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Journal of Chromatography B, 731 (1999) 155–165

JOURNAL OF CHROMATOGRAPHY B

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

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Abstract

The on-line combination of high-performance liquid chromatography with mass spectrometry (HPLC–MS) has become a powerful tool for trace analysis thanks to the developments in interface techniques. However, non-volatile salts such as ion-pairing reagents are considered to be incompatible with HPLC–MS systems; they cause drops in analyte signals because of contamination of mass analyzers and also because of blocking of the capillary transferring ions from atmospheric pressure to the vacuum manifold. In this work, a new type of ion-pairing reagent, di-*n*-butylammonium acetate (DBA), was evaluated for use in HPLC–MS. DBA did not cause these problems to HPLC–MS systems; a possible explanation might be that DBA decomposed to volatile compounds under APCI conditions. In addition, DBA was very useful for obtaining sharp peaks, which resulted in high sensitivity. With this ion-pairing reagent, we developed a procedure for the measurement of five (including internal standard) anticoagulant rodenticides in whole blood and urine samples by SIM detection of $[M-H]^-$ ions. Calibration range, recoveries and precision of the method were examined; detection limits as low as 1–5 ng/ml blood sample or 0.5–2.5 ng/ml urine sample were achieved.

Keywords: Rodenticides

1. Introduction

The on-line coupling of high-performance liquid chromatography with mass spectrometry (HPLC–MS), after more than two decades of development, especially as a result of the breakthrough in interface techniques, has become a robust and routinely applicable analytical tool [1,2]. Among the interfaces for coupling of HPLC with MS, electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) are two common and widely used modes. However, HPLC–MS systems coupled by either of these interfaces are quite intolerant to non-volatile salts such as ion-pairing reagents [1,3,4]. Usually, HPLC separations are needed for samples

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00126-7

with complicated matrices, prior to mass spectrometric detection [5], and ion-pairing reagents are necessary for HPLC separation of ionic compounds. To solve the problem of incompatibility of HPLC–MS with ion-pairing reagents, several research groups have followed different approaches: ion-pairing reagents were removed by membranes from HPLC eluents before the latter enter into the APCI interface and mass analyzer [6]; HPLC eluents were split in flow and a small portion was allowed to enter at a low rate (5–10 μl/min) into the APCI interface [7]; or other type of interfaces and mass analyzers such as inductively coupled plasma (ICP)–MS and field desorption mass spectrometers were used, which tolerate the use of ion-pairing reagents [8,9]. In this paper, we report the direct use of di-*n*-butylammonium acetate (DBA), an ion-pairing reagent, in HPLC–MS without flow split or removal of the reagent prior to the APCI interface.

Anticoagulant rodenticides including warfarin, coumatetralyl, bromadiolone, diphacinone and chlorophacinone (see Fig. 1) used in this study are agricultural chemicals that are readily available and have caused intoxication to humans [10–13]. Detection of these rodenticides in biological samples is useful for the diagnosis of victims and for revealing the causes. Anticoagulant rodenticides have usually been determined by HPLC [14–23]. Diphacinone and chlorophacinone show wide and severely tailed HPLC peaks, but the shape of these peaks can be greatly improved by an ion-pairing reagent such as tetrabutylammonium phosphate [16–18,23]. Unfortunately, a non-volatile ion-pairing reagent is not suitable for use in HPLC–MS. Analysis of warfarin by thermospray and particle beam HPLC–MS has been reported [24,25], but no ESI or APCI HPLC–MS method has been published for the five rodenticides mentioned above. In the present work, we have used a new ion-pairing reagent, DBA, to develop an HPLC–MS method for the measurement of anticoagulant rodenticides, which is useful for identification of these rodenticides for forensic purposes.

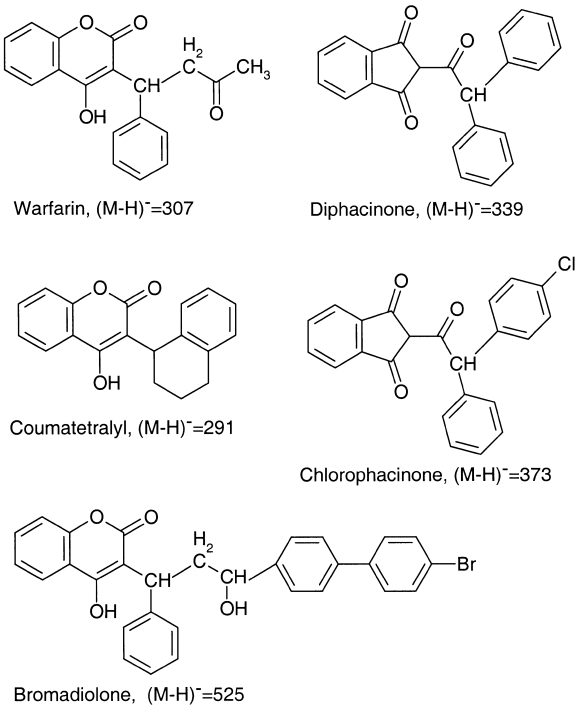

Fig. 1. Chemical structures of the anionic anticoagulant rodenticides used in this study.

