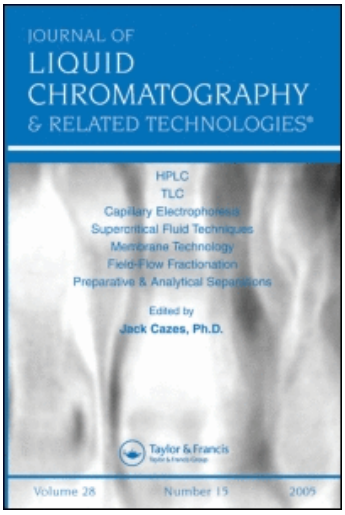

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Determination of Warfarin and Coumatetralyl in Serum by HPLC Coupled with Electrospray Ionization Mass Spectrometry

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Abstract: A sensitive, fast, and accurate method to determine warfarin and coumatetralyl in serum by high performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) was developed. After warfarin and coumatetralyl in serum was extracted by ethyl acetate, a satisfactory separation was achieved on a XDB C_{18} column, 150 mm × 2.1 mm × 5 μm, by using the mobile phase consisting of acetic acid-ammonium acetate (10 mmol/L, pH = 4.5) (20%) and methanol (80%) in isocratic eluent at a constant flow rate of 0.30 mL/min. The molecular ions m/z $[M\text{-}H]^-$ 307 and 291 were selected for the quantification in the selected ion monitoring (SIM) mode for warfarin and coumatetralyl, respectively. The linear range was 0.5 ∼ 100.0 μg/L for warfarin and coumatetralyl. The limit of quantification in serum was found to be 0.5 μg/L for each. The recoveries were between 91.9 ∼ 94.9% for warfarin and 90.9 ∼ 104.0% for coumatetralyl, the intra- and inter-day RSDs for each were less than 10.1%. This method can determine trace warfarin and coumatetralyl in serum and it can be used to diagnose poisoned humans and other animals.

Keywords: Warfarin, Coumatetralyl, Serum, HPLC-ESI-MS

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INTRODUCTION

Warfarin [4-hydroxy-3-(3-oxo-1-phenyl-butyl) coumarin] and coumatetralyl [4-hydroxy- 3-(1,2,3,4-4H-1-naphthyl) coumarin] (Fig. 1), are common agricultural chemicals, which as anticoagulant rodenticides were widely used for pest control in many countries. The increased commercial availability of these compounds had resulted in an increase in accidental and intentional ingestion in both animals and human beings. In the year 1996, Yang[1] reported 189 cases of poisoned humans by coumatetralyl. In 2004, Yi[2] reported 600 cases of goose poisoning owing to the accidental ingestion of the warfarin rodenticide. Methods for rapid identification and determination of these two rodenticides are required both for diagnosis and effective treatment of intoxication and for forensic purposes.

Recently, a variety of techniques have been used for the analysis of warfarin and coumatetralyl in biological matrices, however, high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence detection (FLD) appeared to be the most effective method for the determination of these compounds in serum or other animal organic tissues.[3–10] Nevertheless, owing to the sub-selectivity of HPLC with UV and FLD, a false negative or a false positive could occur and, thus, could have costly consequences for forensic cases. Mass spectrometry (MS) has played an important role in toxicology due to its sensitivity, rapidity, and low levels of sample consumption for the characterization and determination, especially, in combination with a HPLC; it is considered a superior alternative to HPLC/UV or HPLC/FLD for the analysis of many toxic compounds.[11–13]

To our knowledge, there is no literature describing the simultaneous determination of warfarin and coumatetralyl in serum by HPLC coupled with electrospray ionization mass spectrometry (ESI-MS). In the present study, we developed a simple, sensitive, and highly selective HPLC-ESI-MS method for simultaneous determination of warfarin and coumatetralyl in serum.

