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Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 179–185

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Short communication

A method for simultaneous determination of five anticoagulant rodenticides in whole blood by high-performance liquid chromatography

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Received 17 June 1998; received in revised form 25 November 1998; accepted 1 January 1999

Keywords: Anticoagulant rodenticide; Warfarin; Coumatetralyl; HPLC; Analytical toxicology; Whole blood

1. Introduction

Anticoagulant rodenticides of 4-hydroxycoumarin and indandione derivatives are agricultural chemicals widely used for pest control in many countries. Human poisonings, caused by accidental or intentional ingestion of these over-the-counter chemicals, were reported [1–7]. Methods for detection and determination of these rodenticides are required both for diagnosis and effective treatment of the intoxication and for forensic purposes. A number of techniques, such as gas chromatography coupled with mass spectrometry (GC–MS) [8], thin-layer chromatography (TLC) [9] and immunoassay [10], were used for measurements of anticoagulant rodenticides in biological matrices; however, high performance liquid chromatography (HPLC) appears most effective [11–26]. Most of the published methods dealt with a single group of compounds, 4-hydoxycoumarins or indandiones. A few reports described simultaneous determination of both 4-hydoxycoumarins and indandiones in serum [13–15] or animal organ tissue [11,12,18], under different extraction or HPLC conditions; but no reports are available for whole blood samples. In forensic science practices, whole blood analysis is essential especially for hemolyzed blood samples. In this report, a rapid and convenient method is described for simultaneous determination of five common rodenticides in whole blood.

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PII: S0731-7085(99)00108-9

2. Experimental

2.1. Chemicals

Warfarin was purchased from Sigma (St Louis, MO, USA). Coumatetralyl and bromadiolone were obtained from Beijing Institute of Microbiology and Epidemiology (Beijing, PRC); diphacinone and chlorophacinone from Beijing Institute of Pharmacology and Toxicology (Beijing, PRC). Their identity was confirmed by the presence of quasi-molecular ion peaks in atmosphere pressure chemical ionization (APCI) mass spectra, and purity by HPLC with ultraviolet (UV) detection. Di-*n*-butylammonium acetate (DBA, 0.5M aqueous solution, pH 7.4) used as ion-pairing reagent was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan); acetonitrile of HPLC grade from Wako Pure Chemical Industries (Osaka, Japan). Other common chemicals used were of analytical grade. Human whole blood was taken from healthy subjects, and stored in the presence of EDTA–2Na at 4°C.

Warfarin, coumatetralyl, diphacinone and chlorophacinone (1.0 mg ml^{-1} each) were dissolved in acetonitrile, and bromadiolone (1.0 mg ml^{-1}) in methanol (HPLC grade). These stock solutions were stored at 4°C. The working standard mixture (100 μg ml^{-1} for each rodenticide) was prepared by diluting the aliquots of the stock solutions, stored at 4°C, and stable for at least 1 month. More dilute standard mixtures were prepared daily.

2.2. High-performance liquid chromatography

The HPLC system consisted of two LC-10AD pumps, an SPD-10A UV detector, an SCL-10A system controller and a DGU-12A degasser (Shimadzu, Kyoto, Japan). A C-R5A Chromatopac was used for chromatogram recording. A Capcell-Pak column [150 × 2.0 mm internal diameter (i.d.)] (Shiseido, Tokyo, Japan) packed with reversed phase C_{18} (5 μm particle size) was used for HPLC separation. A Rheodyne injector (Rheodyne, Cotati, CA, USA) with an injection loop of 25 μl was employed; the injection volume was 5 μl.

Separations were carried out using gradient elution with mobile phase A, acetonitrile:DBA (5 mM in water) = 20:80, and mobile phase B, acetonitrile:DBA (5 mM) = 70:30. The gradient program expressed as the change in mobile phase A was as follows: 0–3 min, hold at 100%; 3–7 min, a linear decrease to 60%; 7–21 min, a linear gradient to 0% and 21.1–26 min, switch to and hold at initial condition (100%). The mobile phase flow rate was 0.20 ml min^{-1}. The detection wavelength was 315 nm for both 4-hydroxycoumarins and indandiones. HPLC separations were conducted at ambient temperature.

