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DETERMINATION OF WARFARIN IN ANIMAL RELICTA AND FEEDINGSTUFFS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH CONFIRMATION OF IDENTITY BY MASS SPECTROMETRY

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SUMMARY

The determination of warfarin in liver, blood, urine and feedingstuffs by high-pressure liquid chromatography (HPLC) is described. Clean-up is simple and the lower limit of determination is about 0.025 ppm. Recoveries from liver are about 85% over the range 0.025–1.0 ppm; recoveries from blood range from about 67 to 90%.

Chemical ionisation mass spectrometry can be used to confirm identity and, by specific ion monitoring, to detect warfarin in HPLC eluates at levels below the sensitivity of the HPLC detector.

INTRODUCTION

The identification and determination of the anti-coagulant rodenticide warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] in the relicta of animals and in feedingstuffs is required as an aid to diagnosis in cases of suspected poisoning. Methods suitable for determination in biological materials (usually plasma) depending upon absorption[1–3] or fluorescence[4,5] spectrophotometry, gas chromatography (GC)[6–8] and high-pressure liquid chromatography (HPLC)[9] have been described. Of these, the methods of Welling *et al.*[3] and Kaiser and Martin[7] have the necessary sensitivity (0.1 ppm or better) for diagnostic purposes. Both methods were designed to determine warfarin in the plasma of patients treated with the compound however, and the former apparently relies upon comparison of plasma samples before and after treatment for its sensitivity and specificity. The latter requires clean-up of the sample by thin-layer chromatography (TLC) before the determinative step. A sensitive GC method in which TLC clean-up is avoided has recently been developed[10].

A rapid and sensitive HPLC method to complement the GC procedure[10] was desirable because (1) determination by both procedures should provide positive identification if required, and (2) HPLC is potentially suitable for multi-residue determinations of anti-coagulant rodenticides, including compounds that may be insuf-

ficiently volatile for GC. HPLC reversed-phase partition systems have been used to determine warfarin in the plasma of treated patients[9] and in formulations[11]. In the former application, which was published during the development of the present work, neither recoveries nor the lower limit of determination were given, but the method was apparently used in the range 1–10 μg/ml.

This communication describes the analysis of animal tissues, stomach contents, body fluids and feedingstuffs for warfarin by HPLC. An adsorption column is used, giving a lower limit of determination of about 0.025 ppm. The use of chemical ionisation mass spectrometry (CIMS) to confirm identity and of specific ion monitoring to increase the sensitivity of detection are also described.

EXPERIMENTAL

Materials

Technical warfarin, about 99% pure, and difenacoum [3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin] were supplied by Ward, Blenkinsop (Widnes, Great Britain). Coumatetralyl [4-hydroxy-3-(1,2,3,4-tetrahydro-1-naphthyl)coumarin], and chlorophacinone [2-(2-*p*-chlorophenyl-2-phenylacetyl)-indane-1,3-dione] were obtained from Bayer (Bury St. Edmunds, Great Britain) and May & Baker (Dagenham, Great Britain) respectively. A mixture of the diastereoisomeric warfarin metabolites 3-[α-(2-hydroxypropyl)benzyl]-4-hydroxycoumarin (I) was synthesized as described by Trager *et al.*[12] and separated into two fractions (Ia and Ib) by the TLC system of Fishwick and Taylor[2]. The metabolite 2,3-dihydro-2-methyl-4-phenyl-4H-pyrano[3,2-c]benzopyran-5-one (II) was synthesized as described by Chan *et al.*[13] and purified by TLC.

Inorganic compounds and the solvents chloroform, isopropyl alcohol and iso-octane (2,2,4-trimethylpentane) were of analytical reagent grade. Isopropyl alcohol and iso-octane were distilled before use. Chloroform used to dissolve the cleaned-up sample was washed, dried and distilled on the day of the determination.

Apparatus

Liquid chromatograph. The pump[14] and septum injector were built in the laboratory. The column was stainless steel, 1 m × 1.5 mm I.D., packed with Corasil II (Waters Assoc., Stockport, Great Britain). The detector was a UV photometer with grating monochromator (Type CE 212; Cecil, Cambridge, Great Britain).

Mass spectrometer. A Finnigan Model 1015D gas chromatograph–mass spectrometer equipped with a chemical ionisation source and "Promim" multiple ion monitor was used.

An MSE (Catalogue No. 7700A) top-drive homogeniser and a Infotronics (Model CRS-208) digital integrator were also employed.

