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Determination of warfarin in drinking water by high-performance liquid chromatography after solid-phase extraction

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SUMMARY

A method for the determination of 0.1 μg/l of warfarin in drinking water involving concentration by solid-phase extraction is described. A 1000-ml volume of drinking water is aspirated through a solid-phase extraction column and warfarin is eluted to 1.0 ml. The eluate is analysed by reversed-phase high-performance liquid chromatography with UV detection. The detection limit is 0.02 μg/l. Recoveries of greater than 90% were obtained when tap water was spiked with warfarin.

INTRODUCTION

Warfarin, 4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin, is used both as an anticoagulant in man and as a rodenticide. The concentration of warfarin in drinking water must not exceed 0.1 ppb (0.1 μg/l) according to the European Communities guidelines[1]. A method that can detect warfarin at levels down to at least 0.1 ppb was therefore required.

Various techniques have been applied to the determination of warfarin, including gas chromatography[2] and high-performance liquid chromatography (HPLC)[3–9]. HPLC has been used in both normal-phase[3] and reversed-phase modes[4–9]. Wang and Bonakdar[4] used HPLC with electrochemical detection and obtained a detection limit of 40 ppb. Lee *et al.*[5] published an HPLC method with fluorimetric detection. The limit of detection was 18 ppb, and 1.8 ppb when a concentration step was used. Fasco *et al.*[6] described a method for the determination of warfarin and its metabolites in plasma, using solid-phase extraction and HPLC with UV detection. The limit of detection was 30 ppb.

We have developed a method that has a detection limit of 0.02 ppb. Solid-phase extraction with an enrichment factor of 1000 is used prior to analysis by reversed-phase HPLC with UV detection.

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EXPERIMENTAL

Chemicals and reagents

HPLC-grade methanol and acetonitrile were obtained from Labscan (Dublin, Ireland), glacial acetic acid, phosphoric acid (min. 85%) and potassium hydrogenphosphate from Merck (Darmstadt, F.R.G.) and sodium hydroxide from Eka Nobel (Surte, Sweden). The water used to prepare the mobile phase and all aqueous solutions was purified using a Milli-Q water purification system from Millipore (Bedford, MA, U.S.A.). Amorphous sodium warfarin of USP quality was provided as a gift by Chemoswed (Malmö, Sweden).

Extraction columns and vacuum apparatus

Bond-Elut octadecyl-(C_{18}, 200 mg, 3 ml), ethyl-(C_2, 500 mg, 2.8 ml) and phenyl-(PH, 500 mg, 2.8 ml) bonded silica columns were obtained from Analytichem International (Harbor City, CA, U.S.A.). The columns were used with a Vac-Elut 10 sample-processing station from Analytichem International.

Chromatographic equipment

The HPLC system consisted of a Model 590 solvent-delivery pump and a WISP Model 712 autosampler, both from Waters Assoc. (Milford, MA, U.S.A.). A Waters Assoc. Lambda-Max Model 481 variable-wavelength LC spectrophotometer, a Waters Assoc. Model 490 programmable multi-wavelength detector and a Hewlett-Packard (Waldbronn, F.R.G.) Model HP 1046A programmable fluorescence detector were used. A Spectra-Physics (San Jose, CA, U.S.A.) Model SP4270 integrator was used to record chromatograms and calculate peak heights. A Spectra-Physics ChromStation-AT was used for data handling and storage. An HP 79994A HPLC ChemStation was used together with the fluorescence detector.

Standard solution

A solution containing 1 mg/ml of warfarin in deionized water was prepared. This solution was diluted to a concentration of 0.1 μg/ml with acetonitrile–0.04 *M* phosphate buffer (pH 7.4) (1:1).