2. Experimental

2.1. Reagents

Warfarin was purchased from Sigma (St. Louis, MO, USA). Coumatetralyl and bromadiolone were obtained from the Beijing Institute of Microbiology and Epidemiology (Beijing, PRC); diphacinone and chlorophacinone were obtained from the Beijing Institute of Pharmacology and Toxicology (Beijing, PRC). Their identity was confirmed by quasi-molecular ion $[M-H]^-$ peaks in APCI mass spectra, and their purity by HPLC with ultraviolet (UV) detection. DBA (0.5 mol/l aqueous solution, pH 7.4), an ion-pairing reagent, was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan); acetonitrile of HPLC grade was from Wako Pure Chemical Industries (Osaka, Japan). Other common chemicals used were of analytical grade. Human whole blood and urine samples were taken from healthy subjects, and stored at 4°C. The blood sample was kept in the presence of EDTA-2Na. Twice-distilled water was used in the experiments.

Warfarin, coumatetralyl, diphacinone and chlorophacinone (1.0 mg/ml each) were dissolved in acetonitrile, and bromadiolone (1.0 mg/ml) in

methanol (HPLC grade). These stock solutions were stored at 4°C. The working standard mixture (100 μg/ml for each rodenticide) was prepared by diluting the stock solutions, stored at 4°C, and was stable for at least 1 month. More dilute standard mixtures were prepared daily. Ammonium acetate solution (0.50 mol/l) was prepared by dissolving a weighed amount of the compound in water, and its pH was measured to be 7.0.

2.2. Instrumentation

The HPLC–MS consisted of a SpectraSystem P 4000 HPLC system (Thermo Separation Products, Fremont, CA, USA) and a Finnigan Mat TSQ 7000 quadrupole tandem mass spectrometer equipped with a Finnigan Mat APCI interface (San Jose, CA, USA). The LC–MS system was controlled and data were processed by ICIS software (Finnigan Mat) on a Digital DECstation 3000 computer (Maynard, MA, USA).

APCI interface parameters were set as follows: sheath gas, 50 psi ($3.44 \cdot 10^5$ Pa); auxiliary gas, 2 units; vaporizer temperature, 550°C; capillary temperature, 175°C; and corona current, 5 μA. Mass detection was performed by selected ion monitoring (SIM) in the negative ion mode. Time-scheduled SIM conditions were: HPLC time 0–12.0 min, m/z 291 and 307; 12.0–14.5 min, m/z 339; and 14.5–18.0 min, m/z 373 and 525. Mass-to-charge window was $m/z \pm 0.3$ a.m.u.; dwell time was 0.6 s for each selected m/z.

2.3. HPLC conditions

A Capcell-Pak column from Shiseido (Tokyo, Japan), 150×2.0 mm I.D., packed with 5 μm reversed-phase C_{18}, was used for HPLC separation. It was protected by a pre-column (Shiseido, 35×2.0 mm I.D., 5 μm C_{18}). HPLC separations were carried out at ambient temperature, using a binary gradient composed of mobile phase A (20% acetonitrile–80% 5 mmol/l DBA in water, v/v) and mobile phase B (70% acetonitrile–30% 5 mmol/l DBA in water). The aqueous DBA solution (5 mmol/l) was prepared by diluting 0.5 mol/l DBA solution (pH 7.4). The gradient expressed as changes in mobile phase A was as follows: 0–1 min, hold at 100%; 1–5 min, a linear decrease to 60%; 5–14 min, a linear decrase to 0%; 14–16 min, hold at 0%; and 16.1–21 min, switch to and hold at initial condition (100%). The mobile phase flow-rate was 0.20 ml/min. A Rheodyne injector (Rheodyne, Cotati, CA, USA) with an injection loop of 5 μl was employed, and 5 μl of a sample or standard was injected.

2.4. Sample preparation

To 1 ml of human whole blood sample in a 15-ml plastic disposable centrifuge tube, was added an aliquot of the standard rodenticide mixture, and they were well mixed 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 liquid–liquid extraction was repeated once. The combined extracts were evaporated to dryness on a heating block at 50°C under a gentle nitrogen stream. The residue was reconstituted in 60 μl acetonitrile plus 40 μl 10 mmol/l DBA with brief ultra-sonication. Urine samples (2 ml) spiked with the rodenticides were extracted in the same way as the whole blood samples. Five microliters of the final solution was injected into the HPLC system.

2.5. Animal experiments

Male Sprague-Dawley rats weighing about 200 g, under temporary anaesthesia by inhalation of ethyl ether, were orally administered 50 μg diphacinone dissolved in 0.5 ml cooking oil. After administration (3.5 h) a blood sample was taken from the abdominal aorta of the rats under anaesthesia with another inhalation of ethyl ether. The blood samples were stored in the presence of EDTA-2Na at 4°C until analysis. The control group of rats was treated in the same way except that no diphacinone was administered. The blank blood samples from the control group were used to draw calibration curves for quantitation of the dosed rodenticide. Rat blood samples were extracted in the same way as described above for human blood samples. The extracts were reconstituted in 240 μl acetonitrile plus 160 μl 10 mmol/l DBA. Five microliters of the solution was injected onto the HPLC column.

