TECHNICAL NOTE

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Purification and Determination Procedure of Coumarin Derivatives

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ABSTRACT: Anticoagulant coumarin derivatives, bromadiolone, brodifacoum, warfarin, and coumatetralyl were analyzed simultaneously with a modified analytical method. The method includes purification by Sep-Pak cartridge and HPLC analysis using isocratic elution with methanol: 0.8% Acetic acid (8:2). The recoveries of coumarin derivatives from biological samples ranged from 96 to 99%. The concentrations of coumarin derivatives in intoxicated rats' organ were also determined with this analytical procedure.

KEYWORDS: forensic science, forensic toxicology, coumarin derivatives, rodenticides, Sep-Pak cartridge, high-performance liquid chromatography (HPLC), solvent extraction

Recently developed anticoagulant coumarin derivatives, such as bromadiolone, brodifacoum, coumatetralyl, and warfarin have been widely used as rodenticides (1,2). However, because of their high toxicity and easy availability, coumarin derivatives are often involved in many cases of homicide and suicide in Korea. Their oral LD_{50} values in rats are 270 μg/Kg for brodifacoum and 1.2 mg/Kg for bromadiolone (3). Therefore, trace analysis of coumarin derivatives in biological tissue and urine is extremely important in determining the cause of death.

Several selective determination methods have been developed for individual coumarin derivatives. For instance, thin layer chromatography (TLC) (1) gas chromatography (GC) (2) and high performance liquid chromatography (HPLC) (3,4) methods have been employed to determine warfarin. In 1979, Koubek and his group reported the determination of brodifacoum with HPLC (5). Recently Lee et al. showed that a mixture of coumarin anticoagulants could be determined simultaneously by HPLC, (6) employing a gradient elution method. However, HPLC determination of coumarin derivatives in biological samples without adequate purification is not reliable.

Various purification methods have been reported to purify and determine coumarin derivatives: a liquid-liquid extraction purification method was used for plasma analysis (8). HPLC analysis of the 4-hydroxy coumarin anticoagulant phenprocoumon and metabolites in urine after solid phase extraction purification with C_{18} reverse phase materials was reported (9). Lately, a solid phase extraction method has been described for the HPLC analysis of several coumarin derivatives in urine samples using anion exchange stationary phase (10). However, those methods can be applied to biological fluid samples only.

Koubek et al. (5) used the following purification procedure to determine brodifacoum in rat tissue sample: The brodifacoum samples were extracted with chloroform/methanol (9:1) mixed solvent, and subsequently concentrated by evaporation. The solid residues in preconcentrated samples were removed by filtration with glass fiber paper filter. The filtered samples were diluted with 10 mL 15% methylene chloride in cyclohexane, centrifuged and further filtered and separated using GPC columns. However, the recoveries were unsatisfactory. In addition, the risk of contamination is high with this process.

In this paper, we report a new modified method to purify and determine coumarin derivatives in biological samples simultaneously with excellent reproducibility.

Experimental

Materials

Coumarin standards were purchased commercially from various chemical manufacturers: coumarin (Fluka AG), warfarin (ICN Pharmaceuticals Inc.), coumatetralyl (Technical), bromadiolone and brodifacoum (IBL, England). All organic solvents used in HPLC mobile phase and solvent extraction were of HPLC grade. All other reagents were purchased as analytical grade and used without further purification. Water was deionized by Milli Q water purifying system before use.

Chromatography

HPLC (Waters model 990) equipped with a photodiode array detector was used. The column was 20 cm, μ-Bondapak C_{18} (Waters). A methanol and 0.8% acetic acid mixture (8:2) was used as an eluent with a flow rate of 1.0 mL/min. The pump pressure

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was adjusted to 900 psi throughout experiments. The photodiode array detector wavelength was set at 280 nm and on line spectra were recorded in the range 190–610 nm.

Purification Procedure

The coumarin derivative standard mixtures (20 μg/mL each), 5 mL, were adjusted to pH 3–4 with N-sulfuric acid. The coumarin derivatives were extracted three times with 10 mL 10% methanol in chloroform. Extracted samples were purified according to the following methods.

(1) Column chromatography

A glass column (11 × 240 mm) was subsequently packed with 8 g of Florisil (Aldrich Chemical Co.) followed by 5 g silica gel (Merk chemical). The extracted samples were eluted in the column equilibrated with methanol/chloroform (50:1). The eluted samples were concentrated by evaporation at 40°C, and redissolved in methanol. These samples were introduced into the HPLC.

(2) Sep-Pak cartridge

Silica Sep-Pak cartridges(Waters) with 2 g of sodium sulfate were washed with methanol (5 mL) followed by cyclohexane (5 mL). The redissolved sample extracts in cyclohexane were applied to the preconditioned Sep-Pak and eluted with methanol (5 mL).

