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Liquid chromatography-tandem mass spectrometry method for determination of the pyridinium aldoxime 4-PAO in brain, liver, lung, and kidney☆

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ABSTRACT

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was validated and applied to the *in vitro* determination of 4-[(hydroxyimino)methyl]-1-octylpyridinium cation (4-PAO), which can penetrate the blood–brain barrier and reactivate acetylcholinesterase (AChE) inhibited by alkylphosphonate in the brain, liver, lung, and kidney. The limit of detection (LOD) was 0.235 μg cation/g wet weight, and the quantification range and linearity of the calibration curve extended over a range of 0.470–941 μg cation/g wet weight. For the proof of applicability, when 4-PAO was administrated intravenously via the rat tail vein at 10% LD_{50}, we were able to quantify the 4-PAO concentration in the tissues: brain 7.60 ± 1.32 μg cation/g wet weight (mean ± SD, $n = 5$), liver 26.8 ± 2.82 μg cation/g, lung 76.4 ± 24.9 μg cation/g, and kidney 638 ± 266 μg cation/g. In addition, the methods for 4-[(hydroxyimino)methyl]-1-decylpyridinium bromide (4-PAD) and 4-[(hydroxyimino)methyl]-1-(2-phenylethyl) pyridinium bromide (4-PAPE) were partly validated referring to the findings of the 4-PAO full validation. Thus, the LC-MS/MS method described in this study can be useful for quantification of pyridinium aldoxime methiodide (PAM)-type oximes in biological samples.

1. Introduction

In the treatment of organophosphonate poisoning, 2-PAM is a promising agent for the reactivation of inhibited acetylcholinesterase (AChE) [1–6]. Obidoxime, HI-6, and TMB-4 are also known to be efficacious reactivators [6–8]. However, existing reactivators of AChE are not effective against all types of nerve agents [6,9]. Kuca et al. [10–12] therefore attempted to develop various novel effective aldoxime compounds. In 1995, when the Tokyo subway was attacked by terrorist with sarin gas (isopropyl methylphosphonofluoridate) [13–15], 2-PAM was used in some hospitals for the reactivation of inhibited AChE in patients with sarin poisoning. Nagao et al. [16] performed judicial autopsies on four victims within a few days of the attack. They reported that although AChE activities in blood after 2-PAM administration were relatively recovered, the AChE activities in brain cortices were kept low. In 2003, we confirmed that the penetration ratio of 2-PAM through the blood–brain barrier (BBB) was approximately 10% with an *in vivo* rat brain microdialysis technique [17]. Therefore, it was deemed necessary to develop a new reactivator that could easily penetrate the BBB. In 2006, we developed a safe, new method for the preparation of sarin-exposed human erythrocyte AChE using isopropyl *p*-nitrophenyl methylphosphonate (INMP), a stable non-toxic sarin analogue and synthesized 40 known and novel pyridinium aldoxime methiodide (PAM)-type oximes (alkylPAMs) [18]. Next, we examined the reactivation activity of these compounds on INMP-exposed AChE, and the structure–activity relationships were examined. In 2008, we attempted to clarify whether six alkylPAMs, which had relatively high reactivation activity and a highly lipophilic chemical structure, could penetrate the BBB [19]. The results of experiments with *in vivo* rat brain microdialysis and LC-MS/MS confirmed that 4-PAO and 4-[(hydroxyimino)-methyl]-1-(2-phenylethyl)pyridinium bromide (4-PAPE) could penetrate the BBB. In particular, the penetration ratio of 4-PAO across the BBB was approximately 30%, indicating that the intravenous administration of 4-PAO might be effective for reactivation of blocked cholinesterase in the brain.

