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Quantitative determination of pamoic acid in dog and rat serum by automated ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography

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Abstract

Pamoic acid is used as a counter ion to obtain long-acting pharmaceutical formulations of certain basic drugs. In order to investigate the pharmacokinetics of pamoic acid, a simple, sensitive and reliable method has been established for the quantitative determination of pamoic acid in serum from dog and rat. The method uses ion-pair solid-phase extraction followed by ion-pair reversed-phase high-performance liquid chromatograpy. The influence on recovery of the addition of different agents (tetrabutylammonium acetate, methanol, sodium hydroxide) to the serum samples prior to solid-phase extraction was studied and the analytical method was validated. The method was found to be valid for accurate, precise and selective determination of pamoic acid in the tested concentration range of 5–200 ng/ml serum. The overall performance of the HPLC method was found to be satisfactory for the purpose of determining concentrations of pamoic acid in serum samples from pharmacokinetic studies with pamoic acid in dogs and rats.

Keywords: Pamoic acid

1. Introduction

Pamoic acid (embonic acid), an aromatic dicarboxylic acid (see Fig. 1), has been used as a means of masking unpleasant tastes or obtaining prolonged therapeutic action by forming slightly soluble salts with certain basic drugs [1–7].

Although pamoic acid is a well-known substance and is used in salt formulations of several drugs, the literature regarding analysis and, especially, bioanalysis is very limited. For quality control purposes a reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of ampicillin pamoate and amoxycillin pamoate has been described [3]. Likewise, for quality control purposes a reversed-phase HPLC method for the analysis of noscapine hydrogen pamoate has been described [8]. With regard to the bioanalysis of pamoic acid only one article has been published dealing with this subject. Ruuten et al. described a column-switching technique for assaying pamoic acid in human plasma [9]. However, many interfering peaks are seen in the chromatogram and detection limits seem to be rather high (judging from the chromatogram the detection limit is several micrograms per milliliter serum).

Thus, pamoic acid has potential with respect to the development of parenteral depot formulations of

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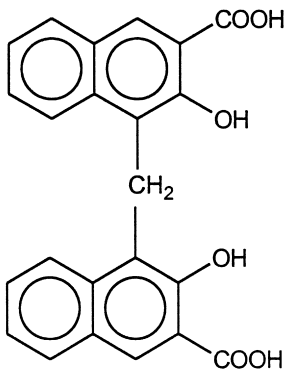

Pamoic acid

Fig. 1. Structural formula of pamoic acid.

basic drugs. In this context a specific and sensitive analytical method for determining serum concentrations of pamoic acid is needed.

Pamoic acid is a difficult compound to handle because of its very limited solubility in almost all solvents [10] except for nitrobenzene, pyridine and dimethylformamide and aqueous solution at pH>7. Therefore, the extraction of pamoic acid from serum is a very difficult task.

In the present work we investigated the use of ion-pair extraction in combination with solid-phase extraction. The protonation constants for the two carboxylate groups of pamoic acid have been estimated to be 4.3 and 7.7 [determined in a water–dioxane mixture (1:1)] [11]. Therefore, theoretically, by the addition of a quaternary ammonium compound and base to the serum sample prior to extraction, ion-pair formation should take place at both carboxylate groups giving rise to a higher extraction ratio.

2. Experimental

2.1. Chemicals and reagents

Pamoic acid was from Fluka (Burchs, Switzerland). Methanol was HPLC-grade from Rathburn (Walkerburn, UK). Acetonitrile was HPLC far UV-grade from Labscan (Dublin, Ireland). Tetrabutylammonium acetate (TBA) was from Sigma (St. Louis, MO, USA). All other chemicals were analytical grade from Merck (Darmstadt, Germany).

A stock solution of pamoic acid was prepared in dimethylformamide and stored at approximately 5°C. Dilutions of the stock solution were prepared in test tubes in 0.1 *M* sodium dihydrogen phosphate buffer adjusted with sodium hydroxide solution to pH 7.0.

