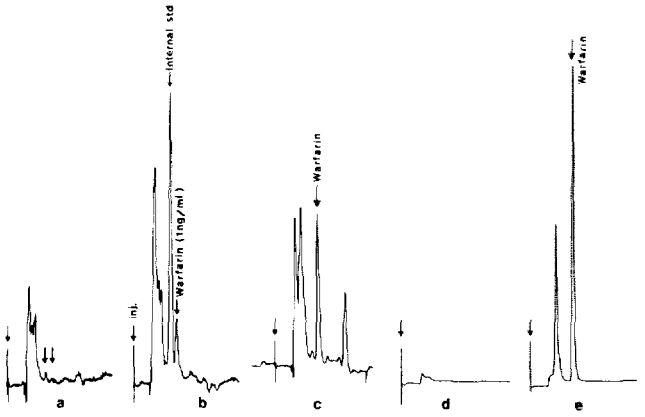


Fig. 4. Chromatograms displaying the absence of interference of warfarin metabolites and endogenous serum components with the assay procedure. (a) Serum ultracentrifugate blank (sensitivity setting 3). (b) Serum ultracentrifugate containing 1 ng/ml warfarin and 25 ng/ml internal standard (sensitivity setting 3). (c) Ultracentrifugate of a patient's serum on maintenance warfarin therapy; total warfarin concentration = 3 μg/ml (sensitivity setting 1); no internal standard. (d) Whole serum blank. (e) Serum extract of patient mentioned in c; no internal standard added.

well from the two peaks of interest. The rest of the drugs investigated showed no interference.

REFERENCES

1 R.A. O'Reilly, P.M. Aggeler and L.S. Leong, Clin. Invest., 42 (1963) 1542.
2 E.S. Vessell and C.A. Shively, Science, 184 (1974) 486.
3 L.T. Wong, G. Solomanraj and B.H. Thomas, J. Chromatogr., 135 (1977) 149.
4 R. Vanhaelen-Fastre and M.J. Vanhaelen, J. Chromatogr., 129 (1976) 397.
5 T.D. Bjornsson, T.E. Blaschke and P.J. Meffin, J. Pharm. Sci., 66 (1977) 142.
6 M.J. Fasco, L.J. Piper and L.S. Kaminsky, J. Chromatogr., 131 (1977) 365.
7 M.J. Fasco, M.J. Cashin and L.S. Kaminsky, J. Liq. Chromatogr., 2 (1979) 265.
8 F.A. De Wolff, C.A.U. Tetteroo-Tempelman and P.M. Edelbroek, J. Anal. Toxicol., 4 (1980) 156.
9 D.E. Mundy, M.P. Quick and A.F. Machin, J. Chromatogr., 121 (1976) 335.
10 C.A. Robinson, D. Mungal and M.-C. Poon, Ther. Drug Monit., 3 (1981) 287.
11 R.A.R. Tasker and K. Nakatsu, J. Chromatogr., 228 (1982) 346.
12 C. Banfield and M. Rowland, J. Pharm. Sci., 72 (1983) 921.
13 S.H. Lee, L.R. Field, W.N. Howald and W.F. Trager, Anal. Chem., 53 (1981) 467.
14 T.D. Bjornson, J.E. Brown and C. TsChanz, J. Pharm. Sci., 70 (1981) 1372.
15 D. Mungall, Y.Y. Wong, R.L. Talber, M.H. Crawford, J. Marshall, D.W. Hawkins and M.L. Ludden, J. Pharm. Sci., 73 (1984) 1000.