2.3. Extraction of blood samples

To 1 ml of human whole blood in a 10-ml centrifuge tube, an aliquot of the standard rodenticide mixture was added, and they were mixed well by brief shaking. Ethyl acetate (4 ml) was placed in the tube and shaken for 2 min. After standing for a few minutes, the upper organic layer was transferred to another tube, and the liquid–liquid extraction was repeated. The combined extracts were evaporated to dryness under a gentle nitrogen stream, on a heating block at 50°C. The residue was reconstituted in 60 μl of acetonitrile plus 40 μl of 5-mM DBA with brief ultra-sonication.

2.4. Animal experiments

Male Sprague–Dawley rats weighing about 200 g were anaesthetized by inhalation of ethyl ether, and were orally administered 1.0 mg of diphacinone suspended in 0.5 ml of cooking oil. Three hours after the administration, blood was taken from the abdominal aorta of the rats under anaesthesia that was effected by another inhalation of ethyl ether, and stored in the presence of anticoagulant citrate dextrose in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) at 4°C until analysis. The control group of rats was treated in the same way except for no diphacinone in the cooking oil administered.

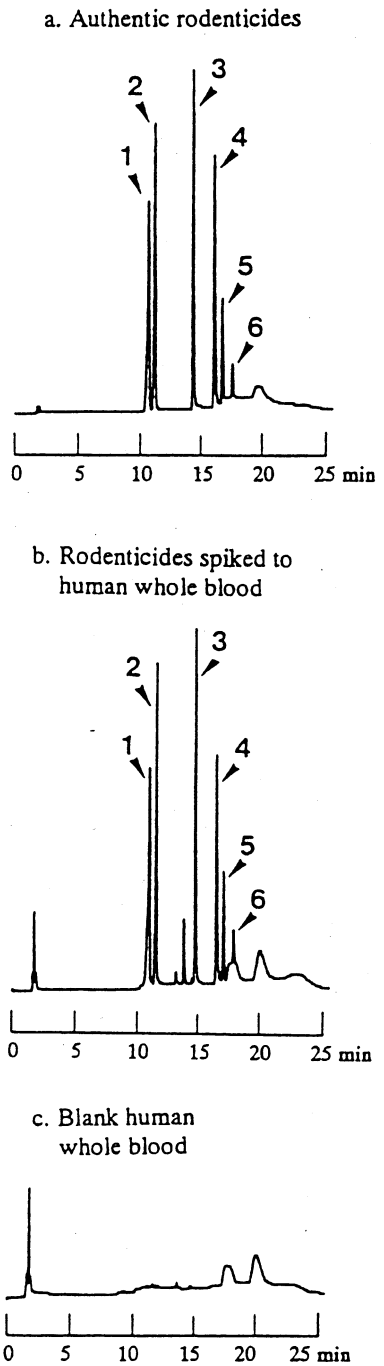

Fig. 1. (*Continued*)

