

Determination of Warfarin in Human Plasma by High Performance Liquid Chromatography and Photodiode Array Detector

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Summary: A simple liquid chromatographic method for the determination of warfarin in human plasma is described. The method involves precipitation of plasma proteins with acetonitril. No further processing of samples is required. The supernatant was analyzed on a short (10 cm) 3- μ m reversed-phase column eluted with 23% acetonitril in 100 mM ammonium formate, pH 3.5. The method takes advantage of the fact that warfarin dissolved in this mobile phase has a characteristic absorbance spectrum with distinct peaks at 271, 281, and 305 nm. Using an online photodiode array detector, the UV spectrum could be recorded during analysis without interrupting the flow of the mobile phase. This spectral information improves identification possibilities and evaluation of the purity of the chromatographic peaks. Warfarin was separated from other UV-absorbing compounds in plasma in <3 min, and there was no interference from numerous drugs given to patients. The standard curve (305 nm) was linear in the concentration range observed after oral intake of the single dose of warfarin and for a time corresponding to several half-lives. The detection limit of the method was about 0.1 μ g/ml when the absorbance was recorded at 305 nm. At this wavelength, the solvent front was small relative to that observed at lower wavelengths. The precision of the method, given as coefficient of variation, was 6.4%. The method was used for the determination of plasma half-lives of R- and S-warfarin in humans. **Key Words:** Warfarin—High performance liquid chromatography—Photodiode array detector.

Warfarin (3-(α -acetylbenzyl)-4-hydroxycoumarin) is an extensively used peroral anticoagulant. This drug has an asymmetric carbon atom and exists in two enantiomeric forms, R- and S-warfarin. The clinical form of the drug is a racemate, but the S-form is more effective as an anticoagulant than the R-form (1). Warfarin undergoes stereoselective hydroxylation catalyzed by hepatic mixed function oxidase (1-3).

The therapeutic effect of warfarin and other coumarin anticoagulants has usually been monitored from

prothrombin times (4). Recently, it has been demonstrated that a significant correlation exists between suppression of prothrombin complex activity and total warfarin in plasma, suggesting that plasma warfarin monitoring may represent a guideline for the design of warfarin dosage regimens (5). In addition, plasma warfarin assays are occasionally required to check patient compliance to therapy, to quantify covert anticoagulant ingestion, to monitor warfarin resistance, and to study warfarin drug interactions (4).

Widely used methods for the determination of warfarin in plasma involve spectrophotometry, thin-layer chromatography, or gas chromatography. These methods suffer from complex sample processing or low sensitivity and specificity (6-11). Recently, several

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convenient high performance liquid chromatography (HPLC) assays for warfarin in plasma have been developed. These methods include extraction of warfarin in plasma into an organic phase, followed by analysis of the extract by reversed-phase liquid chromatography. The absorbance of the effluent is recorded by either a fixed or a variable wavelength detector (12–18).

UV-visible photodiode array detectors for HPLC have recently been introduced. This detector allows continuous recording of absorbance spectra of the compounds eluting from the column within 40 ms without interrupting the flow of the mobile phase. The advantages of this detector in screening for drugs and drug metabolites in biological material have recently been pointed out by Overzet et al. (19).

Warfarin has a rather characteristic absorption spectrum with three absorbance maxima in the range 200–400 nm. The spectral properties of this drug allow the evaluation of the purity and indicate the identity of the warfarin peak in plasma. This makes warfarin an ideal drug for HPLC analysis involving a photodiode array detector.

MATERIALS AND METHODS

Chemicals and Drugs

HPLC-grade acetonitril was purchased from Rarburn Chemicals, Ltd. (Peeblesshire, Scotland). R-warfarin, S-warfarin, and warfarin racemate (sodium salts) were gifts from Chemoswed AB (Malmö, Sweden). O.D.S. Hypersil 3 μm microparticle medium for reversed-phase chromatography was obtained from Shandon Southern Products Ltd. (Cheshire, England) and Pelliquard LC-18 (40 μm) from Supelco, Inc. (Bellefonte, PA, U.S.A.). The analytical column was slurry-packed with O.D.S. Hypersil at 9,000 psi, using a Shandon column packer. The guard column was dry-packed and subjected to mechanical compression, as described previously (20).

Standards

R-warfarin or S-warfarin was dissolved in 100% acetonitril at a concentration of 0.2–10 mg/ml. The compounds were diluted in acetonitril to known concentrations.