Extraction and clean-up

After extraction with chloroform, interfering co-extractives were removed by partition with sodium pyrophosphate as described by Fishwick and Taylor[2].

Liver, stomach contents and feedingstuffs. The sample (10 g) was macerated with anhydrous sodium sulphate (10 g) and chloroform (30 ml), the extract filtered through sintered glass and the residue triturated with a further 15 ml of chloroform. The combined filtrates were concentrated to 10 ml under a stream of nitrogen and

extracted with 2 × 10 ml of aqueous sodium pyrophosphate solution (1% w/v). Emulsions were broken by centrifugation if necessary. The aqueous extract was washed with chloroform (5 ml), acidified with hydrochloric acid (5 *N*, 2.2 ml) and extracted with chloroform (3 × 10 ml). The chloroform extract was dried with anhydrous sodium sulphate, filtered through sintered glass, concentrated to low volume and transferred to a stoppered 10 ml conical centrifuge tube. The remaining solvent was removed under nitrogen and the residue dissolved in 0.1 ml of chloroform.

Body fluids. Plasma or urine (10 ml) was acidified with hydrochloric acid (5 *N*, 2 ml) and extracted successively with 15 and 10 ml of chloroform. The extract was dried with sodium sulphate, filtered and concentrated to 10 ml. Clean-up with sodium pyrophosphate and further treatment followed as described above.

Liquid chromatography

Triplicate 5- or 10-μl aliquots of the final chloroform solution were injected. The eluent was isopropyl alcohol–iso-octane (2:98), and the pressure 60 kg/cm^2, giving a flow-rate of 1 ml/min. The detector was set to measure absorbance at 270 nm. (Of the three maxima in the UV absorption spectrum of warfarin, that at 270 nm was least subject to interference by co-extractives in biological material.) Warfarin was determined by comparison of the peak height with that produced by standard solutions of roughly similar concentration injected before and after the sample. If confirmation of identity by mass spectrometry was required, the eluate fraction containing the warfarin (usually about 1 ml) was collected.

If concentrations of warfarin at levels below the sensitivity of the detector were of interest, repeated injections of 10 μl were made and the fractions which would contain the warfarin if present were collected and combined for examination by specific ion monitoring. (If the entire final chloroform solution was concentrated and applied as a single injection, the warfarin was insufficiently separated from co-extractives.)

Mass spectrometry

The solvent was evaporated from the eluted warfarin fraction under a stream of nitrogen and the residue redissolved in acetone (10–100 μl). This solution or an aliquot of it was transferred to a capillary tube for direct insertion into the mass spectrometer and the acetone evaporated. The capillary was heated in the ion source of the spectrometer with methane as reagent gas at a pressure of 1.2 torr, and the CI spectrum of warfarin was recorded. The rate of heating was adjusted by trial with a standard solution of warfarin so that a record of the ion current due to warfarin as a function of time showed a pronounced peak after about 1 min, at a temperature of about 120°. A satisfactory spectrum was obtained from about 50 ng of warfarin. Lower levels were detected by monitoring the two major ions from warfarin (*m/e* 309, M + H and *m/e* 337, M + C_2H_5) instead of scanning the complete spectrum.

RESULTS AND DISCUSSION

Calibration

Calibration solutions contained 2–1000 ppm of warfarin and 10-μl aliquots were injected. Fig. 1 shows the relation between the quantity of warfarin injected