3. Results and discussion

3.1. Measurements of mass spectra

Warfarin, coumatetralyl and bromadiolone have an acidic enol [26], while diphacinone and chlorophacinone are acids with an acidity close to that of benzoic acid [27]. For these acidic compounds, mass spectrometric measurements of anions would be a first choice. In addition, mass spectrometry in negative ion mode has the benefit of a low level of chemical noise. We compared ESI and APCI, and they gave about the same signal intensity to the analytes. APCI was used in our experiments, because the hot vaporizer of the APCI interface was expected to break down DBA from the HPLC eluent, which otherwise might cause contamination of the mass analyzer.

The APCI mass spectrum of each compound was recorded in the negative ion mode to select the most abundant mass-to-charge ratio (m/z) for further studies. For the five compounds studied, $[M-H]^-$ was the only peak and no fragment peak was observed under the APCI conditions selected. The isotopic $[M-H]^-$ peaks were also observed for diphacinone and bromadiolone, as expected. Warfarin, coumatetralyl and bromadiolone molecules, as shown in Fig. 1, have a phenolic active proton, and the dissociation of this proton under APCI conditions produced an intense $[M-H]^-$ signal. For diphacinone and chlorophacinone, there appears to be no active proton that is able to dissociate, at first glance. In fact, there is a methenyl group attached simultaneously to three carbonyl groups in the molecule, and the hydrogen on this methenyl group becomes active due to the strong electron affinity of the three adjacent carbonyl groups [27]. The dissociation of this active proton resulted in the formation of $[M-H]^-$ peaks.

3.2. Optimization of APCI conditions

There are five parameters affecting the APCI process. The sheath gas assists aerosol formation of the HPLC eluent entering the APCI interface. The vaporizer is a heater for flash vaporization of the aerosols. The corona needle is a device that prompts chemical ionization of sample molecules. The capillary is a channel through which ions pass from the atmospheric pressure region to the vacuum manifold of the mass analyzer. The auxiliary gas, which is optional, helps to focus the aerosols toward the corona needle and the capillary, and helps to keep the spray chamber dry.

The temperature of the vaporizer and the capillary, the sheath gas pressure and auxiliary gas flow were optimized in this work, while the corona current was not, as its variation from 2 to 8 μA has little influence on analyte response [28]. The optimization was carried out by changing one parameter while keeping the other three parameters constant, and started with the parameter settings given in the User's Book of the APCI interface from the manufacturer. At each setting of the parameter examined, each compound (2 ng) was introduced by the flow injection method into the APCI–MS six times, and the peak heights were recorded in the SIM mode and averaged. As shown in Fig. 2a, the effect of vaporizer temperature on the response was compound-dependent; a higher vaporizer temperature was necessary for compounds of higher molecular mass. A vaporizer temperature of 550°C, which was balanced among the five compounds studied, was chosen for further studies.

Fig. 2b shows the effect of capillary temperature on analyte response. It shows a similar trend for all compounds. The optimal temperature was 175°C.

In Fig. 2c there is a trend that higher analyte responses were obtained at lower sheath gas pressure. This perhaps resulted from dilution of the analytes caused by the sheath gas at higher pressure. A lower sheath gas pressure (50 psi) was chosen.

Fig. 2d shows the effect of auxiliary gas. There was a clear pattern that the responses decreased with increase in flow-rate of the auxiliary gas. This was also probably caused by dilution of the analytes at a higher flow-rate of the auxiliary gas. A lower auxiliary gas flow at 2 units was selected.

3.3. Compatibility of DBA as an ion-pairing reagent with HPLC–MS

Non-volatile salts such as conventional ion-pairing reagents used in the HPLC mobile phase may cause two main problems to HPLC–MS systems, blocking of the capillary between atmospheric pressure and