Calibration Curves

Calibration curves for coumarin and its derivatives were obtained by processing standard solutions with known concentration of analytes (2.5–10 μg/mL, each) and internal standard (N,N-diphenylbenzidine, 20 μg/mL) through the analytical procedure. Least square linear regression of the ratio of coumarin and its derivatives peak area/internal standard peak area versus added concentration was used to calculate the calibration factor for each compound. The regression equation values and correlation coefficients for standard calibration curves of coumarin and its derivatives are shown in Table 1.

Analytical Recoveries

The analytical recoveries for each compound were determined by comparing the peak areas of extracts from standard solution and biological samples with added known concentrations, to standard solution of the compounds at the same concentration. We added 1 mL of standards containing warfarin, bromadiolone and brodifacoum (20 μg/mL, each) to 3 mL of rat blood or organ homogenates to evaluate recovery from biological matrices. All analytical procedures were performed as described in the experimental section.

TABLE 1—*Standard calibration curves of coumarin and its derivatives.*

Compound	Regression equation	Correlation coefficient	LOD (μg/mL)
Coumarin	$Y = 0.319X + 0.580$	$r = 0.989$	0.02
Warfarin	$Y = 0.149X + 0.213$	$r = 0.998$	0.025
Coumatetralyl	$Y = 0.153X - 0.090$	$r = 0.999$	0.025
Bromadiolone	$Y = 0.157X - 0.035$	$r = 0.999$	0.025
Brodifacoum	$Y = 0.068X - 0.022$	$r = 0.996$	0.025

NOTE: Sample concentration (2.5 − 10 μg/mL).
Sample injection volume: 10 μL.
Internal standard: N,N-Diphenylbenzidene (20 μg/mL).

Extraction Procedure from Biological Samples

Biological samples of rats' (Sprague Dawley rat, approximately 150 g) organs (heart, lung, liver, kidney and spleen) were ground completely with a crusher (Ultra -turrux T25, IKA). The 3 mL of crushed individual organ or blood samples (in case of the recovery experiments, 1 mL of coumarin derivative mixture was added) were adjusted to pH 3–4 with N-sulfuric acid and extracted three times with 10 mL 10% methanol in chloroform. The sample extracts were concentrated by evaporation under 40°C and redissolved in 5 mL of cyclohexane. In the process, if some solid residues were found, samples were sonicated and centrifuged. These samples were then processed following the purification step with Sep-Pak. The analytical procedure for biological samples is summarized in Fig. 1.

Results and Discussion

The complete separation of standard coumarin derivatives in adequate time (19 min) was obtained with the analytical conditions described in the experimental section. The chromatogram and the corresponding absorption spectra are shown in Fig. 2. This method was optimized in order to simplify the sample purification steps and obtain adequate selectivities, precision, accuracy and analytical recoveries in comparison to previous procedures reported (5–7,9).

We evaluated elution solvents composed of methanol and 0.8% acetic acid at various ratios (9:1, 8:2, and 7:3). The composition of the eluent was that used by Lee et al. (6) in their gradient elution