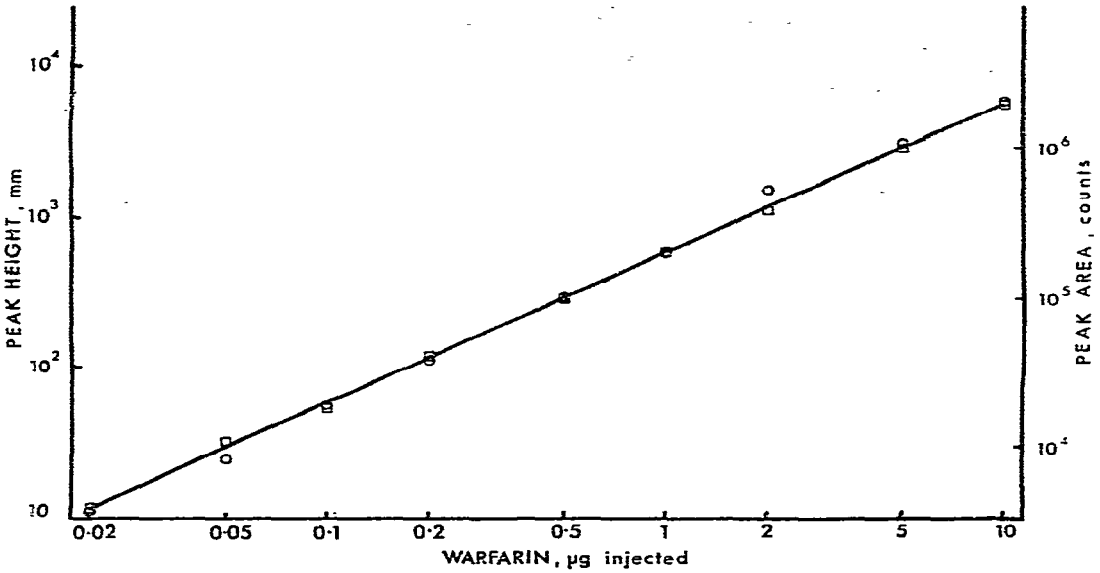

Fig. 1. Calibration curve for warfarin. ○, Measurement of peak height; □, measurement of peak area by digital integration.

and the height and area (measured by digital integration) of the resulting peak. Each point represents the mean of three injections.

It can be seen that the detector responds linearly to the quantity of warfarin injected over the 500-fold range examined. The good agreement between results based on measurements of peak heights and areas shows that measurement of height is satisfactory and is an indication of good peak shape. The lowest point on the curve represents a peak of 4% full-scale deflection (10 mm on the chart used) with a noise level of 2% peak-to-peak. There was some variation from day to day in the response produced by a given quantity of warfarin, so comparison of sample and standard peaks was more reliable than reference to a calibration curve.

Recoveries

Recoveries of warfarin from liver and plasma were studied because these are the most difficult of the diagnostically useful materials to analyse. Pig liver and plasma were used because the pig is particularly susceptible to warfarin poisoning[15].

Recoveries of warfarin added to saline, pig liver and pig plasma are shown in Table I. Each value is derived from a separate portion of fortified saline, liver or plasma and represents the mean of three injected aliquots. Fig. 2 shows a typical chromatogram from liver fortified at 0.1 ppm.

The results show that recoveries from liver were satisfactory over the range examined (0.025–1.0 ppm). Recoveries from plasma were also adequate for diagnostic purposes, although they were significantly lower ($p < 0.001$) at levels below 0.1 ppm.

No peaks in the warfarin position were detectable when extracts of unfortified blood or liver (4 samples of each) were injected. Preliminary experiments had shown that the initial chloroform extract of liver and plasma samples contained too much

TABLE I

RECOVERY OF WARFARIN FROM SALINE, PLASMA AND LIVER BY HPLC

Column, 1 m × 1.5 mm I.D., Corasil II; mobile phase, isopropyl alcohol–isooctane (2:98); pressure, 60 kg/cm²; flow-rate, 1 ml/min. Recoveries: each value represents the mean of triplicate injections from a separate sample of saline, plasma or liver.

Substrate	*Warfarin added (ppm)*	*Recovery (%)*
Saline	0.025	82, 82
	0.05	77, 78
	0.1	80, 83
	1.0	87, 91
Plasma (pig)	0.025	65, 71, 65, 71
	0.05	63, 67, 67, 67
	0.1	80, 83, 83, 80
	1.0	84, 94, 89, 82
Liver (pig)	0.025	88, 84, 79, 93
	0.05	95, 90, 76, 89
	0.1	77, 88, 79, 88
	1.0	79, 102, 91, 89

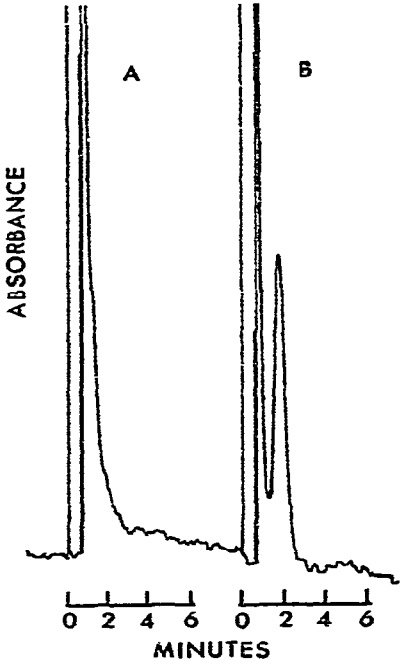

Fig. 2. Liquid chromatograms of extracts from (A) unfortified liver and (B) liver containing 0.1 ppm added warfarin.

interfering material for satisfactory chromatography: the negligible blank values indicate that this is effectively removed by the simple pyrophosphate extraction procedure of Fishwick and Taylor[2].