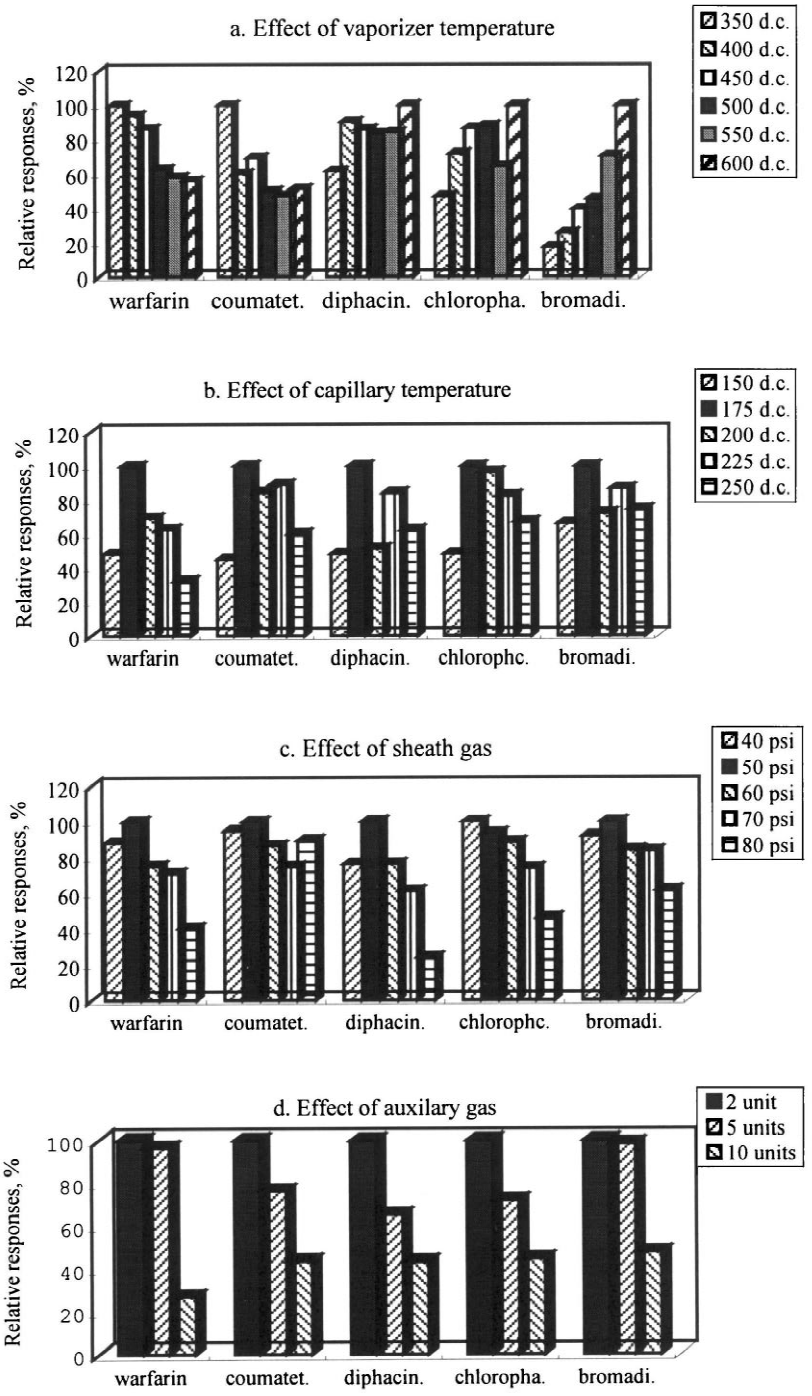

Fig. 2. Optimization of APCI interface parameters. At each setting of a parameter, each compound (2 ng) was injected by flow injection, monitored by SIM, and the peak heights of six duplicate injections were averaged. The relative responses of each compound were calculated as percent peak heights normalized relative to the highest value (100%). (a) Capillary temperature was kept at 200°C, sheath gas pressure at 70 psi ($4.8 \cdot 10^5$ Pa), and auxiliary gas flow 10 units. (b) Vaporizer temperature was held at 550°C, sheath gas pressure at 70 psi ($4.8 \cdot 10^5$ Pa), and auxiliary gas flow at 10 units. (c) Vaporizer temperature was maintained at 550°C, capillary temperature at 175°C, and auxiliary gas flow at 10 units. (d) Vaporizer temperature was kept at 550°C, capillary temperature at 175°C and sheath gas pressure at 50 psi.

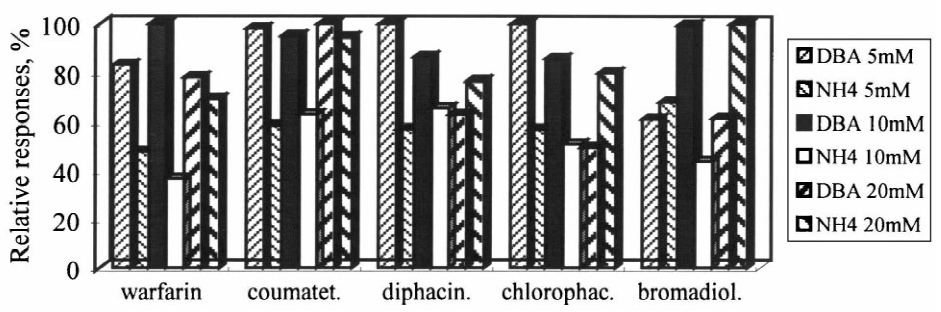

Fig. 3. Comparison of DBA and ammonium acetate (AA) buffers for MS responses of anticoagulant rodenticides. Each analyte was injected into the flow of mobile phases containing different concentrations of DBA and ammonium acetate separately (direct flow method), which entered APCI–MS directly, monitored by SIM, and the peak heights were averaged for six repeated injections. For other conditions, see Fig. 2.

the vacuum manifold, and contamination of the mass analyzers, and thus these cause a drop in m/z signals. In this work, DBA, which had been used as an ion-pairing reagent for HPLC of some anionic compounds [29], was examined for use in HPLC–MS. Fortunately, neither blocking (plug formation) in the capillary, nor contamination of the APCI probe and the mass analyzer was observed over 3 months of routine use of DBA (10 mmol/l) as a mobile phase component in HPLC–MS.

