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Determination of warfarin in the yolk and the white of hens' eggs by reversed-phase high-performance liquid chromatography

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Abstract

A procedure for the determination of warfarin, an anticoagulant rodenticide, in the white and the yolk of hens' eggs, using reversed-phase high-performance liquid chromatography is described. Liquid chromatography was performed on an octadecylsilane cartridge using methanol and ammonium acetate triethylamine buffer as the mobile phase, with UV detection at 281 nm. Samples (5 g) were analysed after liquid-phase extraction using a mixture of acetone and diethyl ether. Linearity, precision and accuracy of the method were determined in the range of 0.5–8.0 μg. Limits of quantitation for warfarin in the white and the yolk were 0.020 and 0.015 μg/g, respectively. Mean recoveries of warfarin from spiked white and yolk samples were 84.6 and 87.4%, respectively. The analytical method was applied to a fourteen-day experimental study conducted in laying hens that had been orally dosed with warfarin. © 1997 Elsevier Science B.V.

Keywords: Eggs; Warfarin

1. Introduction

Warfarin, 3-α-phenyl-β-acetylethyl-4-hydroxy-coumarin, is an anticoagulant rodenticide that has been frequently used for many years to control rodent populations. Its fundamental mechanism of action is the inhibition of the vitamin k epoxide reductase, which causes blood clotting alterations leading to haemorrhages as the ultimate cause of death.

Ready-to-use cereal-based bait containing warfarin may be accidentally ingested by laying hens. As a general rule, hens do not present clinical signs because of the low sensitivity of poultry to anticoagulant rodenticides [1,2]. Nevertheless, the ingestion of hens' eggs containing warfarin residues represents a potential risk for human health. No reports have been published about warfarin residues in hens' eggs.

Several high-performance liquid chromatography (HPLC) methods have been described for the determination of warfarin in bait and animal tissues [3–13]. None of the published HPLC methods appeared to be applicable to the analysis of warfarin in hens' eggs.

The purpose of the present work was to develop a simple and rapid HPLC method for the analysis of warfarin in the white and the yolk of hens' eggs in

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order to determine the residues in eggs laid by hens exposed to warfarin.

2. Experimental

2.1. Reagents

Ammonium acetate (Merck, Darmstadt, Germany), triethylamine (Carlo Erba, Milan, Italy), glacial acetic acid, acetone, anhydrous sodium sulphate, diethylether (Panreac, Barcelona, Spain), acetonitrile and hexane (BDH Chemicals, Toronto, Canada) were analytical-grade reagents. HPLC-grade methanol was obtained from Carlo Erba. Warfarin (100% purity) was provided by Rhône-Poulenc (Lyon, France).

Ammonium acetate triethylamine buffer (pH 5.2) was prepared by mixing 3.85 g of ammonium acetate, 2 ml of glacial acetic acid and 2 ml of triethylamine in water, adjusting the pH to 5.2 with glacial acetic acid and diluting to 1 l.

2.2. HPLC apparatus and chromatographic conditions

The HPLC system consisted of a Varian 5000 chromatograph equipped with a Rheodyne injection valve (Palo Alto, CA, USA), an L 4250 variable-wavelength absorbance detector (Merck) and a C-R4A Chromatopac integrator (Shimadzu, Kyoto, Japan). The analytical cartridge, a 5-μm LiChroSpher 100 RP-18E (125×4.6 mm I.D.) from Merck, was equipped with a 5-μm LiChroSpher 100 RP-18E guard cartridge (4.6×4 mm I.D.).

The mobile phase was methanol–ammonium acetate triethylamine buffer (62:38, v/v). The mixture was filtered using a Sartorius HPLC solvent filtration system (Gottingen, Germany) and 47 mm, 0.22 μm nylon filters (MSI, Westboro, MA, USA). The chromatographic experiments were performed at 24 to 25°C. The operating flow-rate was 1.0 ml/min and the UV detector was set at 281 nm and 0.01 a.u.f.s. The sample volume injected onto the cartridges was 50 μl. The guard cartridge was replaced at intervals of 100 to 150 sample injections. The analytical cartridge was rinsed for 2 h after each day of operation with methanol–water (62:38, v/v) at a flow-rate of 0.2 ml/min.

2.3. Preparation of standard solutions

A stock standard solution of warfarin was prepared in acetone (1 mg/ml) and was stable for one month when stored at +4°C. The working standard solutions were prepared by diluting aliquots of the stock standard solution with mobile phase immediately before use. A 50-μl volume of the working standard solution (0.50 and 1.00 μg/ml) was injected onto the HPLC cartridge at the beginning of each day of operation.