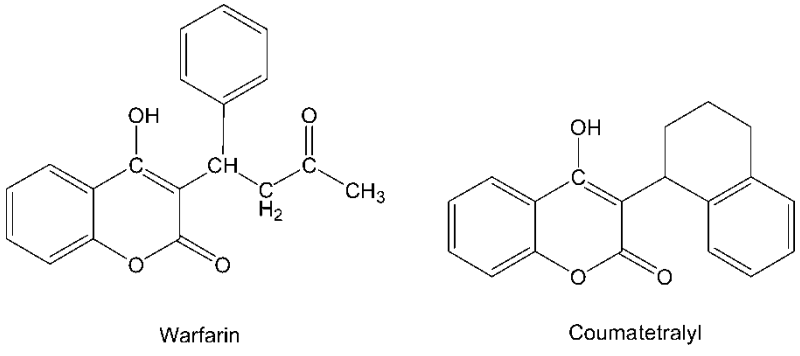

Figure 1. Chemical structures of warfarin and coumatetralyl.

EXPERIMENTS

LC-MS System

The method for development and validation was performed on an Agilent 1100 series LC/MSD Trap SL System (Agilent Technologies, Germany), consisting of a quaternary pump (G1311A), a column thermostat (G1316A), a degasser unit (G1379A), an autosampler (G1313A), a diode array detector (1315B), and an ion trap mass spectrometer with an ESI interface. The HPLC-ESI-MS system was controlled, and data were analyzed, on a computer equipped with LC/MSD Trap Software 4.2 (Bruker). All tubing used for connections was PEEK (0.25 mm I.D., Agilent Technologies, Germany).

Solvents and Materials

The acetonitrile (MeCN), methanol (MeOH), acetic acid, ethyl acetate (Merck, Darmstadt, Germany) used were HPLC grade. Water employed was supplied by a Milli-Q water purification system from Millipore (Molsheim, France). Warfarin (>99%) and coumatetralyl (>98%) were purchased from Sigma (St. Louis, MO, USA). Acetone, diethyl ether, dichloromethane, and ammonium acetate were obtained from Shanghai Reagent Company (Shanghai, China). Drug-free human serum was supplied by Ningbo Blood Center (Ningbo, Zhejiang, China). Serum samples were provided from Ningbo Veterinary Station (Ningbo, Zhejiang, China).

Preparation of Standard Stock Solutions

Authentic standards of warfarin and coumatetralyl were accurately weighed, transferred to volumetric flasks, and dissolved in MeOH to make individual stock solutions of 1.0 mg/mL. These solutions were thoroughly mixed and stored at 4°C in tightly closed bottles until use, which were stable for at least a month. Interim diluted solutions of warfarin and coumatetralyl were prepared with MeOH at the concentration of 10.0 μg/mL, which was used for spiking serum.

Preparation of Spiked Serum Samples

Appropriate amounts of the diluted standard solutions (10.0 μg/mL) of warfarin and coumatetralyl were taken to a 10 mL polypropylene centrifuge tube, and evaporated to dryness under a gentle stream of nitrogen. Then the residues were reconstituted with 0.5 mL of drug-free human serum, which was thawed to room temperature in advance, to give final warfarin and

coumatetralyl concentrations of 1.00, 15.00, and 100.00 μg/L. These spiked human serum solutions were considered as the quality control (QC) samples.

Preparation of Sample

Serum samples (0.50 mL), thawed to room temperature in a 10 mL polypropylene centrifuge tube before analysis, were vortex mixed and extracted using 2.0 mL ethyl acetate for 3 min. After centrifugation for 5 min at 7800 rpm, the upper organic layer was transferred to a disposable glass tube and liquid-liquid extraction was repeated once. The organic layer was combined and evaporated to dryness under a stream of nitrogen on a heating block at 50°C in a disposable glass tube. Prior to analysis, the residues were dissolved in 100 μL mobile phase. The tubes were briefly sonicated in a KQ 500DB ultrasonic cleaning bath (Kunshan Ultrasonic, Jiangsu, China) to facilitate dissolution and centrifugation before injection. All experiments were performed in duplicate.