3. Results and discussion

3.1. High-performance liquid chromatography conditions

4-Hydroxycoumarin rodenticides (warfarin, coumatetralyl and bromadiolone) are phenolic-type weak acids, and indandione rodenticides (diphacinone and chlorophacinone) are relatively strong acids like benzoic acid [27], although there is no carboxyl or phenolic proton in their molecules. 4-Hydroxycoumarins could be resolved without difficulties by mobile phases composed of methanol, acetonitrile, and pH buffers in the gradient or isocratic mode on a C_{18} column; while indandiones gave tailing and very broad peaks under the conditions just mentioned. To make the chromatographic peaks of indandiones narrow and sharp, we added trifluoroacetic acid to a elution system; the peaks of indandiones became less broad but still showed tailing. Then an ion-pairing reagent DBA was tested for the purpose of improving peak shapes of indandiones, since this class of reagent like tetraalkylammomium phosphate was reported to be useful in HPLC separation [13–15,18]. With the addition of DBA to the mobile phase, indandiones gave sharp and narrow peaks, as shown in Fig. 1. 4-Hydroxycoumarins also yielded well-shaped peaks in this elution system, and all five rodenticides tested were baseline-resolved (Fig. 1). Bromadiolone usually exists as a mixture of two stereo isomers [15,19], and they were separated into two peaks under the present conditions.

By using the mobile phase selected above, 4-hydroxycoumarins could also be detected by fluorescence detection with higher sensitivity.

Fig. 1. High-performance liquid chromatography (HPLC) chromatograms of (a) anticoagulant rodenticides (50 ng of each) in an authentic mixture; (b) rodenticides (0.60 μg of each) spiked to 1 ml of human whole blood and (c) blank blood. Peak identities: 1, warfarin; 2, coumatetralyl; 3, diphacinone; 4, chlorophacinone; 5, bromadiolone; 6, stereo-isomer of bromadiolone. For other conditions, see Section 2. The extra peak just before the diphacinone peak in (b) was considered to be resulting from an active endogenous compound in fresh human blood, which disappeared after the blood was stored at 4°C for a few days.

Vitamins K_1, K_2 and K_3 are clinically used as antidotes to treat patients poisoned by anticoagulants. They did not overlap the peaks of the latter under the present HPLC conditions.

3.2. Selection of an extraction method

Acetonitrile or chloroform–acetone had been used for extraction of anticoagulant rodenticides from serum [13,15], and ethyl acetate for extraction of chlorophacinone from the same matrix [2]. In this study, ethyl acetate, chloroform, ethyl ether, and C_{18} cartridges were assessed for extraction of the rodenticides from whole blood. Preliminary experiments indicated that ethyl acetate gave the highest recoveries of the analytes, while the C_{18} cartridges the lowest. Recoveries of five rodenticides by ethyl acetate extraction measured at two concentrations are shown in Table 1. They were not lower than 65%; it was highest for warfarin, and lowest for bromadiolone. For warfarin and coumatetralyl, the recoveries were nearly the same at two different concentrations. For diphacinone, chlororphacinone and bromadiolone, however, the recoveries varied with analytes concentration, for which the reason was unknown.

Ethyl acetate extraction gave not only good recoveries of the analytes but also a clean chromatogram of blank blood as shown in Fig. 1c. There were no interfering peaks around those of the analytes.

3.3. Calibration curve, detection limit and reproducibility

When the chromatographic peak areas of the rodenticides were plotted versus the concentrations, straight lines were obtained. Typical linear regression parameters for five rodenticides in human whole blood are shown in Table 2. The quantitation range was from 0.1 to 10 μg ml^{-1}. The detection limit (signal-to-noise ratio = 3) was 0.05 μg ml^{-1} of whole blood for all rodenticides.

Within-day and day to day precision data for analysis of the anticoagulant rodenticides at two concentrations are summarized in Table 3. Within-day variations were examined with blood from one person as well as from five persons. The coefficients of variation (CVs) ranged from 2.4 to 7.6% for the former, and from 8.1 to 16% for the latter. Day to day CVs measured with one-person's spiked blood were 2.8–11%.