We also examined whether the use of DBA in the HPLC mobile phase causes MS signals of analytes to drop. The signals obtained with DBA by the flow injection method were compared with those obtained with ammonium acetate (AA), since the latter is known to be compatible with HPLC–MS and is extensively used in HPLC–MS measurements but is not an ion-pairing reagent. As shown in Fig. 3, compared with ammonium acetate, DBA at low concentrations (5 and 10 mmol/l) did not decrease the signals of the analytes, while at high DBA concentration (20 mmol/l) the signals of some analytes were reduced, which was compound-dependent and could not be considered as a result of contamination of the APCI interface or mass analyzer by DBA. Thus, the concentration of 10 mmol/l was chosen. At this concentration or lower (5 mmol/l), DBA was effective as an ion-pairing reagent for HPLC separation of anionic analytes, giving sharp peaks. Even at a lower concentration (1 mmol/l), DBA has been reported to be effective [29].

On the basis of the above results, we conclude that DBA, unlike other ion-pairing reagents, does not cause any problem and can be used in HPLC–MS measurements. The APCI interface with counter-current drying gas (curtain gas) was reported to show, to some extent, tolerance to non-volatile mobile phase constituents [1,30]. In this work, we used an APCI interface without counter-current drying gas. This would rule out the possibility that the HPLC–MS instrument itself tolerates the use of DBA.

We have proposed a possible mechanism to explain the compatibility of DBA with HPLC–MS. As is known, ammonium acetate is compatible with HPLC–MS, and it is considered to result from the fact that this compound decomposes to NH_3 and acetic acid at about 200°C or higher. DBA is composed of secondary ammonium and organic acetate, and it may be quite reasonable to consider that DBA decomposes to di-*n*-butylamine (boiling point 159–160°C [31]) and acetic acid in the vaporizer of the APCI interface heated to above 350°C. The decomposition products are volatile and can easily be pumped out. Thus, DBA causes no problems to the mass analyzer. We predict that ion-pairing reagents composed of uni-, di- or tri-alkyl ammonium and organic formate or acetate may also be used in HPLC–APCI–MS.

3.4. Improvement of the chromatographic profiles of anionic rodenticides by DBA

DBA and ammonium acetate were further compared for their effects on HPLC peak shapes of anionic compounds, as shown in Fig. 4; the results