Extraction procedure

Drinking water and drinking water spiked with 0.1 ppb of warfarin were allowed to stand for 24 h before being acidified to pH 4.3 with acetic acid. Warfarin was extracted from the water using octadecyl-bonded silica columns (Bond-Elut C_{18}, 200 mg, 3 ml). The columns were held in a Vac-Elut 10 processing station operated at 10 in.Hg pressure. The columns were conditioned with 5 ml of acetonitrile followed by 5 ml of water (pH adjusted to 4.3 with acetic acid). A small volume of water was left in each column to prevent the sorbent from drying before the samples were applied. PTFE tubes of 1/8 in. I.D. were immersed in the sample solutions and connected to Bond-Elut adaptors. The siphon effect was used to fill the columns with sample solutions. The Bond-Elut adaptors were connected to the columns and 1000 ml of the sample solutions were aspirated through the columns by vacuum. The columns were washed with 20 ml of acetonitrile–water (pH 4.3) (20:80). Warfarin was eluted with acetonitrile–0.04 *M* phosphate buffer (pH 7.4) (1:1) to 1.0 ml.

Chromatography

The standard solution and the eluates from the solid-phase extraction columns were analysed by reversed-phase HPLC on a 150 × 3.9 mm I.D. Nova-Pak C_{18} (4 μm) column (Waters Assoc.). The mobile phase was prepared by mixing 620 ml of methanol, 380 ml of water and 10 ml of glacial acetic acid. The mixture was filtered through a 0.45-μm nylon-66 membrane filter and degassed before use. The flow-rate was 1.0 ml/min and the column temperature was ambient. The injection volume was 30 μl. Warfarin was detected at both 282 and 306 nm. Quantification was based on peak heights. Before use, a number of suitability tests were performed in order to evaluate the chromatographic system. The standard solution was injected six times. The number of theoreticl plates (n) and the tailing factor (T) were calculated according to USP XXI[10]. Typical values of n were in the range 3000–3100 and of T in the range 1.3–1.6. The relative standard deviation of the peak heights of warfarin from six successive injections was also calculated. Typical values were in the range 1.2–3.0%.

The eluate from the drinking water was injected and the peak height of warfarin was compared with that obtained when the standard solution was injected directly. The peak height of warfarin from spiked drinking water was compared with that obtained when the standard solution was injected directly and with that of the eluate from unspiked drinking water, in order to establish the recovery of warfarin from the drinking water in question.

RESULTS AND DISCUSSION

Solid-phase extraction

Solid-phase extraction columns with different phases (octadecyl-, ethyl- and phenyl-bonded silica) were tested. Use of the ethyl column gave eluates that were as clean as those from the octadecyl column, whereas the eluates from the phenyl column were not as clean. The recoveries were identical with all the columns. Octadecyl-bonded silica columns were used in all subsequent experiments. Washing of the extraction columns prior to elution was necessary in order to obtain suffciently clean eluates. Volumes of 5 ml of different concentrations (10–40%) of acetonitrile in acidified water (pH 4.3) were used to wash the extraction columns after sample application. Warfarin was eluted when an acetonitrile concentration higher than 25% was used. Different volumes (5–20 ml) of 20% acetonitrile in acidified water (pH 4.3) were used for the column washing. All volumes gave the same recovery of warfarin, but the chromatograms were cleaner when 20 ml was used instead of 5 ml.

An additional wash with a mixture of dichloromethane in hexane was tested. The sorbent was dried for 5 min before hexane was sucked through the column and then mixtures of dichloromethane in hexane. A wash with 5 ml of dichloromethane–hexane (5:95) resulted in elution of 50% of the warfarin. It was possible to wash with 1 ml of dichloromethane–hexane (5:95) without warfarin being eluted. However, the eluates were no cleaner when this additional wash was used.

Chromatograms of the standard solution injected directly and of spiked tap water analysed according to the described method are shown in Fig. 1. Chromatograms are shown both with and without the washing procedure. The typical retention time of warfarin was 5.0 min.