HPLC-ESI-MS Analysis

The separation was performed on a Zorbax Eclipse XDB C_{18} column (150 mm × 2.1 mm, 5 μm particle size, Agilent Technologies, USA) using acetic acid-ammonium acetate (5 mmol/L, pH = 4.5)/methanol (20:80) as mobile phase in isocratic eluent, at a constant flow rate of 0.30 mL/min. Detection was carried out on an Agilent 1100 series LC/MSD Trap SL *mass* spectrometer in the negative mode with a full scan mass spectra over the m/z range 100 ~ 400 u, using a cycle time of 1 s and a peak width of 0.1 s, a capillary voltage of 2.3 kV, a capillary exit voltage of −136 V, a dry temperature of 350°C, a high purity nitrogen (99.999%) dry gas of 9.0 L/min, a nitrogen nebulizer pressure of 35.0 psi, and a dwell time of 200 ms. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. Analytes were detected with an ESI in selected ion monitoring (SIM) mode. The quantification ions were selected from their molecular ions, m/z [M-H] 307 for warfarin, and m/z [M-H] 291 for coumatetralyl, respectively. The SIM peak areas were integrated for quantification.

RESULTS AND DISCUSSION

Optimization of the Extraction Solvent

Extraction of the interested components in a biological matrix before HPLC-ESI-MS is a prerequisite for successful analysis. In published methods, liquid–liquid extraction with chloroform-acetone (50:50, V/V),[8]

acetone-diethylether (90:10, V/V),[9] and acetonitrile[10] as a solvent, had been used for extraction of warfarin and coumatetralyl from serum samples. The disadvantages of these solvents were either that they resulted in poor separation from serum endogenous interferences or low extraction efficiencies (not more than 80%). We observed that ethyl acetate was better than chloroform-acetone, acetone-diethylether, and acetonitrile because it extracted warfarin and coumatetralyl efficiently from the serum samples, the recoveries were more than 90.9%, as shown in Table 1. Ethyl acetate extraction gave not only good recoveries of the analytes but also a clean chromatogram of the drug-free serum and no interfering peak near the retention times of warfarin and coumatetralyl, as shown in Fig. 2. This result was in agreement with that reported by Guan,[4] who thought ethyl acetate was the best solvent for extraction of the anticoagulant rodenticides from the serum.

Development of HPLC-ESI-MS Method

Optimization of the Mobile Phase

To optimize the chromatographic conditions, the effects of the organic component and mobile phase modifier were explored. Use of MeCN as the organic component of the mobile phase did not result in adequate sensitivity and selectivity. MeOH as the organic component resulted in better sensitivity but variation of the amount of MeOH in the mobile phase affected resolution and run time. It was found that the retention time of both warfarin and coumatetralyl was reduced with the increase of MeOH concentration. In addition, in order to improve the reserved capability of the two target compounds on the XDB C_{18} column, we found that an acidic system, e.g., acetic acid-ammonium acetate buffer (5 mmol/L, pH = 4.5), which was extensively used in HPLC-MS measurements, could prolong the retention times of both warfarin and coumatetralyl. As shown in Fig. 2 (B–C), compared with water/MeOH (20/80, V/V), the acetic acid-ammonium acetate system obtained good separation, while the water/MeOH (20/80, V/V) system obtained little separation and little reserved capability on the column; the retention times of both warfarin and coumatetralyl were 2.5 min as well as the dead time. However, the SIM technique did not require good separation for the determination of compounds, suitable separation could usually increase the precision of the method owing to the complicate matrices of the serum. Thus, the buffer system was chosen, which was effective for HPLC separation of the analytes and obtained good precision for the method.