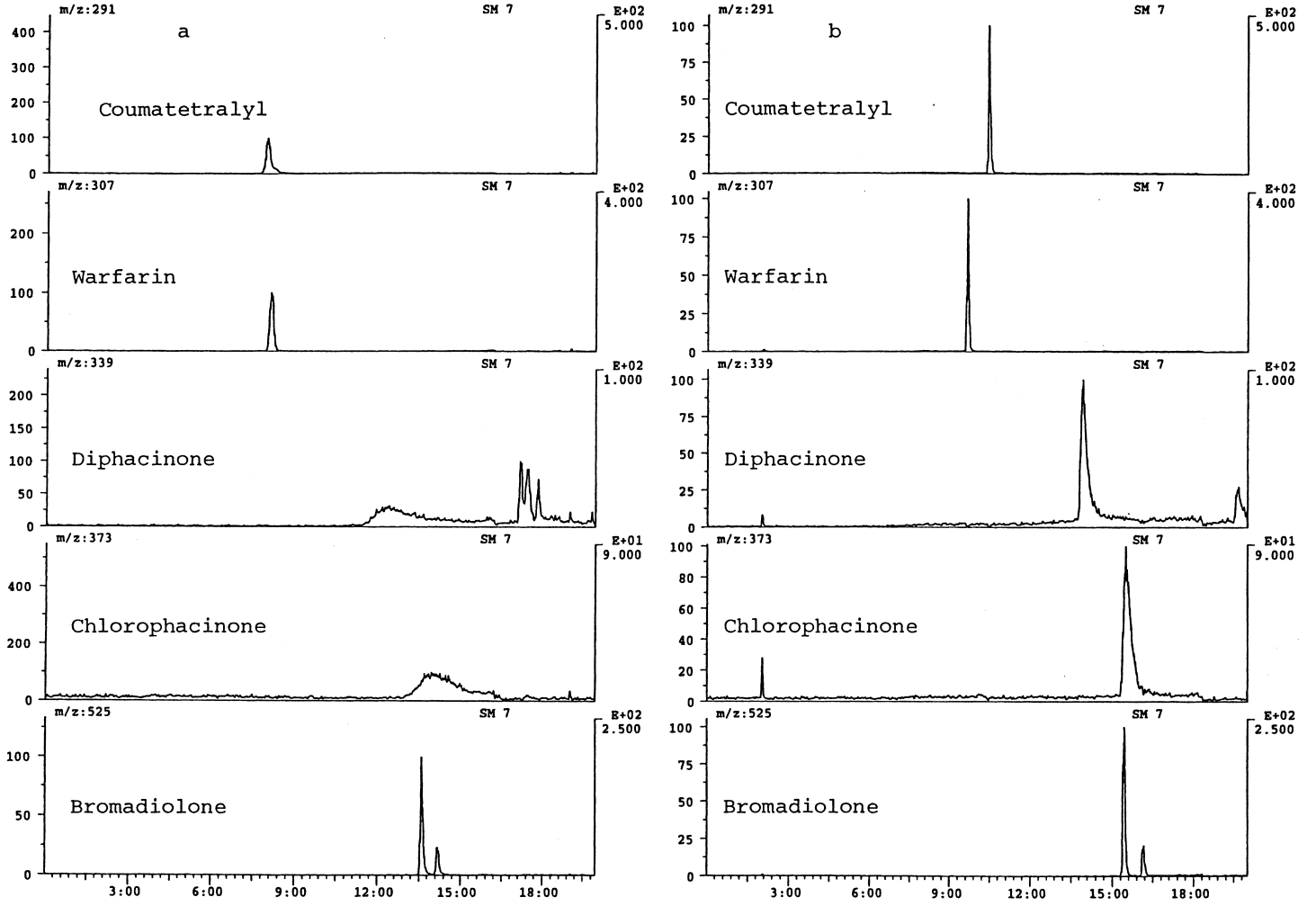

Fig. 4. Effect of DBA on HPLC peak shapes of anionic anticoagulant rodenticides. SIM chromatograms of five analytes (1.0 ng each) were obtained with 10 mmol/l ammonium acetate, pH 7.0 (a) and 10 mmol/l DBA, pH 7.4 (b) added to the mobile phase. The left vertical axes indicate the relative percentage intensity of peaks, while the right vertical axes indicate the 'absolute' intensity recorded by the mass spectrometer. The horizontal axes show the HPLC running time (min). The same HPLC mobile phase gradient was used in (a) and (b), as indicated in the Experimental section. The $[M-H]^-$ ion of each analyte was monitored throughout a whole run. Bromadiolone has two diastereoisomers (not in equal amounts) and thus showed two peaks [21]. For other conditions, see the Experimental section.

Table 1
Recoveries of rodenticides from human whole blood and urine samples by ethyl acetate extraction ($n=8$)[a]

	Blood sample			Urine sample	
	Added (ng)	Recovery (%) ($X\pm$SD[b])	CV (%)	Recovery (%) ($X\pm$SD[b])	CV (%)
Warfarin	10	64±12	20	79±15	19
	50	87±4.9	6	98±13	13
Coumatetralyl	10	54±14	26	75±13	17
	50	77±4.3	6	99±11	11
Diphacinone	10	–[b]		–[b]	
	50	67±8.8	13	65±13	20
Chlorophacinone	10	–[b]		–[b]	
	50	74±6.6	9	70±12	17
Bromadiolone	10	48±21	43	57±15	26
	50	62±5.2	8	66±10	15

[a] The recoveries were measured by external standard calibration.
[b] The recoveries of diphacinone and chlorophacinone at 10 ng/ml were not measured, because this concentration was close to their detection limits.

indicate that DBA greatly improves the peak shapes of anionic analytes, which would be beneficial to HPLC–MS determination of the analytes. It should be noted that, in Fig. 4a, with the use of ammonium acetate, HPLC–MS measurements of chlorophacinone and diphacinone were impractical because of the poor peak shapes. In contrast, the well-defined peaks of these compounds, achieved with the assistance of DBA, were considered to result from two effects: the ion-pairing action of DBA and its blockage of the undesirable interactions between the analytes and silanol groups on the surfaces of the column packing particles [29].

3.5. Validation of the method

Human whole blood and urine samples spiked with rodenticides were extracted with ethyl acetate; the recoveries were estimated by external standard calibration, as shown in Table 1. They ranged from 48 to 99% for the different rodenticides.

SIM chromatograms for the spiked blood and urine samples are shown in Fig. 5. There was no interference of impurities with the analyte peaks.

The quantitation of rodenticides was conducted by HPLC–MS/SIM, using internal standard calibration; coumatetralyl, one of the five rodenticides studied,

Table 2
Calibration curves and detection limits for rodenticides spiked in human whole blood and urine samples

Compound	Calibration equation[a]	Correlation coefficient (r)	Concentration range (ng/ml)	Detection limit (ng/ml)
Blood sample				
Warfarin	$y=2.56\times10^{-2}x-1.02\times10^{-1}$	0.994	1–100	1
Diphacinone	$y=5.49\times10^{-3}x-2.56\times10^{-2}$	0.987	10–100	5
Chlorophacinone	$y=2.58\times10^{-3}x+2.16\times10^{-2}$	0.986	10–100	5
Bromadiolone	$y=5.30\times10^{-3}x-1.38\times10^{-2}$	0.965	1–100	1
Urine sample				
Warfarin	$y=2.02\times10^{-2}x+6.86\times10^{-3}$	0.994	0.5– 50	0.5
Diphacinone	$y=1.29\times10^{-2}x-1.35\times10^{-2}$	0.990	5– 50	2.5
Chlorophacinone	$y=5.92\times10^{-3}x-9.75\times10^{-3}$	0.987	5–50	2.5
Bromadiolone	$y=1.13\times10^{-2}x-2.41\times10^{-2}$	0.999	0.5–50	0.5

[a] Ratio of peak area versus the concentration of spiked compounds.