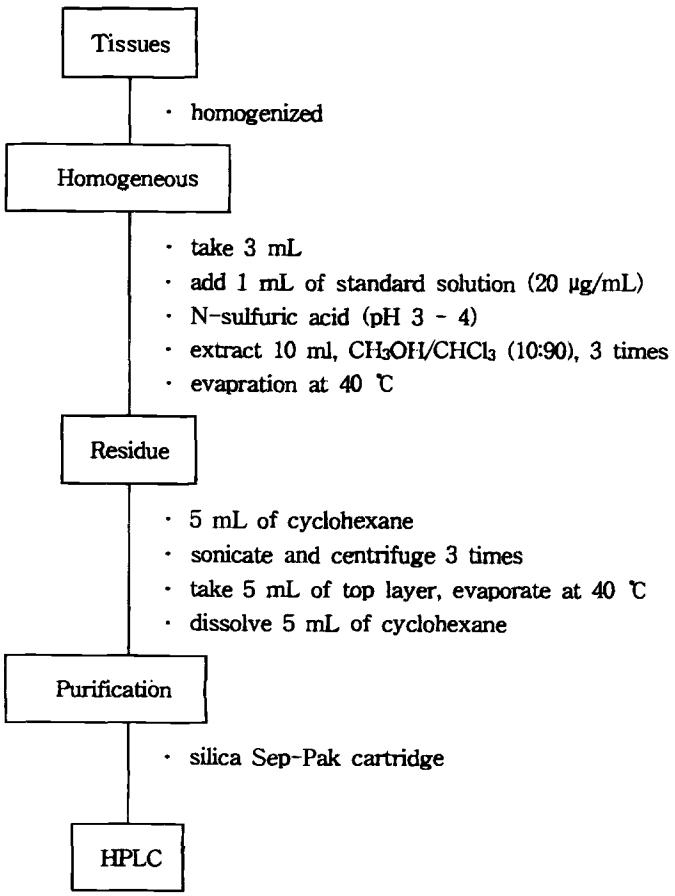
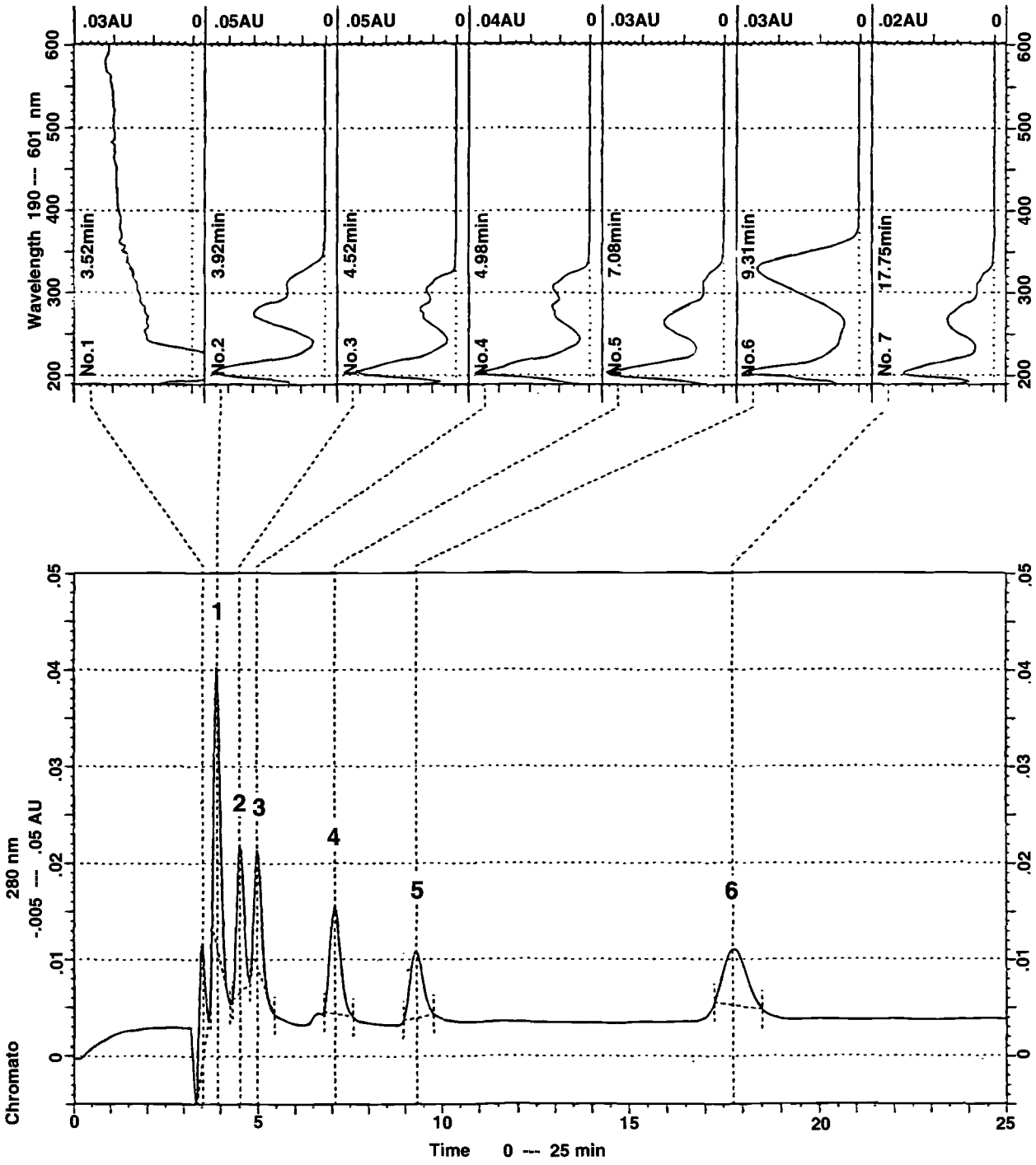