To date, analysis of alkylPAMs has generally been performed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection or electrochemical detection (ECD) [7,17,20–24]. Recently, capillary zone electrophoresis (CZE) has also been used to determine 2-PAM concentration in urine, serum, brain, and cerebrospinal fluid (CSF) [25,26]. On the other hand, we were the first to use an LC-MS/MS technique for the detection of 6 alkylPAMs in dialysate and blood [19]. The LC-MS/MS method was suitable for the detection of the alkylPAMs in dialysate and blood, from trace

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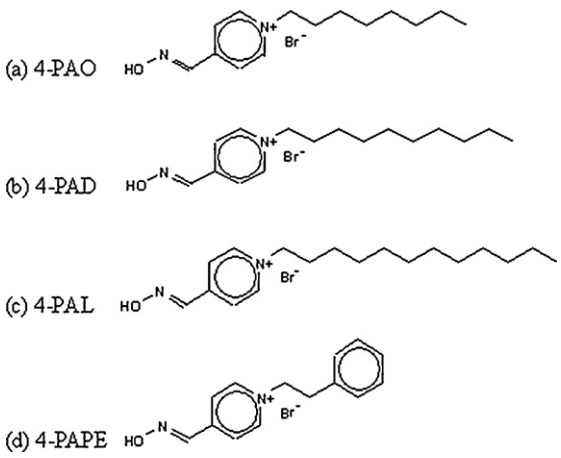

Fig. 1. Chemical structures of alkylPAMs used in this experiment.

amounts to high levels. The quite low limit of detection (LOD) and the wide quantification ranges over four digits is potentially useful for various other matrices and alkylPAMs.

In the present study, an LC-MS/MS assay for the determination of PAM-type oximes was presented in different matrices than blood and dialysate. As a proof of applicability of the validated method, after intravenous administration of 4-PAO to rats, the levels in brain, liver, lung, and kidney were measured with the LC-MS/MS method.

2. Experimental

2.1. Chemicals

The following alkylPAMs were synthesized in our laboratory as previously described [18]: 4-[(hydroxyimino)methyl]-1-octylpyridinium bromide (4-PAO), 4-[(hydroxyimino)methyl]-1-decylpyridinium bromide (4-PAD), 4-[(hydroxyimino)methyl]-1-laurylpyridinium bromide (4-PAL, which was used as an internal standard [IS]), and 4-[(hydroxyimino)methyl]-1-(2-phenylethyl)pyridinium bromide (4-PAPE). The chemical structures are shown in Fig. 1. HPLC-grade methanol, reagent grade ammonium acetate and formic acid (96%) were purchased from Wako Pure Chemicals Co. (Tokyo, Japan). Ultra-pure water was prepared in-house using a Milli-Q water purifying system (Millipore Corp., Bedford, MA, USA). Ultrafree-MC Centrifugal Filter Unit (0.45 μm poly(vinylidene fluoride) filter, 0.5 mL capacity) was purchased from Millipore Corp. (Bedford, MA, USA).

2.2. Animal treatment

Male Wistar strain rats weighing 300–350 g were housed under conditions of constant temperature and humidity and a 12-h dark cycle. A solution of PAM-type oximes in saline was administered intravenously via the rat tail at a concentration amounting 10% of the LD_{50}. After approximately 3 h, the rats were sacrificed under anesthesia induced by diethyl ether, and then the brain, liver, lung, and kidney were immediately removed without washed-out from blood using saline isotonic perfusion. The organ samples taken were stored at −80 °C until use. The animal experiments were carried out in accordance with the guidelines for the care and use of laboratory animals established by the Committees of the National Research Institute of Police Science, which conforms to the NIH guidelines.

2.3. Preparation of rat tissue samples

Samples (200 mg) of brain, liver, lung, and kidney were dissected from the rats, suspended in 400 μL of 4 mM ammonium acetate adjusted to pH 3 with 0.1% formic acid, and homogenized by moving a Potter-type homogenizer (AS ONE, Tokyo, Japan) up and down about ten times. Then, the mixture was again homogenized in the presence of 400 μL of 8 nM 4-PAL/MeOH as the IS. The mixture was centrifuged at 12,000 × *g* for 10 min. The supernatant was filtered through an Ultrafree-MC Centrifugal Filter Unit. After the filtrate was left overnight at −20 °C, the supernatant was again filtered through an Ultrafree-MC Centrifugal Filter Unit in order to remove proteins completely, and a portion (10 μL) of the filtrate was injected into the LC-MS/MS apparatus.