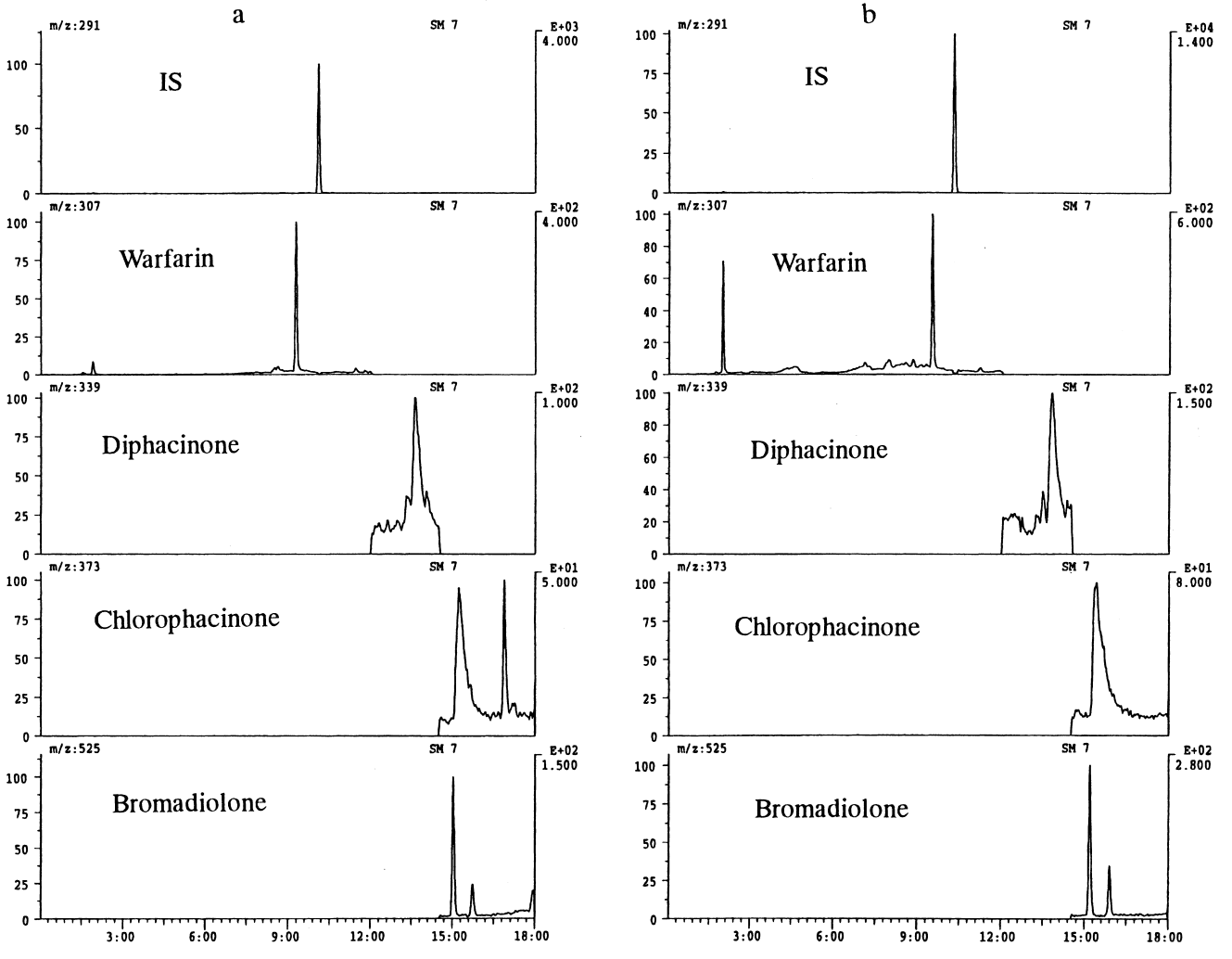

Fig. 5. Time-scheduled SIM chromatograms for blood and urine samples spiked with anticoagulant rodenticides. (a) Blood sample (1 ml) spiked with 10 ng of each of warfarin, diphacinone, chlorophacinone and bromadiolone and 50 ng of coumatetralyl (IS). (b) Urine sample (2 ml) spiked with 10 ng of each of warfarin, diphacinone, chlorophacinone and bromadiolone and 100 ng of coumatetralyl (IS). The left and right vertical axes and horizontal axes are the same as those in Fig. 4. For other conditions, see the Experimental section.