Spectrophotometry

Warfarin (5 mg/ml racemate) was dissolved in 23% acetonitril in 100 mM ammonium formate, pH 3.5,

or in acetonitril. The absorbance spectrum was recorded between 190 nm and 600 nm using a recording spectrophotometer from Kontron, model 810.

Sample Processing

Plasma was mixed with equal volumes of 100% acetonitril. The precipitated protein was removed by centrifugation, and the supernatant was transferred to sample vials for HPLC analysis.

Instruments

A Spectra-Physics SP 8700 solvent delivery system was connected to a Perkin-Elmer ISS 100 autosampler for HPLC, equipped with a coolable sample tray. The column outlet was connected to a photodiode array detector, model HP 1040 A, from Hewlett Packard. In some experiments a variable wavelength detector, model Spectroflow 773 from Kratos, was used. The chromatographic profiles were routinely recorded and integrated by a reporting integrator (Spectra Physics model SP 4270 or Hewlett Packard model HP 3390A). The absorbance spectra of the upslope, top, and downslope of the warfarin peaks were monitored and stored on floppy discs (model HP 82901 M from Hewlett Packard). The spectra (and the chromatograms) were recorded by a X-Y plotter (model HP 7470 A from Hewlett Packard).

HPLC

Samples of 25 μl were injected into a 3 μm O.D.S. Hypersil column (10 cm) equipped with a guard column (2.5 cm). The sample tray was cooled (0–2°C) by circulating 20% ethanol. The column was eluted isocratically at ambient temperature with a mobile phase composed of 23% acetonitril in 100 mM ammonium formate, pH 3.5. The flow rate was 2 ml/min, corresponding to a back pressure of 3,500 psi. The retention time was 2.6 min for both R- and S-warfarin.

RESULTS

Extraction of Warfarin from Plasma

Human plasma was supplemented with warfarin (racemate) at concentrations in the range found in plasma from patients receiving a single dose (about 0.3 mg/kg) of the drug. Warfarin was extracted from plasma by mixing plasma with equal volumes of acetonitril. The recovery of warfarin, determined by

TABLE 1. Recovery of exogenously added warfarin from plasma

Concentration of warfarin ($\mu\text{g/ml}$)	Recovery (%)
10	101.2 \pm 1.1
2	100.4 \pm 1.9
0.2	95.0 \pm 2.1

A concentrated solution (1 mg/ml) of warfarin in acetonitril was diluted in plasma to 10 $\mu\text{g/ml}$ and was further diluted with plasma to the concentrations given in the table. In a parallel experiment, the same concentrated standard was diluted according to the same procedure, except that plasma was replaced with distilled water. The recovery of warfarin from plasma was determined by comparison of the peak area of standards in water vs. standard extracted from plasma. The values are given as mean \pm SEM of four determinations.

comparison of the peak area of standard in water versus standard extracted from plasma, was 95% (Table 1).

Absorbance Spectrum

The absorbance spectrum of racemate warfarin was identical with that of the separate enantiomers and was dependent on the medium in which the drug was dissolved (data not shown). Warfarin in a solution with the same composition as the mobile phase (23% acetonitril in 100 mM ammonium formate, pH 3.5) showed a characteristic spectrum with three distinct maxima (at 271, 281, and 305 nm) and a shoulder at 317 nm. This spectrum (Fig. 1), obtained by conventional scanning photometer equipped with a deuterium and tungsten lamp, was equal to that obtained by HPLC and a photodiode array detector (Fig. 3).

Reversed-Phase Liquid Chromatography

The reversed-phase column was routinely subjected to refilling and mechanical compression (20) each 100 injections to prevent peak splitting during unattended analysis.

Figure 2 shows chromatograms of plasma from a patient treated with warfarin (panels to the left) or plasma from the same patient before treatment (panels to the right). The absorbance was recorded simultaneously at eight different wavelengths, and the profiles obtained at 254, 271, 281, and 305 nm are shown in the figure. Warfarin eluted as a single sharp peak at a retention time of 2.6 min. Warfarin was separated from interfering material at 271, 281, and 305 nm, but a peak eluted close to warfarin at 254 nm. The solvent front was less pronounced at higher wavelengths (Fig. 2).