2.4. Sample preparation

A 5-g amount of egg white or yolk was placed in a 25-ml polypropylene tube. For the validation assay, the samples were spiked at this point with the working standard solutions. A 2-g amount of anhydrous sodium sulphate was added to the tube and warfarin was extracted twice with 15 ml of a mixture of acetone–diethyl ether (90:10, v/v). After homogenization for 5 min (Rotator Drive, Heidolph, Keilheim, Germany) and centrifugation at 10 000 *g* for 5 min at −8°C (MR 1822 centrifuge, Jouan, Saint Herblain, France), the combined supernatants were evaporated to dryness in a rotavapor Büchi 461 (Flawil, Switzerland) at +40°C. The dried extract was reconstituted with 3 ml of acetonitrile. This sample was washed twice with 3 ml of hexane and centrifuged at 10 000 g for 5 min at −8°C (MR 1822 centrifuge). The hexane phases were discarded and the acetonitrile phase was evaporated to dryness under nitrogen in a +40°C dry bath. The dried extract was reconstituted with 1 ml of mobile phase and filtered through a Millex HV 4 mm, 0.45 μm filter (Millipore, Bedford, MA, USA). A 50-μl volume of the filtrate was injected into the HPLC system.

2.5. Validation assay

The specificity of the method was studied by analysing the chromatograms of the working standard solutions with those of spiked samples (spiked and extracted egg whites or yolks) and those of blank samples (unspiked and extracted egg whites or yolks).

The calibration curves for warfarin (range: 0.5, 1.0, 2.0, 4.0 and 8.0 μg) were obtained by analysing

two replicates of each spiked 5.0-g sample (white or yolk of hens' eggs) for three days [14]. They were drawn by plotting the known warfarin amounts against the warfarin peak heights. These data were used to study linearity, regression, precision, accuracy and extraction recoveries. The extraction recoveries of warfarin were determined by comparing the results of the analysis of the spiked samples with those of the working standard solutions [14]. The limits of detection were calculated as the lowest warfarin concentrations that gave a signal-to-noise ratio greater than three [14]. The limits of quantitation were defined as the lowest warfarin concentrations for which the method was validated with an accuracy and a precision that was within the range recommended [15,16].

2.6. Experimental conditions and device

Lohmann Brown laying hens were used in the study. No clinical signs of disease were apparent in the hens. The animals were housed in individual cages and acclimatized for five days in the laboratory, during which time they were clinically examined. Room temperature and illumination were maintained at 20 to 22°C and 15 h a day respectively. Five hens with correct clinical parameters, weighing 1.5 to 2.4 kg, were used. Food and water were supplied ad libitum.

Warfarin was orally administered in a single dose of 10 mg/kg body weight, diluted in sunflower oil. Eggs were collected over fourteen days and the white and the yolk of each egg were separated after each collection. The yolk was punctured with a needle and then taken up with a 5-ml polypropylene syringe, to avoid the yolk and the white mixing. The white and the yolk were immediately stored at −20°C until analysis.

3. Results and discussion

3.1. Chromatographic conditions

Reversed-phases have often been used for the HPLC determination of warfarin in bait and animal tissues [4–13]. A LiChroSpher 100-RP 18E prepacked cartridge was used as the stationary phase. This end-capped reversed-phase cartridge was chosen because of its low content of residual silanol groups [13].

All previous workers using reversed-phase systems added an organic modifier (methanol or acetonitrile) to the aqueous eluent. Comparisons of the two organic modifiers indicated that methanol led to higher resolution and symmetry factors than acetonitrile [5,8,12,13]. When disodium phosphate or potassium phosphate monobasic or dibasic were used as aqueous eluents, baseline drift and severe peak tailing were always noted. When ammonium acetate and triethylamine were used as aqueous eluents, the chromatograms were free of interfering peaks and neither tailing nor baseline drift were observed.

An isocratic system was used and different proportions of methanol and ammonium acetate triethylamine buffer were tested. When using 62% methanol and UV detection at 281 nm, the retention time of warfarin was constant (3.1 min) and chromatograms were free of additional peaks that could have resulted from impurities or degradation products and could interfere with the warfarin peak (Fig. 1). Under the operating conditions, the capacity, symmetry and resolution factors were 0.43, 0.08 (white) or 0.05 (yolk) and 2.66 respectively.

The guard cartridge was replaced after 100 to 150 injections because of its saturation with egg components, which gave rise to a drifting baseline. At the end of each day of operation, the analytical cartridge was rinsed for 2 h with methanol–water (62:38, v/v), which increased the cartridge's life. The number of theoretical plates of the analytical cartridge after the injection of 1500 samples into the HPLC system was about 90% of its initial value.