Specificity

The chromatographic separation of warfarin from likely metabolites and from other anti-coagulant rodenticides with which it might be confused was examined. Under the conditions described above (with 2% isopropyl alcohol in iso-octane as eluent) coumatetralyl, difenacoum, chlorophacinone and metabolite II were eluted within half the retention volume of warfarin and the alcohol metabolites Ia and Ib were eluted together after a volume about 1.5 times that of warfarin. The phenolic compounds 6- and 7- hydroxywarfarin (which are known to be metabolites in the rat and man[12]) were not chromatographed but could be assumed to be more strongly adsorbed than the alcohols. Evidently warfarin is well separated from these compounds and it appears that the proposed method gives sufficient separation for quantitative and for many qualitative purposes. Added evidence of identity can be obtained if necessary by GC determination of the methyl derivative[10] or by mass spectrometry.

Confirmation of identity and detection at low levels by CIMS

Although Deckert reported the GC of warfarin without prior conversion to a derivative[16,17], we have beeen unable to elute the free compound at low levels from the stationary phases he used or from any of several others examined. Eluted HPLC fractions were therefore examined by direct insertion into the mass spectrometer. The electron-impact mass spectrum of warfarin is complex[12], but the methane CI

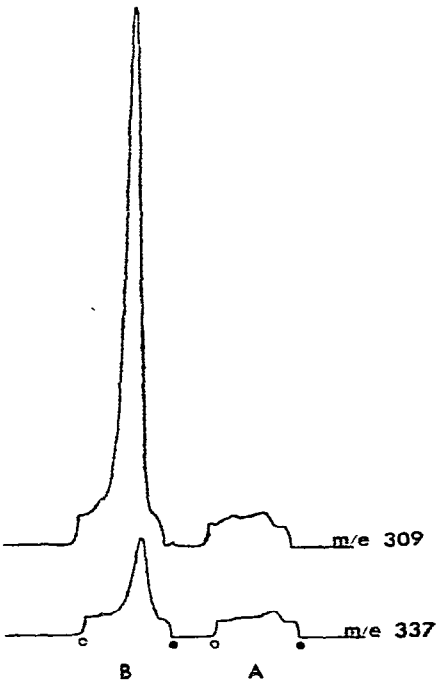

Fig. 3. Specific ion monitoring of warfarin heated in probe of mass spectrometer. Chemical ionisation with methane. *m/e* 309 and *m/e* 337 ion currents, as function of time, from (A) unfortified blood and (B) blood containing 0.005 ppm warfarin. ● = Heater and ioniser on; ○ = heater and ioniser off.

spectrum consists essentially of three peaks at *m/e* 309 (M + H, base peak), 337 (M + C_2H_5, about 16% of base peak) and 349 (M + C_3H_5, about 4%). When warfarin was heated in the ion source as described above, it volatilized sharply after an interval whose reproducibility approached that of a GC retention time. Positive identification of warfarin therefore depended upon its HPLC retention volume, the "retention time" of its ion-current peak and the occurrence of the *m/e* 309, 337 and 349 ions in the correct intensity ratio. A level of about 0.05 ppm in the original sample was required for a satisfactory spectrum.

Specific detection at lower levels was possible by monitoring the *m/e* 309 and 337 ion currents as a function of time. To test the sensitivity attainable, the extracts from 10 ml of normal plasma and 10 ml of plasma fortified with 50 ng (0.005 ppm) of warfarin were injected (as 10 × 10 μl injections) into the liquid chromatograph. No peaks which could be definitely attributed to warfarin were seen in either chromatogram, but the fractions of eluate emerging at the retention time of warfarin were combined and concentrated, and a 1/5 aliquot of each concentrate was volatilized in the spectrometer. Fig. 3 shows the ion currents due to *m/e* 309 and 337. It is clear that warfarin was easily detected at this level. The absence of peaks from the extract of unfortified blood suggests that substantially lower levels could be detected and semi-quantitatively estimated, but the possibilities were not examined further.

CONCLUSIONS

It is concluded that warfarin can be determined conveniently, with sufficient sensitivity and specificity for most diagnostic purposes, by the proposed HPLC method. Identity can be confirmed by CIMS or by GC of the eluted warfarin after methylation[10]. Specific ion monitoring can be used to identify warfarin in HPLC eluates at levels below the sensitivity of the HPLC detector.

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