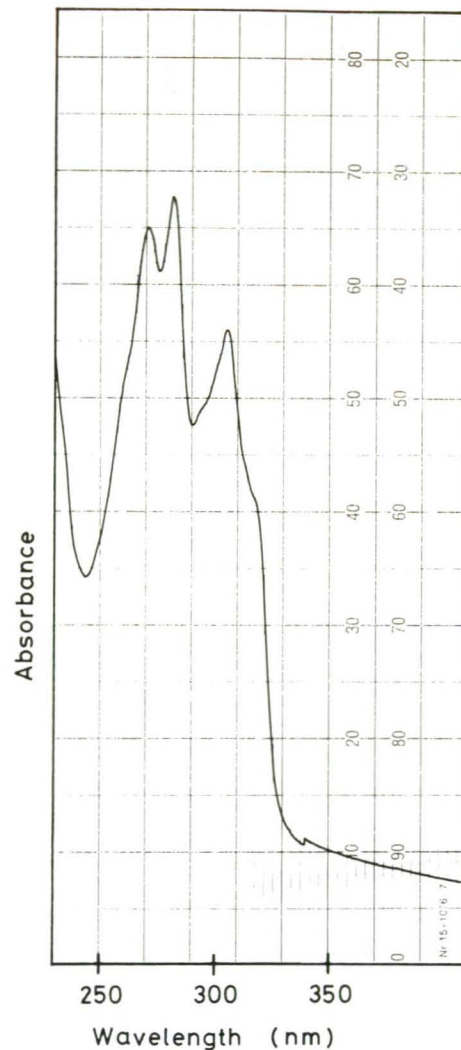


FIG. 1. The absorbance spectrum of warfarin. Warfarin (racemate) was dissolved in 100 mM ammonium formate, pH 3.5, containing 23% acetonitril. The absorbance was recorded between 230 and 400 nm using a conventional recording spectrophotometer equipped with a deuterium and tungsten lamp.

Identification and Purity of the Chromatographic Peak

The warfarin peak in plasma obtained from persons receiving single doses of warfarin p.o. cochromatographed with authentic warfarin (racemate) in several systems (15–30% acetonitril in 100 mM ammonium formate, pH 3.5).

The absorbance spectrum of warfarin in plasma from patients receiving this drug was routinely recorded. Figure 3 shows the spectrum at the upslope, maximum, and downslope of the warfarin peak in plasma. The chromatogram of the same plasma is shown in Fig. 2. The spectra were identical with each

File: **WARF**
 Date: 04/15/1985
 Inj. Time: 13:07

B 2, 8:	5.0 (126.1)	MAU 10%
C 4, 8:	5.0 (253.5)	MAU 10%
D 5, 8:	5.0 (362.9)	MAU 10%
E 6, 8:	5.0 (78.4)	MAU 10%

hp 1040A

Wavelength	
1. 218, 4	
2. 254, 4	
3. 280, 4	
4. 271, 4	
5. 281, 4	
6. 305, 4	
7. 308, 4	
8. 550, 100	

File: **WARF2**
 Date: 04/15/1985
 Inj. Time: 14:16

B 2, 8:	5.0 (136.1)	MAU 10%
C 4, 8:	5.0 (267.8)	MAU 10%
D 5, 8:	5.0 (378.6)	MAU 10%
E 6, 8:	5.0 (80.6)	MAU 10%

hp 1040A

Wavelength	
1. 218, 4	
2. 254, 4	
3. 280, 4	
4. 271, 4	
5. 281, 4	
6. 305, 4	
7. 308, 4	
8. 550, 100	