3.2. Sample preparation

Many organic solvents, e.g., acetone, chloroform, diethyl ether, dichloromethane, methanol and acetonitrile, have been used to extract warfarin from bait and animal tissues [3–13,17]. A mixture of acetone and diethylether was found to be the most suitable solvent for extracting warfarin from egg. This solvent gave clean extracts, possibly resulting from the degradation of some proteins of the egg [8,13,17].

The removal of lipids was very important in the clean-up procedure for the egg extracts, as the HPLC mode involved the use of a polar and aqueous mobile

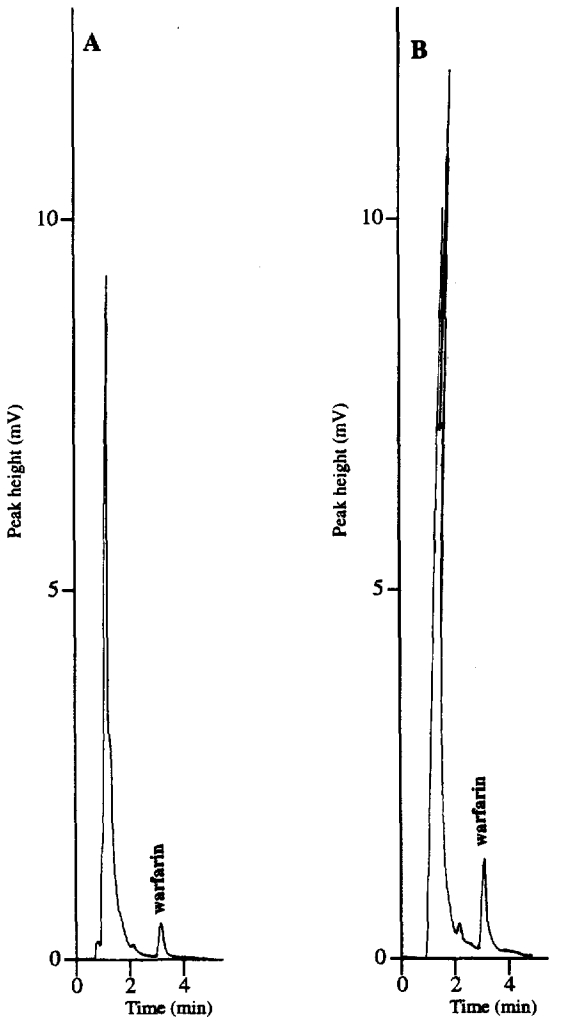

Fig. 1. (A) HPLC chromatogram of an egg white containing warfarin at a concentration of 0.15 μg/g; (B) HPLC chromatogram of an egg yolk containing warfarin at a concentration of 0.40 μg/g. Conditions: mobile phase, methanol–ammonium triethylamine buffer (62:38, v/v); analytical cartridge, 125×4.6 mm, C_{18E} (5 μm); flow-rate, 1.0 ml/min; wavelength, 281 nm; recorder sensitivity, 0.01 a.u.f.s.; injection volume, 50 μl.

phase. The egg extracts were reconstituted in acetonitrile and the lipids were removed using hexane. The best extraction recoveries were obtained by washing the acetonitrile phase twice with 3 ml of hexane.

The extraction recoveries of warfarin from the white and the yolk of egg were calculated for each of the five fortification levels (Table 1). They did not differ significantly from one fortification level to another at the 0.05 level (data not shown). Therefore, the mean extraction recoveries were calculated as the mean of the recoveries obtained for each of the five fortification levels. They were 84.6 and 87.4% for the white and the yolk of hens' eggs, respectively, and were very close to those determined in liver tissues [5–13,17].

3.3. Validation assay

The chromatograms of the blank samples were always free of additional peaks that could interfere with the warfarin peak. In addition, the retention time of warfarin remained constant when the working standard solutions or the spiked samples were chromatographed. Therefore, the analytical method could be considered to be specific.

A linearity and regression study was performed for each calibration curve separately (Table 2). The high values of the correlation coefficients (0.997 to 1.000) indicated good correlations between the amount of warfarin and the peak height. Moreover, for each calibration curve, the statistical data showed that the slope was significantly different from 0 at the 0.05 level [14].