Development of MS Method

Warfarin and coumatetralyl are coumarin compounds which have phenolic hydroxyl and have some characteristics of the phenolic compounds, which

Table 1. The recoveries and RSDs of warfarin and coumatetralyl spiked with three different concentrations (n = 5, $\bar{x} \pm s$)

Added (μg/L)	Found (μg/L[a])		Recovery (%)		RSD (%)			
					Warfarin		Coumatetralyl	
	Warfarin	Coumatetralyl	Warfarin	Coumatetralyl	Intra-day[b]	Inter-day[c]	Intra-day	Inter-day
1.00	0.94 ± 0.05	1.04 ± 0.06	94.0	104.0	5.3	7.3	5.9	7.9
15.00	14.24 ± 1.22	13.63 ± 1.31	94.9	90.9	8.5	9.3	9.6	10.1
100.00	91.82 ± 5.96	95.32 ± 9.05	91.9	95.3	6.5	9.5	7.2	9.9

[a]Determined in one day.
[b]n = 5.
[c]n = 5 Replicates × 5 days within a 14-day period.

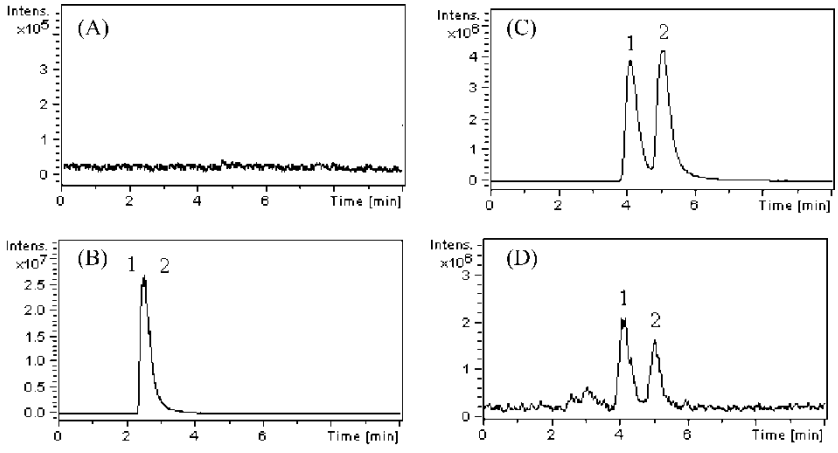

Figure 2. Typical SIM chromatograms of: (A) drug-free serum in 2.6 analytical conditions; (B) serum spiked with 0.50 mg/L warfarin and coumatetralyl, each, in 2.6 analytical conditions except using water/MeOH (20/80, V/V) as mobile phase; (C) serum spiked with 0.50 mg/L warfarin and coumatetralyl, each, in 2.6 analytical conditions; (D) serum spiked with 0.50 mg/L warfarin and coumatetralyl, each, in 2.6 analytical conditions except using ESI in the positive mode. Peak identification: (1) warfarin and (2) coumatetralyl.

could correspondingly easily dissociate active proton under the ESI conditions to form pediocratic anions, which would be the first choice in the negative mode for mass spectrometric measurements. In addition, mass spectrometry in negative ion mode has the benefit of a low level of chemical noise. In this study, we compared ESI in the positive mode with ESI in the negative mode for the determination of warfarin and coumatetralyl, and they gave the significantly differential mass signal intensity to the analytes, as shown in Fig. 2 (C–D). As can be seen, ESI (−) was more sensitive than ESI (+) for both compounds. ESI (−) was used in our experiments, because the ESI interface was expected to be have a low flow rate from the HPLC eluent, not more than 0.5 mL/min, which was compatible with the narrow C_{18} column (2.1 mm I.D.), and otherwise might cause the decrease of the sensitivity and waste of the solvent. The ESI mass spectrum of each compound was recorded in the negative ion mode to select the most mass-to-charge ratio (m/z) for further studies. For either warfarin or coumatetralyl, an intense $[M\text{-}H]^-$ signal was the only peak and no fragment peak was observed under the ESI (−) conditions selected, as shown in Fig. 3.