2.4. Preparation of stock solutions

Stock solutions of 4-PAO, 4-PAD, 4-PAPE and the IS (4-PAL) were initially prepared as 470, 526, 454 and 582 μg cation/mL, respectively (2000 nM each) methanol solutions and stored at −20 °C. The working solutions were freshly prepared by diluting stock solutions with methanol and were fortified with blank rat brain, liver, lung, or kidney to verify the linearity and limit of detection.

2.5. LC-MS/MS conditions

LC separation was carried out using an Alliance 2695 series liquid chromatograph with autosampler (Waters, Milford, MA, USA), equipped with an Inertsil ODS-4 column (2.1 mm I.D. × 150 mm, 3.0-μm particle size; GL Sciences Inc., Tokyo, Japan). Mobile phase A was methanol and B was 4 mM ammonium acetate adjusted to pH 3 with 0.1% formic acid. Linear gradient elution with mobile phases A and B was employed using A at 40% at 0 min, and A at 95% for 12–20 min. The flow rate was 0.2 mL/min and the column temperature was maintained at 40 °C. The injection volume was 10 μL.

Mass spectrometric analysis was performed on a Micromass Quattro-micro API (Waters, Milford, MA, USA). Electrospray ionization (ESI) in positive mode was used for ion production. The optimized cone voltages for the compounds are shown in Table 1. The capillary voltage was 3.5 kV, the ion-source temperature was 120 °C, and the desolvation temperature was 350 °C. The cone gas flow and desolvation gas flow were 50 and 600 L/h, respectively. Detection, quantification, and confirmation were carried out in multiple reaction monitoring (MRM) mode. For all compounds, molecular ions of the cation (M^+) were used as precursors. The product ions for monitoring, the optimized cone voltages, and the collision energies are also summarized in Table 1.

2.6. Brain, liver, lung, and kidney calibrators

Blank brain, liver, lung, and kidney (200 mg each) were dissected, suspended in 400 μL of 4 mM ammonium acetate adjusted to pH 3 with 0.1% formic acid, and homogenized by moving a Potter-type homogenizer (AS ONE, Tokyo, Japan) up and down about ten times. To this was added 200 μL of, 0.235, 0.470, 0.941, 2.35, 4.70, 9.41, 23.5, 94.1, 235, and 941 μg cation/mL solution of 4-PAO, 0.263, 0.526, 1.05, 2.63, 5.26, 10.5, 26.3, 105, 263, and 1053 μg cation/mL solution of 4-PAD, or 0.227, 0.454, 0.908, 2.27, 4.54, 9.08, 22.7, 90.8, 227, and 908 μg cation/mL solution of 4-PAPE, and 200 μL of 1165 μg cation/mL (4000 pM) IS solution. Then, the mixture was again homogenized and centrifuged at 12,000 × *g* for 10 min. The supernatant was filtered through an Ultrafree-MC Centrifugal Filter Unit. After the filtrate was left overnight at −20 °C, the supernatant was again filtered through an Ultrafree-MC Centrifugal Filter Unit in order to remove proteins completely. The resulting filtrates were

Table 1
Optimized MRM conditions for alkylPAMs.

AlkylPAMs	Precursor ion (M^+)	Cone voltage (V)	Transition 1 (detection/quantification)		Transition 2 (confirmation)	
			Product ion	Collision energy (V)	Product ion	Collision energy (V)
4-PAO	235.18	38	123.06	20	79.04	36
4-PAD	263.21	40	123.06	22	57.07	24
4-PAL	291.24	44	123.06	24	57.07	26
4-PAPE	227.12	30	105.07	18	77.04	48

used as calibrators of 1, 2, 4, 10, 20, 40, 100, 400, 1000, and 4000 pM of alkylPAMs, and portions (10 μL) of them were injected into the LC-MS/MS apparatus.