FIG. 1—*Analytical procedure for coumarin derivatives in biological samples.*



1. Coumarin 2. Warfarin 3. Coumatetralyl

4. Bromadiolone 5. N,N,-Diphenylbenzidene(INSTD) 6. Brodifacoum

FIG. 2—*HPLC chromatogram and corresponding UV-VIS spectra of coumarin and its derivatives.*

experiments. The chromatographic results are summarized in Table 2. These experiments were conducted to determine the optimum mobile phase composition to separate the coumarin derivatives under isocratic condition. The 9:1 mixture yielded poor resolution between coumarin derivatives. The 7:3 mixture showed relatively long retention times and low sensitivities due to band broadening despite very good separation between peaks. The chromatographic results of isocratic elution with 8:2 mixed solvent were similar to or better than the results reported by Lee who employed gradient elution, in terms of analytical time (16.6 min) and resolution. The coumarin derivatives' calibration curves were measured in a range of 2.5–10 μg/mL, each. Calibration curves were linear (r = 0.989

TABLE 2—*Influence of resolution and sensitivity by mobile phase composition.*

Compound	Mobile Phase (Methanol/0.8% Acetic acid in water) 90:10 t_o	90:10 h	80:20 t_o	80:20 h	70:30 t_o	70:30 h
Coumarin	3.4	0.06	3.9	0.04	4.0	0.03
Warfarin	3.4	0.06	4.5	0.02	4.7	0.02
Coumatetralyl	3.4	0.06	5.0	0.02	5.3	0.02
Bromadiolone	3.4	0.06	7.1	0.01	8.2	0.01
Internal Standard	3.9	0.03	9.3	0.01	11.4	0.01
Brodifacoum	4.5	0.04	17.8	0.01	21.0	0.01

NOTE: t_o = Retention time (min).
h = Peak height (absorbance units at 280 nm).
Sample concentration: 10 μg/mL.
Sample injection volume: 10 μL.
Internal standard: N,N-Diphenylbenzidene (20 μg/mL).

− 0.999) with less than 3% coefficient of variable (CV) values. The limits of detection for coumarin derivatives are similar to those of Lee (6).

Koubek (5) reported a purification method for the analysis of brodifacoum from biological tissue samples. However, his procedure contained more than seven steps that are liable to cause experimental error and contamination. Therefore, we attempted to find a simpler way of purifying samples. The mixed standards of bromadiolone and brodifacoum were applied to column chromatography or Sep-Pak column for purification procedures. Although both methods showed acceptable recoveries between 98–100%, the Sep-Pak method is simpler and faster than the column chromatographic technique. In order to adsorb samples in Sep-Pak, we chose cyclohexane, which has the best distribution coefficient with silica gel of the solvents tested (acetone, 3.0, 2.0; cyclohexane, 778, 777; chloroform, 90, 2; methanol, 0.08, 0 for bromadiolone and brodifacoum, respectively). Methanol was used to elute adsorbed samples from Sep-Pak cartridges.

The recoveries are listed in Table 3. The coumarin derivatives showed recoveries of 97–98% with good reproducibilities (CV = −2%). These results indicate that coumarin derivatives were not bound to protein or other cellular materials. The results of our recovery studies were much better than those of Koubek's (6) results of 92–107% and Yeun's (7) 88–114% for brodifacoum, and De Vries' (9) 85–98% for phenprocoumon.

TABLE 3—*Recoveries of coumarin derivatives added to biological samples.*

Rats blood and organs	Warfarin Found μg	Warfarin Recovery (%)	Bromadiolone Found μg	Bromadiolone Recovery (%)	Brodifacoum Found μg	Brodifacoum Recovery (%)
Blood	19.4	97.0	19.1	95.5	19.2	96.0
Liver	19.3	96.5	19.2	96.0	19.6	96.5
Lung	19.5	97.5	19.6	98.0	19.8	99.0
Heart	19.6	98.0	19.3	96.5	19.7	98.5
Kidney	19.2	96.0	19.4	97.0	19.8	98.5
Spleen	19.4	97.0	19.5	97.5	19.8	99.0

NOTE: Standard coumarin derivatives 1 mL (20 μg/mL) added to 3 mL of grounded rats' blood and tissue samples.
Purification method: Sep-Pak cartridge.

TABLE 4—*Distribution of warfarin in rats' blood and tissues (μg/g).*

Blood	Liver	Lung	Heart	Kidney	Spleen
22.5	104.8	29.0	42.3	32.8	24.7

NOTE: Dose: 12.9 mg per day, total 5 day (64.5 mg).

One of the coumarin derivatives, warfarin in intoxicated rats' organs was also determined. Warfarin is the most frequently used rodenticides among the coumarin derivatives in Korea. Warfarin (12.9 mg/day) was injected into Dawley rats for five days; its concentrations in organs ranged from 22 to 104 μg/g (Table 4).

The advantages of the present method consist in the simplicity of the solid phase extraction step with Sep-Pak and the use of isocratic elution of HPLC with photodiode array detector; also, adequate analytical time and higher analytical recoveries, calibration curve linearities, reproducibilities and limits of detection were compared with those obtained with previous methods reported.

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