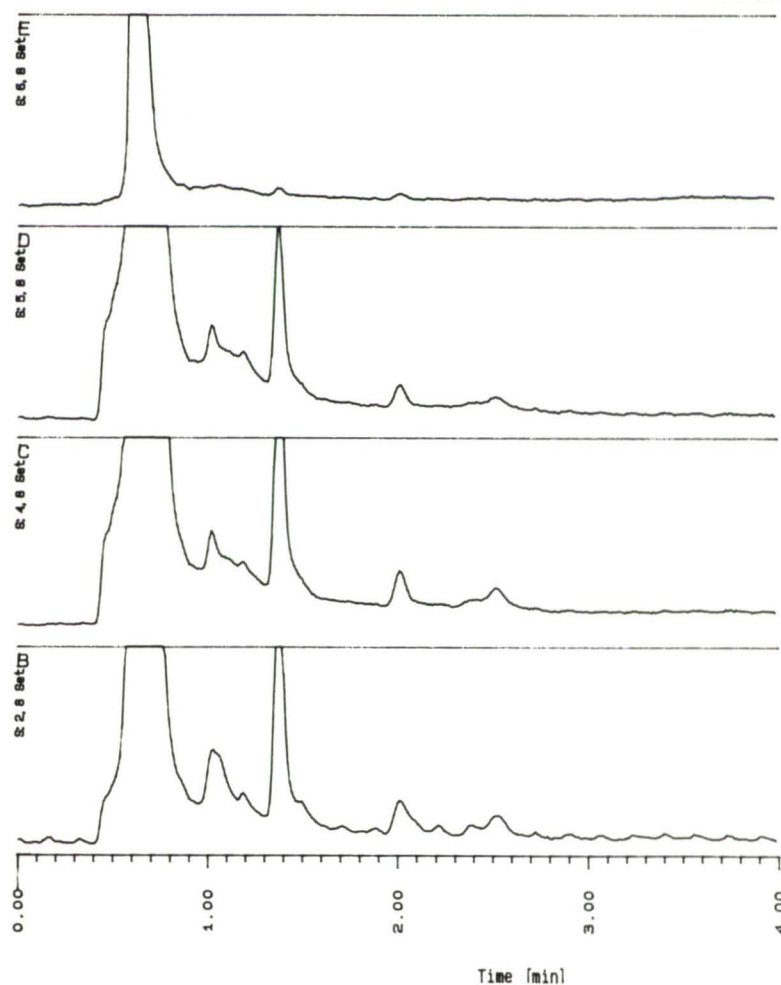
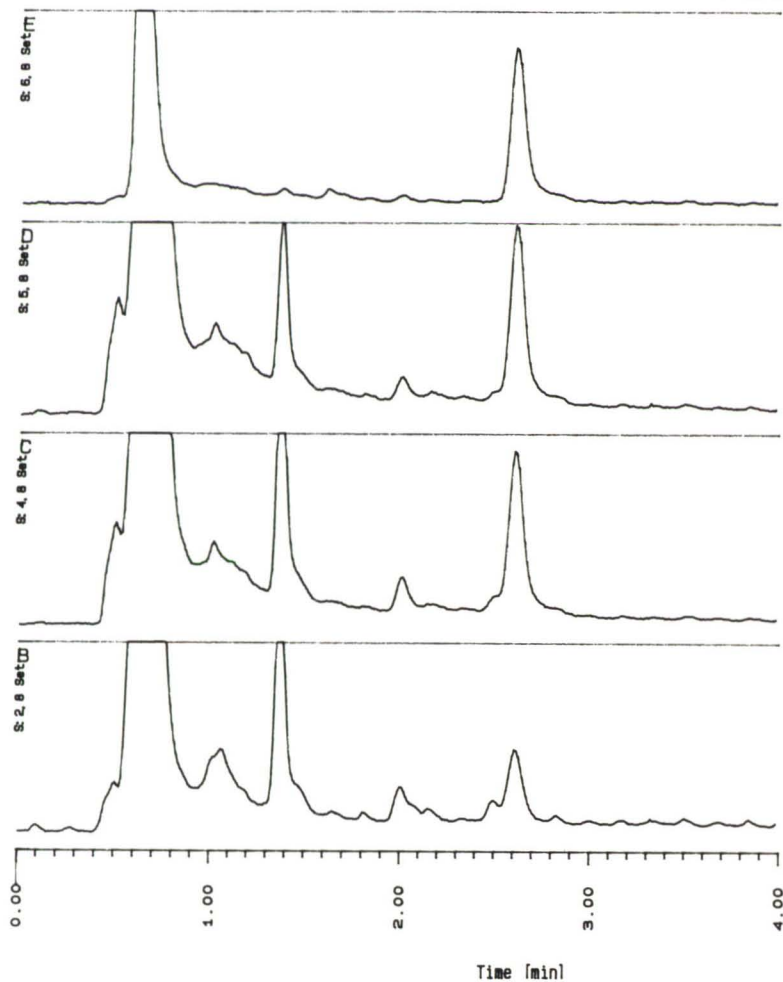


FIG. 2. Multiple signal plot of plasma containing warfarin (panels to the left) or containing no drug (panels to the right). Plasma from a patient was subjected to analysis by reversed-phase liquid chromatography, before and after initiation of warfarin treatment. The absorbance of the effluent was recorded by a photodiode array detector simultaneously at the eight wavelengths given in the upper right corner of each set. The chromatographic profiles obtained at 254 nm (lower panels, **B**), 271 nm (panels **C**), 281 nm (panels **D**) and 305 nm (upper panels, **E**) are shown.