It was reported that there was no risk of blood-bleeding syndrome associated with intoxication of chlorophacinone when blood chlorophacinone level dropped to below 1 μg ml^{-1} in humans [2]. The toxic blood (or serum) concentration of warfarin in humans was reported to be 10 μg ml^{-1} [28]. Basing on these data, we might conclude that the present method was sensitive enough for de-

Table 1
Recoveries of rodenticides from human whole blood by ethyl acetate extraction ($n = 6$)

Rodenticides	Added (μg ml^{-1})	Found[a] (μg ml^{-1})	Recoveries (%) ($X \pm SD$)	CV (%)
Warfarin	0.50	0.46 ± 0.022	92 ± 4.4	4.8
	2.0	1.8 ± 0.065	90 ± 3.3	3.7
Coumatetralyl	0.50	0.44 ± 0.015	88 ± 3.0	3.4
	2.0	1.7 ± 0.040	85 ± 2.2	2.6
Diphacinone	0.50	0.40 ± 0.020	80 ± 4.0	5.0
	2.0	1.4 ± 0.032	70 ± 1.6	2.3
Chlorophacinone	0.50	0.38 ± 0.019	76 ± 3.8	5.0
	2.0	1.4 ± 0.038	70 ± 1.9	2.7
Bromadiolone	0.50	0.37 ± 0.031	74 ± 6.2	8.4
	2.0	1.3 ± 0.050	65 ± 2.5	3.8

[a] The extracted rodenticides were quantitated with external standard calibration.

Table 2
Calibration equations and detection limits of rodenticides in human blood

	$y = mx + b$[a]						Detection limits[b] (μg ml^{-1})
	(0.10, 0.20, 0.40, 0.60, 0.80, 1.0 μg ml^{-1})			(1.0, 2.0, 4.0, 6.0, 8.0,10 μg ml^{-1})			
	m[c]	b[c]	r^2	m[c]	b[c]	r^2	
Warfarin	(1.33 ± 0.06) × 10^5	(1.56 ± 3.35) × 10^3	0.993	(1.34 ± 0.01) × 10^5	(2.20 ± 8.28) × 10^3	0.999	0.05
Coumatetralyl	(1.51 ± 0.05) × 10^5	(3.50 ± 3.20) × 10^3	0.995	(1.45 ± 0.02) × 10^5	(7.14 ± 10.5) × 10^3	0.999	0.05
Diphacinone	(1.64 ± 0.03) × 10^5	(2.64 ± 1.73) × 10^3	0.998	(1.43 ± 0.04) × 10^5	(2.46 ± 2.69) × 10^4	0.996	0.05
Chlorophacinone	(1.17 ± 0.01) × 10^5	(1.69 ± 0.85) × 10^3	0.999	(9.67 ± 0.28) × 10^4	(2.46 ± 1.72) × 10^4	0.996	0.05
Bromadiolone	(5.17 ± 0.11) × 10^4	(6.95 ± 6.37) × 10^2	0.998	(4.39 ± 0.12) × 10^4	(1.31 ± 0.74) × 10^4	0.996	0.05

[a] y was the peak area of an anticoagulant rodenticide, x was its concentration in whole blood and r^2 was the square of the correlation coefficient.

[b] Whole blood containing a series of different concentration of the analytes was extracted and chromatographed, and the lowest concentration, at which an analyte produced a signal three times higher than baseline noise, was designated to be the detection limit.

[c] The value of 'm' and 'b' were expressed as 'average ± SD'. For diphacinone, chlorophacinone and bromadolone, the slope of the calibration line was lower in the high concentration range than in the low concentration range, which might be caused by a lower extraction recovery at higher concentration of the analyte.