3. Results

3.1. Product ion scan spectra

Fig. 2 shows the product ion scan spectra of the alkylPAMs. Linear side-chain PAM analogues, 4-PAO, 4-PAD, and 4-PAL, primarily generated the cation of *m*/*z* 123 that had lost the side chain (Fig. 2a–c); 4-PAPE formed an ion of *m*/*z* 105, and this ion was thought to be generated from the side chain of 4-PAPE as C_6H_5-CH_2-CH_2^+. This primary carbocation is adjacent to benzyl position, thus more stable benzyl cation C_6H_5-CH^+-CH_3 would be formed by rearrangement of the benzyl proton, and some or many of the benzyl cations would undergo further rearrangements to form methyltropylium cations (Fig. 2d). These cations were the most abundant ions for the alkylPAMs and were used for their detection and quantification. At higher collision energies, *n*-butyl cation (*m*/*z* 57) or pyridine cation radical (*m*/*z* 79) were primarily generated from 4-PAO, 4-PAD, and 4-PAL. Phenyl cation (*m*/*z* 77) was mainly primarily generated from 4-PAPE (Fig. 2e). Thus, these cations were used for confirmation of the alkylPAMs.

3.2. LC separation

The MRM chromatograms of 941, 1053, 1165, and 908 μg cation/mL (4 nM each) mixed solutions of the alkylPAM standards, 4-PAO, 4-PAD, 4-PAL, and 4-PAPE in the LC mobile phase of 0 min are shown in Fig. 3(a). The peaks were well separated, and the analytes could be determined simultaneously. No interference from organ matrices was observed (from Fig. 3(b) to (e)), all analytes were eluted within 20 min. Both MRM of transition 1 (for detection/quantification) and 2 (confirmation) were shown in the chromatogram with the identical *y*-axis scale. 4-PAL was used only as the IS in this experiment; 4-PAL itself is also a reactivator for inhibited AChE that penetrates the BBB and might be used as an analyte in future experiments. Therefore, the MRM condition for confirmation was also established for 4-PAL.

3.3. Calibration curves

The LOD (S/N = 3), the limit of quantification (LOQ), the quantification range, and linearities of the calibration curves of the alkylPAMs in brain, liver, lung, and kidney obtained by the LC-MS/MS method are summarized in Table 2. LOQ was defined as the lowest level in which the intra-assay and inter-assay precision were within 20%. The upper limit of the quantification range was determined by the upper limit of the sample concentration of the LC