Validation of HPLC-ESI-MS Method

Calibration curves were obtained for warfarin and coumatetralyl using a series of standard solutions over the concentration range of 0.5 ∼ 100.0 μg/L. Three

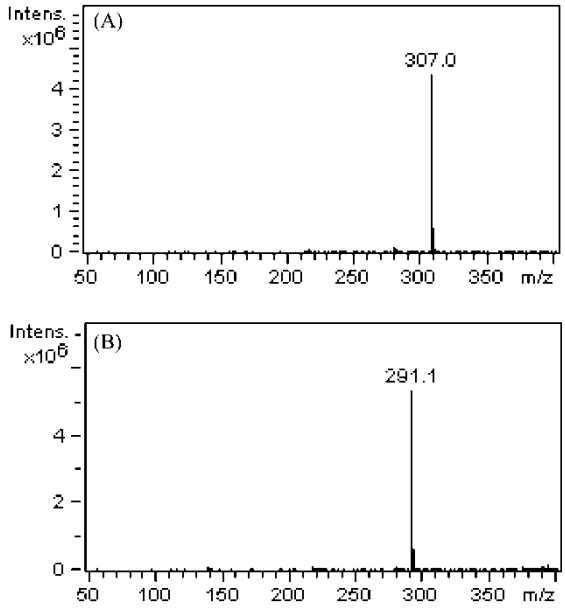

Figure 3. The mass spectra of warfarin (A) and coumatetralyl (B) in the negative mode.

replicate injections of standards at each concentration were performed. All calibration curves were linear over the concentration ranges tested, with coefficients of determination $r^2 > 0.992$. The peak area (A) was then used in conjunction with the calibration curve to derive the concentration (C, $\mu g/L$) in serum. The regression equations were $A = 2.78 \times 10^5 C + 7.72 \times 10^4$ and $A = 2.25 \times 10^5 C + 5.78 \times 10^4$ for warfarin and coumatetralyl, respectively.

The limits of quantification for warfarin and coumatetralyl were determined using a drug-free human serum sample spiked at 2.0 $\mu g/L$ standard solution, extraction with ethyl acetate, detection in SIM mode, and evaluation by the criterion that the signal to noise ratio (S/N) should be >10 for quantification purposes. The limits of quantification were 0.5 $\mu g/L$ for both warfarin and coumatetralyl.

The intra-day precision was evaluated by performing five replicates of three spiked QC samples (1.00, 15.00, and 100.00 $\mu g/L$) including extraction procedures. The intra-day precision (RSD) on the basis of peak area was less than 8.5% and 9.6% for warfarin and coumatetralyl, respectively, as shown in Table 1.

The inter-day precision was also evaluated by performing five replicates of three spiked QC samples (1.00, 15.00, and 100.00 $\mu g/L$) including extraction procedures each day on five different days within a 14-day period. Inter-day precision (RSD) on the basis of peak area was less than 9.5% and 10.1% for warfarin and coumatetralyl, respectively, as shown in Table 1.

Table 2. The results of the serum suspected samples for coumatetralyl ($n = 6$, $\bar{x} \pm s$)

Sample	A	B	C	D	E	F
Content (μg/L)	3.3 ± 0.2	58.9 ± 5.4	70.4 ± 3.6	92.8 ± 9.0	12.8 ± 1.0	31.5 ± 1.7

The recoveries were estimated by external standard calibration, drug-free human serum samples spiked with warfarin and coumatetralyl at three different concentrations (1.00, 15.00, and 100.00 μg/L) were extracted with ethyl acetate, as shown in Table 1. They were 94.0%, 94.9%, 91.9%, and 104.0%, 90.9%, 95.3% for warfarin and coumatetralyl, respectively.

Application to the Real Serum Samples

The method described in this paper has shown to be useful in the diagnoses of several intoxications in humans and animals. During our method validation, we received a set of serum samples from Ningbo Veterinary Station (Ningbo, Zhejiang, China) requesting confirmation of suspected anticoagulant rodenticides poisioning. We found that the molecular ion $[M\text{-}H]^-$ 291 and the retention time ($t_R = 5.0$ min) for the suspected sample were the same as that of the coumatetralyl standard, so we thought there was coumatetralyl in the suspected serum samples, which were contaminated by coumatetralyl. Table 2 shows that the results of the total concentration of coumatetralyl in serum samples were from 3.3 to 92.8 μg/L.