2.2. Instrumentation

A HPLC system consisting of the following components, all from (Merck) Hitachi (Tokyo, Japan), was used: L-7100 pump, L-7200 autosampler, L-7480 fluorescence detector, L-7300 column oven, D-7000 interface and HSM Data System. Separation was performed on a 150 mm×4.6 mm I.D. YMC basic 5 μm column (YMC, Japan). A guard column (20 mm×4.6 mm I.D.) and a saturation column (150 mm×4.6 mm I.D) of the same type were used.

An ASPEC™ sample processor for solid-phase extraction with 721 keypad software, version 1.11, from Gilson (Villiers-le-Bel, France) was used. The sample work-up was performed on SPEC® C_{18} 3 ml microcolumns from Ansys (Irvine, CA, USA). The elution solvent was evaporated on a TurboVap® LV Evaporator from Zymark (Hopkinton, MA, USA).

2.3. Chromatographic conditions

The mobile phase used for the ion-pair reversed-phase chromatographic method consisted of a mixture of acetonitrile and a solution containing 12.5 m*M* sodium dihydrogen phosphate and 12.5 m*M* cetyltrimethylammonium hydrogen sulphate, adjusted with sodium hydroxide solution to pH 7.0 (40:60, v/v). Before use, the mobile phase was degassed for 10 min in an ultrasonic bath. The flow-rate was 1.0 ml/min. The injection volume was 50 μl. The column oven temperature was 40°C. The saturation column was connected to the HPLC system in front of the injection port and placed in the column oven. This was to ensure saturation of the mobile phase with silica before it reaches the analytical column. By use of the saturation column, dissolution of silica in the analytical column is prevented. Fluorescence detection was used with excitation at 240 nm and emission at 523 nm.

2.4. Solid-phase ion-pair extraction

2.4.1. Influence of TBA, methanol and sodium hydroxide on recovery

The influence of TBA (added in excess compared to the concentration of pamoic acid), methanol (corresponding to a concentration of about 24 vol.%) and sodium hydroxide (to provide alkaline conditions) added to serum prior to extraction on the recovery of pamoic acid was investigated in eight experiments (see Table 1 for details). To a sample of 500 μl dog serum spiked with 100 ng pamoic acid was added either none of the three above-mentioned reagents (experiment 1), sodium hydroxide alone (experiment 2), methanol alone (experiment 3), TBA alone (experiment 5), sodium hydroxide and methanol (experiment 4), TBA and sodium hydroxide (experiment 6), TBA and methanol (experiment 7) or all three reagents (experiment 8). In experiments 1–4, 1000 μl in 0.1 *M* sodium dihydrogen phosphate buffer adjusted with sodium hydroxide solution to pH 7.0 was added instead of TBA solution.

2.4.2. Influence of volume of methanol added on recovery

Initial experiments showed that the volume of methanol added to the serum samples prior to extraction had a very pronounced influence on the recovery of pamoic acid from serum, especially from rat serum. To investigate the influence of the volume of methanol added on recovery, to rat and dog serum samples (500 μl) spiked with 100 ng pamoic acid were added TBA (1000 μl, 10 m*M*), sodium hydroxide (20 μl, 2 *M*) and 0, 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 μl methanol.

2.4.3. Final extraction method (used for validation)

Serum samples (500 μl) were processed using an extraction procedure performed as follows: 1500 μl of the following solution was added: 10 m*M* tetrabutylammonium acetate (TBA)–methanol–2 *M* sodium hydroxide (10:7:0.2), corresponding to about 870 μl 10 m*M* TBA solution, 610 μl methanol and 17 μl 2 *M* sodium hydroxide solution. The test tube contents were mixed on a whirlimixer for 2 min and centrifuged for 15 min at approximately 5000*g* after which the samples were carefully transferred to clean ASPEC™ tubes. The tubes were corked, placed in the ASPEC™ sample processor and extracted in the sequential mode using the following procedure.