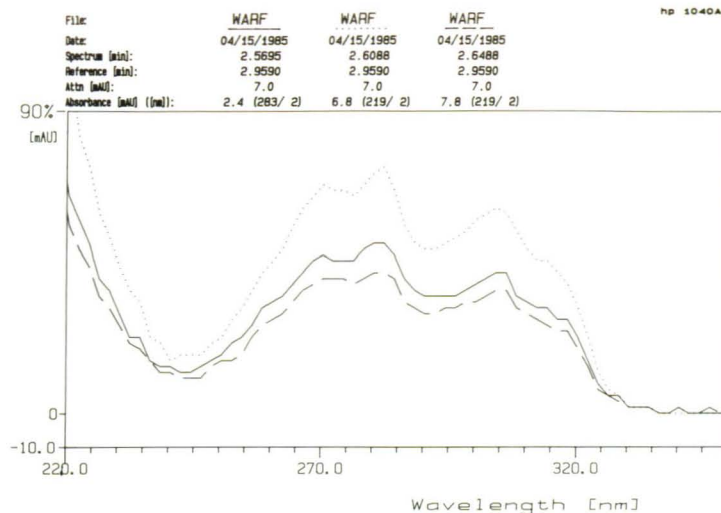


FIG. 3. Absorbance spectra of the warfarin peak in plasma. The absorbance spectrum of the chromatographic peak tentatively identified as warfarin in Fig. 2 was recorded at the upslope (solid line), apex (dotted line) and downslope (interrupted line) of the peak.

other (Fig. 3) and with the spectrum obtained by injection of warfarin standard (data not shown). The comparison of the spectra of the various parts of the chromatographic peak was facilitated by computerized overlay of the separate spectra. In Fig. 3, the attenuations were selected to slightly separate the spectra.

The data in Fig. 3 show that the sensitivity of the photodiode array detector allows the recording of an absorbance spectrum of high quality of warfarin in plasma from a patient treated with this drug. The main details of the spectrum were also apparent in chromatograms of plasma 2–3 half-lives after the patient received a single dose (about 20 mg) of warfarin (data not shown).

Standard Curves, Detection Limit, and Precision of the Method

The standard curves of warfarin in plasma were constructed at three wavelengths, corresponding to the absorbance maxima of warfarin, i.e., at 271, 281, and 305 nm (Fig. 4). Linear standard curves were obtained at these wavelengths, and the detection limit was about 0.07 μg warfarin per ml plasma.

Experiments were carried out to compare the photodiode array detector with a conventional variable wavelength detector, characterized by low noise. The detection limit of the latter detector was about the same at all wavelengths corresponding to the absorbance maxima of warfarin (271, 281, and 305 nm), and the detection limit was about 20% of that observed with the photodiode array detector (data not shown). Because of the small solvent front at 305 nm and no interference at this wavelength (Fig. 2), the pilot signal was routinely recorded at 305 nm.

The within-run precision of the method was determined by analyzing 10 samples of the same plasma containing warfarin (2 $\mu\text{g}/\text{ml}$). The coefficient of variation was 6.4%.

Interference with Various Drugs

Interference with various drugs was tested for by analysis of plasma from patients taking therapeutic doses of these drugs, but not warfarin. None of the drugs listed in Table 2 caused interference with the warfarin assay (305 nm).

Plasma Concentration Curves

Patients were given a single dose of either R-warfarin (0.33 mg/kg) or S-warfarin (0.20 mg/kg), and

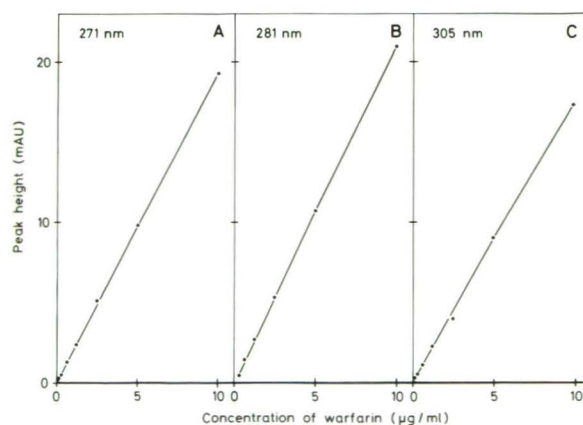


FIG. 4. Standard curves for warfarin at various wavelengths. The standard curve for warfarin was constructed from the absorbance (peak height) obtained at 271 nm (A), 281 nm (B), and 305 nm (C) using a photodiode array detector. The band width was 4 nm.