CONCLUSIONS

This method provides a selective, reliable, and precise measurement for the rapid determination of warfarin and coumatetralyl in serum samples. The sensitivity, simple extraction with no additional cleanup procedures, and small sample requirements make this method appropriate for use in forensic toxicology or clinical diagnoses of poisoned humans and animals.

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REFERENCES

1. Yang, J.; Mi, H.W. Therapy for acute poisoning by coumatetralyl (189 cases). Chin. J. Vector Bio. & Contr. **2002**, *13* (5), 390–391.

2. Yi, Y.Z.; Zhou, K.J.; Hang, H.S. Treatment for goose poisoning by warfarin. Chin. Poultry Husband. & Dis. Contr. **2004**, *6* (1), 5–5.
3. Guan, F.Y.; Liu, L.; Luo, Y. High-performance liquid chromatographic determination of seven anticoagulant rodenticides in plasma. Chin. J. Anal. Chem. **1995**, *23* (2), 159–161.
4. Guan, F.Y.; Akira, I.; Hiroshi, S.; Kanako, W.; Takeshi, K.; Osamu, S. A method for simultaneous determination of five anticoagulant rodenticides in serum by high-performance liquid chromatography. J. Pharm. Biomed. Anal. **1999**, *21* (1), 179–185.
5. Paula, R.R.; James, M.B. Validation of a method for the determination of (R)-warfarin and (S)-warfarin in human plasma using LC with UV detection. J. Pharm. Biomed. Anal. **2000**, *22* (3), 573–581.
6. Zhang, Z.Y.; King, B.M.; Wong, Y.N. Quantitative liquid chromatography mass spectrometry mass spectrometry warfarin assay for *in vitro* cytochrome P450 studies. Anal. Biochem. **2001**, *298* (1), 40–49.
7. Ufer, M.; Kammerer, B.; Kirchheiner, J.; Rane, A.; Svensson, J.O. Determination of phenprocoumon, warfarin and their monohydroxylated metabolites in human plasma and urine by liquid chromatography-mass spectrometry after solid-phase extraction. J. Chromatogr. B **2004**, *809* (2), 217–226.
8. Eugenie, A.P.; Jan den, H.; Jean, F.S.; Frederik, A.W. A method for the simultaneous identification and quantitation of five superwarfarin rodenticides in human serum. J. Anal. Toxicol. **1995**, *19* (3), 557–562.
9. Fauconnet, V.; Pouliquen, H.; Pinault, L. Reversed-phase HPLC determination of eight anticoagulant rodenticides in animal liver. J. Anal. Toxicol. **1997**, *21* (3), 548–553.
10. Thongchai, C.; Lawrence, J.F.; Michanel, J.M. Simultaneous determination of eight anticoagulant rodenticides in blood serum and liver. J. Anal. Toxicol. **1993**, *17* (1), 56–61.
11. Marquet, P. Progress of liquid chromatography–mass spectrometry in clinical and forensic toxicology. Ther. Drug. Monit. **2002**, *24* (2), 255–276.
12. Jin, M.C.; Chen, X.H.; Pan, B.X. Simultaneous determination of 19 chlorophenols in water by liquid chromatography mass spectrometry with solid-phase extraction. J. Liq. Chromatogr. & Rel. Technol. **2006**, *29* (9), 1369–1380.
13. Guan, F.Y.; Ishii, A.; Seno, H.; Watanabe-Suzuki, K.; Kumazawa, T.; Suzuki, O. Use of an ion-pairing reagent for high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry determination of anionic anticoagulant rodenticides in body fluids. J. Chromatogr. B **1999**, *731* (1), 155–165.

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