The relative standard deviations of repeatability for spiked eggs were between 3.5 and 5.2% (Table 3). The relative standard deviations of reproducibility were between 4.1 and 6.7% (Table 3). The confidence limits of the mean relative bias at the 0.05

Table 1
Recoveries of warfarin from the white and the yolk of hen's eggs (5.0 g samples) spiked with from 0.5 to 8.0 μg of warfarin (mean±standard deviation in %, $n=6$)

	Recovery (%)				
	0.5 μg	1.0 μg	2.0 μg	4.0 μg	8.0 μg
Egg white	86.5±6.0	84.0±5.2	82.3±4.8	83.5±3.8	86.7±6.2
Egg yolk	89.5±5.9	87.0±4.3	85.8±3.9	84.9±6.8	89.8±4.7

Table 2
Linearity and regression data for the calibration curves obtained from the white and the yolk of hens' eggs (5.0 g samples) spiked with warfarin from 0.5 to 8.0 μg

	Slope	Intercept	Correlation coefficient	Slope existence (F test)
Egg white				
Day 1	2952.50	47.65	0.997	5352.98[a]
Day 2	3041.23	59.35	0.999	8223.52[a]
Day 3	2878.95	35.24	0.998	7012.58[a]
Egg yolk				
Day 1	3104.21	28.41	0.999	7407.55[a]
Day 2	2985.14	47.80	0.998	7001.86[a]
Day 3	3240.52	39.51	1.000	13 481.44[a]

$y=ax+b$ where y=peak height (μV); x=amount of warfarin (μg); a=slope and b=intercept; $n=12$.
[a] Slope is significantly different from 0 at the 0.05 level.

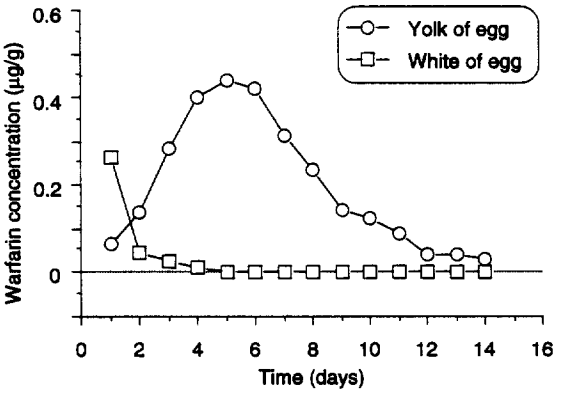

Fig. 2. Graphic representation of the mean concentrations of warfarin in the white and the yolk of eggs after a single oral dose of warfarin (10 mg/kg of body weight) to laying hens. For further details, see text.

level was within the range of −20 and +20% and those of the extraction recoveries at the 0.05 level were within the range of 80 and 120% [18]. Therefore, in the examined range (0.5 to 8.0 μg), the method was accurate and precise.

The limits of detection and quantitation for warfarin in egg white were 0.006 and 0.020 μg/g, respectively. Those in the yolk were 0.005 and 0.015 μg/g, respectively. The limits of quantitation were accepted (relative standard deviations <20.0% with $n=10$) because the mean peak heights were significantly different from the intercepts at the 0.05 level and they were greater than three standard deviations [15,16]. The limits of detection for warfarin in eggs were lower than those determined in liver tissues [5–13,17]. The limits of quantitation for warfarin were suitable for studying residues in hens' eggs.

3.4. Experimental conditions and device

The results of the experimental study are shown in Fig. 2. In egg white, the warfarin concentrations decreased from the first to the third day of the experiment and remained below the limit of detection of the analytical method from the fourth day onwards. In egg yolk, the warfarin concentrations increased, reaching a peak five days after the oral administration of the anticoagulant and then decreased up to the fourteenth day. Fourteen days after the oral administration of warfarin, the mean con-

Table 3
Precision data obtained from the white and the yolk of hens' eggs (5.0 g samples) spiked with warfarin from 0.5 to 8.0 μg ($n=6$)

	0.5 μg	1.0 μg	2.0 μg	4.0 μg	8.0 μg
Egg white					
R.S.D.[a] of repeatability (%)	3.5	4.2	4.5	4.6	4.1
R.S.D.[a] of reproducibility (%)	5.3	4.2	5.3	6.0	6.5
Egg yolk					
R.S.D.[a] of repeatability (%)	4.1	4.8	5.2	3.8	4.6
R.S.D.[a] of reproducibility (%)	4.1	5.5	5.8	4.9	6.7

[a] R.S.D.=relative standard deviation.

centration in the yolk was still 0.016 μg/g. The results of this study and of some other experiments will be fully discussed elsewhere [19].

4. Conclusion

The analytical method was specific, linear, precise and accurate in the range 0.5 to 8.0 μg/g and had low limits of quantitation. Because the extraction and clean-up procedure only involved centrifugation and liquid-phase extraction, the method was simple, rapid and not too expensive. An analyst familiar with the method could easily process fifteen samples a day. The proposed HPLC method may be used to investigate warfarin residues in the white and the yolk of hens' eggs.

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