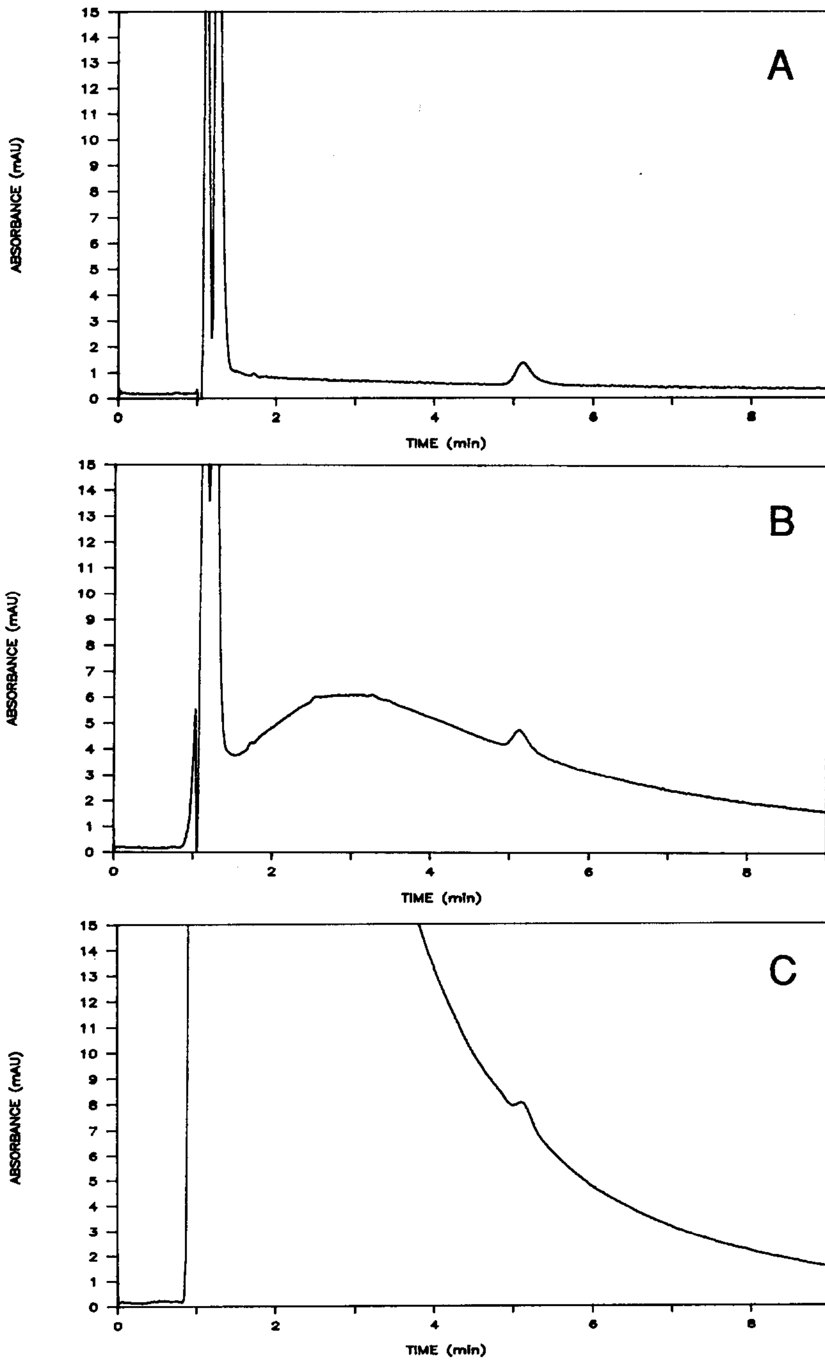

Fig. 1. Chromatograms of (A) the standard solution injected directly and of eluates of tap water spiked with 0.1 ppb of warfarin, (B) with the washing procedure and (C) without washing. Details of the washing procedure for the solid-phase extraction columns and the HPLC conditions are given under Experimental.