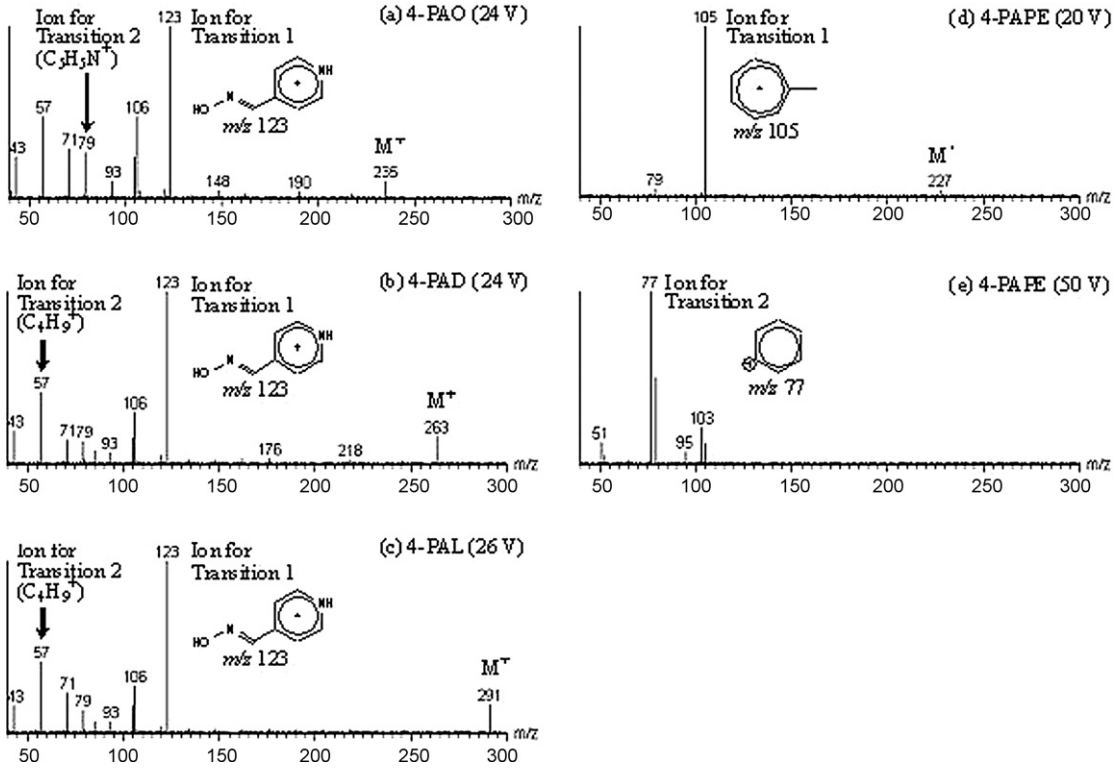

Fig. 2. Product ion scan spectra of (a) 4-PAO, (b) 4-PAD, (c) 4-PAL, (d) 4-PAPE (20 V), and (e) 4-PAPE (50 V). Assignments of ions used for MRM experiments are shown in each spectrum. Cone voltage of each spectrum is shown in Table 1. Collision energies of the spectra are shown in each spectrum.

Table 2
Limit of detection (LOD), limit of quantification (LOQ), quantification range, and linearity of the calibration curves of the alkylPAMs in rat brain, liver, lung, and kidney.

Matrix	AlkylPAMs	LOD (μg/g)	LOQ (μg/g)	Quantification range (μg/g)	r^2
Brain	4-PAO	0.235	0.470	0.470–941	0.9967
	4-PAD	1.05	2.63	2.63–1053	0.9899
	4-PAPE	0.0908	0.227	0.227–908	0.9988
Liver	4-PAO	0.235	0.470	0.470–941	0.9980
	4-PAD	2.63	5.26	5.26–1053	0.9989
	4-PAPE	0.227	0.454	0.454–908	0.9972
Lung	4-PAO	0.235	0.470	0.470–941	0.9852
	4-PAD	2.63	5.26	5.26–1053	0.9876
	4-PAPE	0.227	0.454	0.454–908	0.9772
Kidney	4-PAO	0.235	0.470	0.470–941	0.9998
	4-PAD	1.05	2.63	2.63–1053	0.9987
	4-PAPE	0.227	0.454	0.454–908	0.9989

system (1 μg/mL) so that the severe carryover would be prevented. A low LOD (0.0908–2.63 μg cation/g wet weight) and wide quantification ranges over three or four orders of magnitude were obtained for all matrices and alkylPAMs. The recoveries of 4-PAO, 4-PAD and 4-PAPE from liver were determined to be 48.4 ± 3.88% (mean ± SD, n = 5), 29.9 ± 5.47% and 36.9 ± 4.05%, respectively when spiked at 23.5, 26.3 and 22.7 μg cation/g wet weight (100 pM each). The relatively low recoveries might be due to high lipophilicities of the alkyl side chain of the alkylPAMs, and it suggests that it is difficult to separate the highly lipophilic alkylPAMs completely from lipid-rich organs such as livers with this simple pre-treatment method. The recoveries from other organs were not available there were not sufficient blank organs for further experiment.