Table 3
Within-day and day-to-day variations

	Within-day					Day to day	
	Blood from one person[a]			Blood from five persons[b]		Blood from one person [c]	
	Added (μg ml^{-1})	Found (X ± SD)	CV (%)	Found (X ± SD)	CV (%)	Found (X ± SD)	CV (%)
Warfarin	0.50	0.52 ± 0.027	5.1	0.55 ± 0.055	10	0.59 ± 0.063	11
	2.0	2.0 ± 0.077	3.7	2.1 ± 0.17	8.1	2.1 ± 0.10	5.0
Coumatetralyl	0.50	0.55 ± 0.020	3.7	0.58 ± 0.047	8.3	0.59 ± 0.066	11
	2.0	2.2 ± 0.054	2.5	2.3 ± 0.19	8.3	2.3 ± 0.090	4.0
Diphacinone	0.50	0.53 ± 0.026	5.0	0.53 ± 0.49	9.2	0.55 ± 0.015	2.8
	2.0	2.1 ± 0.049	2.4	2.2 ± 0.22	10	2.3 ± 0.17	7.3
Chlorophacinone	0.50	0.54 ± 0.027	4.9	0.54 ± 0.061	11	0.55 ± 0.026	4.8
	2.0	2.1 ± 0.063	3.1	2.2 ± 0.24	11	2.2 ± 0.17	8.0
Bromadiolone	0.50	0.58 ± 0.044	7.6	0.53 ± 0.084	16	0.53 ± 0.013	2.5
	2.0	2.0 ± 0.084	4.2	2.1 ± 0.31	15	2.0 ± 0.15	7.6

[a] Six aliquots of one-person's blood were spiked with rodenticides and analyzed with external calibration.

[b] Five aliquots of blood from five persons were spiked and analyzed.

[c] Spiked blood was kept at 4°C and analyzed on six separate days, with one sample each day.

tection and determination of the five rodenticides in blood from poisoned victims.

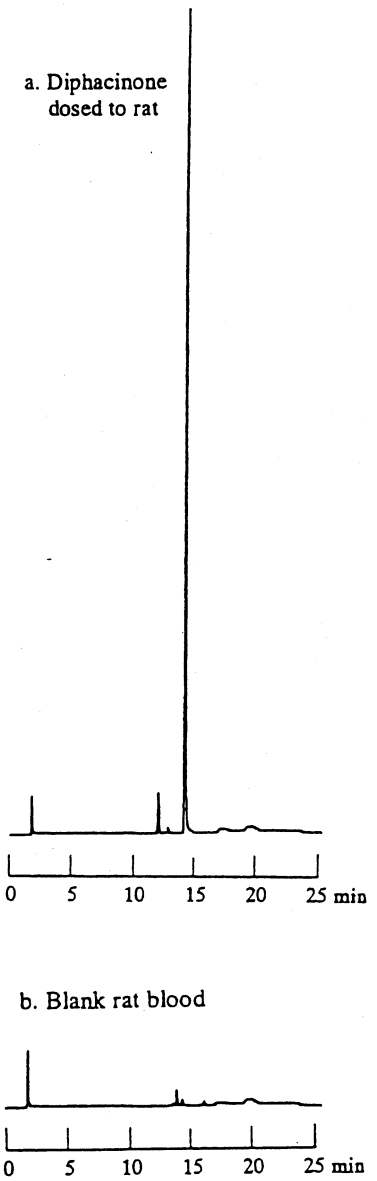

Fig. 2. High-performance liquid chromatography (HPLC) chromatograms of (a) diphacinone (1.0 mg) dosed to a rat and (b) blank rat blood. For other conditions, see Section 2.

3.4. Measurements of a dosed rodenticide in rat blood

To validate the method developed above, we administered orally diphacinone (1.0 mg) to five rats and sampled their blood for determination of the dosed rodenticide. Rat whole blood was extracted in the same way as human blood was. Typical chromatograms for the rat blood are shown in Fig. 2. The blood concentration of diphacinone 3 h after the administration was 7.8 ± 2.6 μg ml^{-1} of blood ($n = 5$ rats). The result suggests that the present method is workable with real blood samples.

4. Conclusion

A method for determination of anticoagulant rodenticides in whole blood has been developed, and validated by successful measurements of a dosed rodenticide in rat blood. To our knowledge, it is the first report for whole blood samples. It is recommendable for detection and determination of anticoagulant rodenticides in clinical and forensic analytical toxicology.

Acknowledgements

Fuyu Guan is grateful to the Ministry of Education, Science and Culture of Japan for its financial support with the fellowship.

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