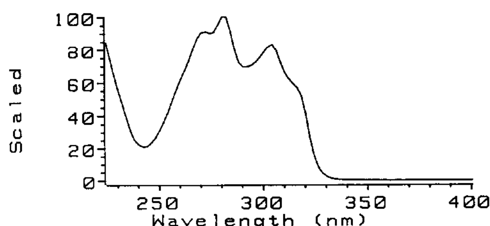

Fig. 2. UV spectrum of warfarin dissolved in mobile phase.

Detection

A UV spectrum of warfarin dissolved in the mobile phase is shown in Fig. 2. Warfarin showed UV absorption maxima at 272, 282 and 306 nm. It was deteced at both 282 and 306 nm and the absorbance ratio was used to confirm the identity of the warfarin peak; the ratio obtained was 1.2.

Fluorimetric detection was tested to see whether a lower detection limit could be achieved. A lower detection limit would mean that smaller volumes of the sample solution could be applied to the solid-phase extraction column. Lee *et al.*[5] reported that the fluorescence of warfarin was quenched under acidic conditions. The mobile phase used in our method was acidic. A post-column acid–base manipulation procedure to enhance the fluorescence of warfarin has been described[5,9]. In order to increase the pH we used a post-column pump that added 1.0 ml/min of methanol–0.5 *M* sodium hydroxide (60:40) to the column effluent through a Valco low-dead-volume T-piece. Warfarin was injected and trapped in the flow cell of the detector by stopping the flow. Excitation and emission scans were collected. Warfarin had three excitation wavelength maxima, at 241, 290 and 328 nm. The emission wavelength maximum was about 386 nm for the three excitation wavelengths. An excitation wavelength of 241 nm gave the highest fluorescence. The limit of detection for warfarin was about the same when fluorimetric detection was used instead of UV detection. It was surprising that fluorimetric detection did not give a lower limit of detection. However, the chromatographic parameters were not optimized for fluorimetric detection. A higher degree of selectivity and a more reliable peak identification are possible advantages of fluorimetric detection.

Validation of the method

The linearity was determined by analysing solutions of warfarin in deionized water with concentrations between 0.02 and 0.30 ppb. Warfarin was concentrated by solid-phase extraction, the eluates were injected onto the HPLC column and the warfarin peak heights were measured. The regression line was calculated by the method of least squares. The results from the linear regression analysis are given in Table I.

The peak height was linear between 0.02 and 0.30 ppb at both wavelengths. A solution of 0.02 ppb of warfarin in deionized water gave a peak with a signal-to-noise ratio of 3. Hence it follows that the detection limit of the method was 0.02 ppb.

The precision and accuracy were determined by spiking tap water with sodium warfarin to give concentrations of 0.05, 0.10 and 0.20 ppb of warfarin and analysing these solutions according to the method on each of three days. The results obtained are

TABLE I

LINEAR REGRESSION ANALYSIS

Parameter	*282 nm*	*306 nm*
Slope	5964	5344
Intercept	5.6	−6.7
Correlation coefficient	0.9995	0.9997

given in Table II. The relative standard deviation of the results for each concentration ranged from 4 to 13%. The highest value was obtained for the lowest concentration. The mean recovery for each concentration ranged from 97 to 103%.

In conclusion, the method was found to be selective, linear, precise and accurate.

TABLE II

PRECISION AND ACCURACY

Concentration (ppb)	*282 nm*			*306 nm*		
	Recovery (%)	*Mean recovery (%)*	*R.S.D.[a] (%)*	*Recovery (%)*	*Mean recovery (%)*	*R.S.D.[a] (%)*
0.05	92	100	13	92	103	11
	87			114		
	111			102		
0.10	100	100	4	94	97	9
	104			106		
	96			90		
0.20	94	99	5	98	99	5
	104			104		
	99			94		

[a] Relative standard deviation.

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