Table 3
Within-day and between-day precision and accuracy of the method for spiked blood and urine samples ($n=8$)

	Added (ng/ml)	Blood sample				Urine sample			
		Within-day[a]		Between-day[b]		Within-day[a]		Between-day[b]	
		Found ($X\pm$SD)	CV (%)	Found ($X\pm$SD)	CV (%)	Found ($X\pm$SD)	CV (%)	Found ($X\pm$SD)	CV (%)
Warfarin	10	9.9±0.9	8.7	10±1.7	17	10±0.49	5.1	9.9±0.56	5.6
	50	47±3.2	6.9	45±3.3	7.0	51±3.8	7.6	53±3.6	6.8
Diphacinone	10	–[c]	–	–	–	–	–	–	–
	50	55±7.7	14	59±14	24	48±5.4	11	50±6.6	13
Chlorophacinone	10	–[c]	–	–	–	–	–	–	–
	50	39±7.8	20	62±17	27	55±9	16	56±7.9	14
Bromadiolone	10	10±1.4	14	12±3.1	27	13±1.6	12	12±1.7	14
	50	66±4.8	7.3	57±11	20	67±7.6	11	61±5.9	9.7

[a] Within-day CVs were calculated from measurements of eight spiked samples on the same day.
[b] Spiked blood and urine samples were kept at 4°C and analyzed on eight separate days, with one sample each day.
[c] Not measured, since this concentration was close to the detection limit.

was chosen as internal standard. The calibration curve was linear in the range 1–100 ng/1 ml blood sample or 2 ml urine sample for warfarin and bromadiolone, or 10–100 ng/1 ml blood sample or 2 ml urine sample for diphacinone and chlorophacinone, as shown in Table 2.

Detection limits were 1 ng/1 ml blood sample or 2 ml urine sample for warfarin and bromadiolone, which corresponds to 50 pg injected onto the column, and 5 ng/1 ml blood sample or 2 ml urine sample for diphacinone and chlorophacinone. These limits are far below the therapeutic or intoxication levels of these rodenticides [32,33].

Within-day and between-day precision and accuracy of the method were examined with spiked blood and urine samples, as shown in Table 3. Within-day

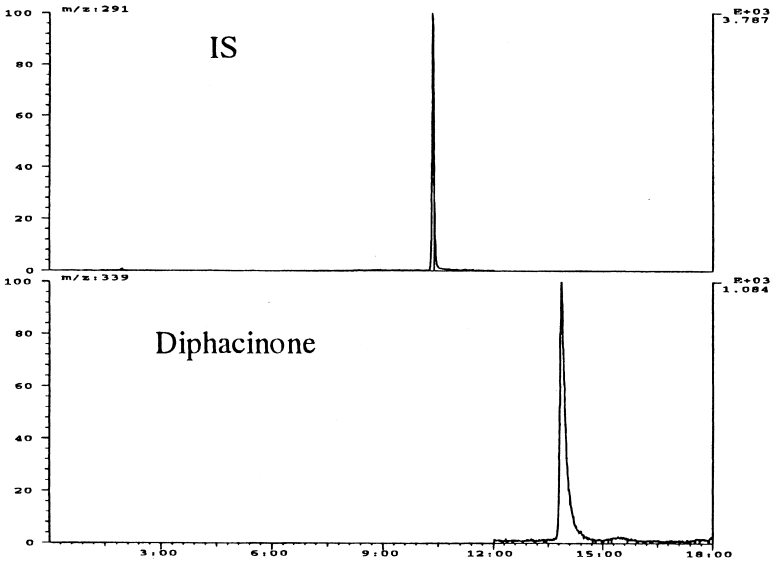

Fig. 6. Time-scheduled SIM chromatograms for a blood sample from a rat dosed with 50 μg of diphacinone. The blood sample was taken 3.5 h after administration, and 500 ng of coumatetralyl (IS) was added to 1 ml of the blood sample prior to liquid–liquid extraction. The left and right vertical axes and the horizontal axes are the same as those in Fig. 4. For other conditions, see the Experimental section.

precision, expressed as the coefficient of variation (CV), was not greater than 20 and 16% for blood and urine samples, respectively. Between-day CVs were not greater than 27 and 14% for blood and urine samples, respectively. The accuracy ranged from 87 to 130%. For the determination of warfarin, the precision and accuracy were better than those for the other three rodenticides, due to the fact that the chromatographic retention time of warfarin was close to that of the IS.

3.6. Application of the method

We administered orally 50 μg of diphacinone to six rats and sampled their blood for the determination of rodenticides. Typical chromatograms for the rat blood sample are shown in Fig. 6. The blood concentration of diphacinone 3.5 h after administration, determined by internal standard calibration, was 320 ± 51 ng/1 ml whole blood sample ($n=6$).

4. Conclusion

Conventional ion-pairing reagents consisting of quaternary ammonium and inorganic acid are known to be incompatible with HPLC–MS. In this paper, however, we report an ion-pairing reagent, DBA, composed of di-*n*-butylammonium and organic acetate, for HPLC–MS measurements of anionic anticoagulant rodenticides. Fortunately, it could be used in HPLC–MS without causing any problems. DBA may be applicable to HPLC–MS analysis of other anionic compounds, broadening the application of HPLC–MS.

Acknowledgement

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

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