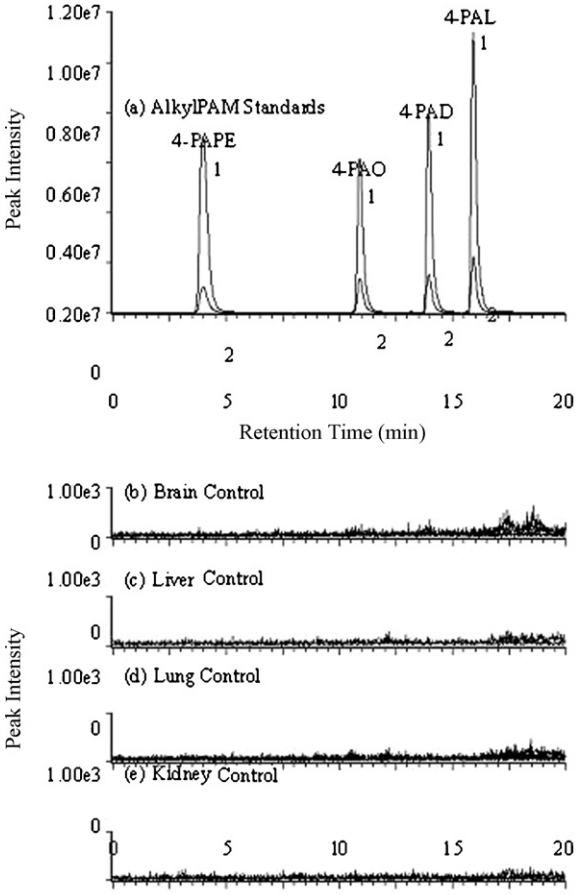

Fig. 3. (a) The MRM chromatograms of the alkylPAM standards, 4-PAO, 4-PAD, 4-PAL, and 4-PAPE (941, 1053, 1165 and 908 μg cation/mL, respectively (4 nM each)). Each chromatogram consists of MRM of transition 1 (shown as "1" beside each peak) for detection/quantification and transition 2 for confirmation (shown as "2" beside each peak), and the MRM chromatograms of (b) blank brain extract, (c) blank liver extract, (d) blank lung extract, and (e) blank kidney extract. Instrument: Waters Alliance LC and Micromass Quattro-micro API. Ionization: ESI+; cone voltages and collision energies: see Table 1; column: GL Sciences Inertsil ODS-4 (2.0 mm × 150 mm, 3.0 μm); mobile phase: A: MeOH, B: 4 mM $AcONH_4$–0.1% HCO_2H (pH 3); 0 min: A 40%, 12–20 min: A 95%. Linear gradient, flow rate: 0.2 mL/min, injection volume: 10 μL, column temp: 40 °C.

3.4. Precision and accuracy

Intra-day precision was determined by measuring the means of two replicates of liver samples five times at two different concentrations (23.5 μg cation/g wet weight (100 pM) and 2.35 μg cation/g wet weight (10 pM)) on the same day. Inter-day precision was determined by measuring the means of two replicates of liver samples (23.5 and 2.35 μg cation/g wet weight) on five different days. The intra- and inter-day precisions are summarized in Table 3. The RSD intra-day precision at 23.5 and 2.35 μg cation/g wet weight were 14.4% (24.7 ± 3.57 μg cation/g wet weight, mean ± SD, n = 5) and 18.6% (2.42 ± 0.449 μg cation/g wet weight), respectively. The RSD inter-day precision at 23.5 and 2.35 μg cation/g wet weight were 16.8% (24.2 ± 4.07 μg cation/g wet weight, mean ± SD, n = 5) and 18.0% (2.29 ± 0.412 μg cation/g wet weight), respectively.

The intra- and inter-day accuracies are also summarized in Table 3. The intra-day accuracy was 105% at 23.5 μg cation/g wet weight and 103% at 2.35 μg cation/g wet weight. The inter-day accuracy was 103% at 23.5 μg cation/g wet weight and 97.2% at 2.35 μg cation/g wet weight.

3.5. LC-MS/MS analysis of the brain, liver, lung, and kidney samples after 4-PAO administration

A solution of 4-PAO (0.889 mg/kg) [19] in saline was administered intravenously via the rat tail vein (n = 5) at a concentration amounting to 10% of the LD_{50}. The brain, liver, lung, and kidney were removed at approximately 3 h after administration of 4-PAO, and the amount of 4-PAO in each tissue was measured by the LC-MS/MS. The MRM chromatograms of 4-PAO extracted

Table 3
Precision and accuracy of 4-PAO-spiked liver tissue extract.

Spiked concentration	Intra-day (n = 5)		Inter-day (n = 5)	
	23.5 μg/g	2.35 μg/g	23.5 μg/g	2.35 μg/g
Mean concentration	24.7	2.42	24.2	2.29
Standard deviation	3.57	0.449	4.07	0.412
RSD (%)	14.4	18.6	16.8	18.0
Accuracy (%)	105	103	103	97.2