TABLE 2. *Drugs tested for interference with the warfarin assay*

Phenoxymethylpenicillin
Nitrazepam
Glibenclamid
Dexamethasone
Digitoxin
Furosemide
Hydrochlorothiazide
Piroxicam
Propranolol
Indomethacin
Chlorpromazine
Methadone chloride
Morphine chloride
Naproxen
Tamoxifen
Paracetamol
Codeine phosphate
Prednisone
Flunitrazepam
Buprenorphine chloride
Diazepam

the amount of drug in plasma was determined at various time points. The terminal plasma half-lives were ~40 h for R-warfarin and 20 hours for S-warfarin (Fig. 5). These data are in accordance with the half-lives for warfarin published by others (1).

DISCUSSION

The present report describes a simple liquid chromatographic method for the determination of warfarin in human plasma. No extraction of plasma with organic phase was required, and the plasma samples were only deproteinized with acetonitril prior to HPLC analysis. This simple sample processing ensures quan-

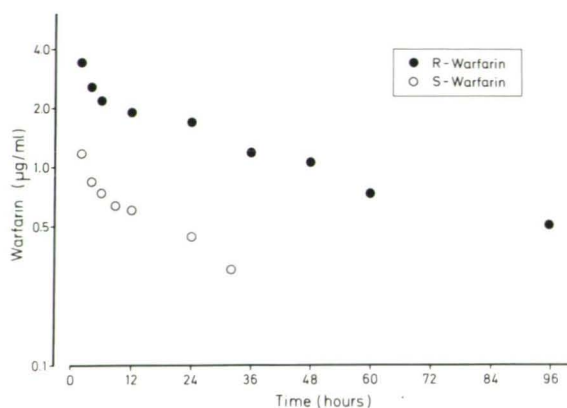


FIG. 5. Plasma concentration curves for R-warfarin and S-warfarin after oral administration (0.33 mg/kg R-warfarin; 0.20 mg/kg S-warfarin, single dose).

titative transfer of the drug to the solution subjected to HPLC, and no internal standard was required.

Published HPLC methods for warfarin include more or less laborious extraction procedures to remove interfering material (14,16-18). We observed no interference from compounds present in normal plasma when the absorbance of the effluent was recorded at 305 nm (Fig. 2). This may be related to both the mobile phase (low pH and high ionic strength) and the column (short microparticulate O.D.S. column) used. However, routine analysis of crude plasma extract should include continuous evaluation of the purity of the chromatographic peak. This possibility is offered by the photodiode array detector recording the spectral properties of the warfarin peak in plasma. Because of the rather complex and characteristic absorbance spectrum of warfarin (Figs. 1 and 3), this spectral information confirms the identity and purity of the warfarin peak. Thus, determination of warfarin in plasma is an example of the versatility of the photodiode array detector in the analysis of drugs in biological material.

The sensitivity of the present method equalled that obtained with other HPLC assays and ordinary UV detectors (14,17,18) and allows the determination of warfarin in plasma and the elimination curves of the drug (Fig. 5). A new generation of variable wavelength detectors for HPLC, characterized by low noise (about 2×10^{-5} absorbance units full scale at 0.1 s response time), has recently been introduced. The detection limit with this type of detector is 1/5 to 1/3 of that obtained with the photodiode array detector, but multiple injections or stopped flow are required to obtain the absorbance spectrum of a particular peak.

We have used the present warfarin assay for the assessment of the induction of drug metabolism, as judged by half-lives of R- and S-warfarin (Fig. 5), in patients receiving various dosage regimens with aminoglutethimide against advanced breast cancer. Such investigations require a large number of analyses over a wide concentration range for the construction of numerous plasma elimination curves. On a few occasions, the method has been used for the diagnosis of covert warfarin ingestion.

In conclusion, UV detection using a photodiode array detector for HPLC is the basis of a rapid HPLC assay for warfarin in plasma, which includes simple sample processing and fast analysis (retention time of <3 min). Several hundred samples could be subjected to unattended analysis per day, and the number of samples was in fact only limited by the capacity of

the sample tray (100) or the mechanical stability of the reversed-phase column.

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