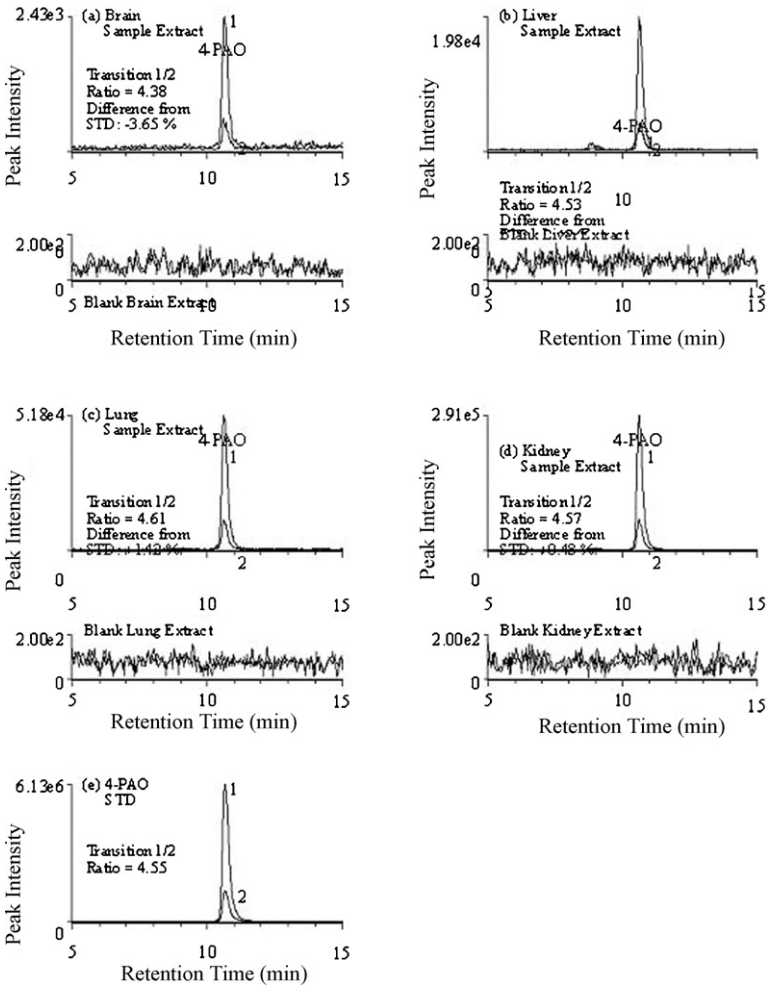

Fig. 4. MRM chromatograms of extracts of (a) brain sample (top) and blank brain (bottom), (b) liver sample (top) and blank liver (bottom), (c) lung sample (top) and blank lung (bottom), and (d) kidney sample (top) and blank kidney (bottom) of rats administered 4-PAO (0.889 mg/kg, 3 h), and (e) 4-PAO standard consisted of transition 1 (shown as "1" beside each peak) for detection/quantification and transition 2 for confirmation (shown as "2" beside each peak).

from the organ samples are shown in Fig. 4. In each organ sample, 4-PAO was easily detected without any interference from organ matrices. The ratio of the peak areas of transitions 1 and 2 in the four organ samples (shown in Fig. 4a–d) coincided with the standard 4-PAO with an error of less than ±3.65%, indicating that the detected peaks originated in 4-PAO and did not contain any interference peaks. The results of quantification are also shown in Table 4. The concentrations of 4-PAO in brain, liver, lung, and kidney were 7.60 ± 1.32 μg cation/g wet weight (mean ± SD, $n = 5$), 26.8 ± 2.82 μg cation/g wet weight, 76.4 ± 24.9 μg cation/g wet weight, and 638 ± 266 μg cation/g wet weight, respectively.

4. Discussion

Generally, 2-PAM, obidoxime, and HI-6 can be analyzed using HPLC-UV, which we also used previously for determination of 2-PAM concentration in rat blood and striatal extracellular fluid [17]. Gyenge et al. [24] measured the concentration of a bisquaternary asymmetric pyridinium aldoxime-type cholinesterase reactivator K-27 in rat brain, cerebrospinal fluid, serum, and urine with HPLC-ECD. They reported that the calibration curve was linear through the range of 10–250 ng/mL and that the accuracy, precision, and LOD were satisfactory. Kalász et al. [26] reported that measurement by CZE was a fast and reliable method for monitoring blood–brain and blood–cerebrospinal fluid penetration of pyridinium aldoxime-type antidotes. They noted that the calibration curves covered the ranges from 0.3 to 200 μg/mL, 0.3 to 7 μg/mL, and 0.1 to 7 μg/mL for serum, brain, and cerebrospinal fluid, respectively. In 2008, we first used the LC-MS/MS method to examine the penetration the BBB by six novel alkylPAMs, which had little UV absorbance [19]. In that study, the LOD and quantification range of 4-PAO in both dialysate and blood were 0.4 pM and 2–4000 pM, respectively. This indicated that the LC-MS/MS method could be a reliable method with high precision and accuracy for quantification of alkylPAMs in dialysate and blood.

In the present study, we aimed to quantify the concentration of alkylPAMs using this LC-MS/MS method in various biological samples, namely, brain, liver, lung, and kidney. The calibration curves of 4-PAO which showed approximately 30% penetration into the BBB [17], were established using spiked samples. The LOD and quantification range of 4-PAO in all organs were 1 and 2–4000 pM, respectively. Intra- and inter-day precision C.V.s in

Table 4
4-PAO concentrations in brain, liver, lung, and kidney samples taken from rats 3 h after intravenous 4-PAO administration at 0.889 mg/kg each.

Organ	Administered alkylPAM	n	Mean (μg/g)	SD
Brain	4-PAO	5	7.60	1.32
Liver	4-PAO	5	26.8	2.82
Lung	4-PAO	5	76.4	24.9
Kidney	4-PAO	5	638	266

SD: standard deviation.

spiked liver samples at 100 pM were 14.4% and 16.8%, respectively. The relatively high RSD intra- and inter-day for precision might be reduced by using a more appropriate IS or employing solid-phase extraction methods for sample cleanup. The intra- and inter-day accuracies with the 100 pM spiked concentration of liver samples were 105% and 103%, respectively. These values were considered to be sufficient for rapid determination of 4-PAO levels in liver tissues. On the other hand, other tissues were too small to determine these parameters, and therefore we determined them only on liver. However, these parameters should be essentially determined in each tissue. And also, it will be more correct to use three concentrations for determined these parameters: one low (<9.41 μg cation/g wet weight (40 pM)), one medium (between 23.5 μg cation/g wet weight (100 pM) and 94.1 μg cation/g wet weight (400 pM)) and one high level (upper 235 μg cation/g wet weight (1000 pM)). On the basis of these data, we determined the 4-PAO concentrations in brain, liver, lung, and kidney at approximately 3 h after administration of 4-PAO at 10% LD_{50}. Since dialysis of each tissue with saline was not performed, blood might not have been completely removed from each tissue. Therefore, the values of each tissue could have been affected by the presence of blood. However, the LC-MS/MS method was sufficiently reliable and sensitive for monitoring 4-PAO levels of different biological samples. Furthermore, the LOD, LOQ, the quantification range, and linearities of the calibration curves of 4-PAD and 4-PAPE in brain, liver, lung, and kidney showed that this method is reliable and sensitive enough for monitoring 4-PAD and 4-PAPE. In our previous study [19], although we failed to correctly prepare the striatal extracellular dialysate samples of 4-PAD and therefore the BBB penetration of 4-PAD remained unclear, it was revealed that 4-PAPE could penetrate the BBB slightly. The validation is ongoing and the preliminary data presented suggest that the method will work comparably with that of 4-PAO, presented.

At present, little is known about the pharmacokinetics of most alkylPAMs in organophosphate poisoning. Moreover, the mechanism of alkylPAMs toxicity has not been fully examined. In 2009, we investigated the effects of oximes on mitochondrial oxidase activity [27]. That preliminary study showed that the toxicities of oximes may be related to their inactivation of mitochondrial oxidase enzymes and generation of reactive oxygen species. However, further research is required to determine the effects of oximes on mitochondrial activity. Thus, to obtain more detailed data, it is very important to develop much better techniques for quantification of alkylPAMs. The LC-MS/MS method described in this study is a reliable method for quantification of alkylPAMs in various biological samples.

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