The SPEC® C_{18} columns were preconditioning with 500 μl methanol. The samples (2.0 ml) were then loaded onto the extraction columns (loading rate 3 ml/min) and washed with 1000 μl water–methanol (90:10) (washing rate 1 ml/min). The extraction columns were dried with 3.0 ml of air. Finally, the samples were eluted with 500 μl water–methanol (10:90) (elution rate 3 ml/min). The eluates were evaporated under a gentle stream of nitrogen (about 30 min at 40°C) using a TurboVap® LV Evaporator. The samples were reconstituted in the mobile-phase buffer (12.5 m*M* cetyltrimethylammonium hydrogen sulphate, 12.5 m*M* sodium dihydrogen phosphate adjusted with sodium hydroxide solution to pH 7.0) by whirlimixing for 10 min. After centrifugation at approximately 5000 *g* for 15 min, aliquots of 150 μl

Table 1
Influence of tetrabutylammonium acetate (TBA), methanol and sodium hydroxide on recovery of pamoic acid from dog serum

Experiment	TBA (1000 μl, 10 m*M*)	Methanol (500 μl)	Sodium hydroxide (20 μl, 2 *M*)	Recovery (%)		
				Experiment 1	Experiment 2	Mean (n=2)
1	−	−	−	1	1	1
2	−	−	+	2	3	3
3	−	+	−	2	2	2
4	−	+	+	3	1	2
5	+	−	−	16	16	16
6	+	−	+	73	65	69
7	+	+	−	74	75	75
8	+	+	+	97	92	95

were transferred to HPLC sample vials. Aliquots of 50 μl were injected onto the HPLC system.

2.4.4. Preparation of calibration standards, test samples and blank serum samples

Calibration standards and test samples for dog and rat sera were processed for analysis as described above.

2.4.4.1. *Calibration standards*. Calibration standards at seven concentrations (2–200 ng/ml) were prepared by spiking 500 μl dog or rat serum with pamoic acid standard solution.

In addition, 0.1 *M* sodium dihydrogen phosphate buffer, adjusted with sodium hydroxide solution to pH 7.0, was added to give a total buffer content of 200 μl.

2.4.4.2. *Test samples*. Test samples were prepared as 5.0 ml pools by spiking control serum pools with pamoic acid standard solution. In addition, 0.1 *M* sodium dihydrogen phosphate buffer, adjusted with sodium hydroxide solution to pH 7.0, was added to give a total buffer content of 200 μl per sample.

2.4.4.3. *Blank serum samples*. The blank serum samples were prepared by adding 200 μl 0.1 *M* sodium dihydrogen phosphate buffer, adjusted with sodium hydroxide solution to pH 7.0, to 500 μl dog or rat control serum.

2.5. Validation of the analytical method

The final analytical method was tested on three different working days assaying dog and rat serum samples. On each validation day, calibration standards (in duplicate), test samples and blank serum samples were prepared, extracted and analysed. Calibration standards, blank serum samples and test samples were analysed in sample volumes of 0.5 ml.

The accuracy and precision of the analytical method was determined from analysis of test samples (i.e. dog and rat samples spiked with known amounts of pamoic acid) at four concentrations (2, 5, 40 and 160 ng/ml for dog serum and 5, 10, 40 and 160 ng/ml for rat serum). Accuracy was calculated as a percentage of the measured versus the known concentration. Precision was determined as the coefficient of variation, i.e. the ratio between the mean of the found concentration and its standard deviation. The lowest concentration (2 and 5 ng/ml for dog and rat serum, respectively) was chosen as the signal-to-noise ratio and was approximately 10 times that of the baseline noise. The test samples were prepared as pools and six replicates of each test samples were analysed on each of three working days.

All analytical work on each day of analysis was performed in batch sizes of 80 samples. Blank serum samples were included in a number necessary to achieve the 80 samples.

Concentrations of pamoic acid in the test samples found by analysis were calculated from calibration curves obtained on the actual day of analysis. Peak area was used for calculation.

3. Results and discussion

3.1. LC conditions

Because of the very limited solubility of pamoic acid in acidic aqueous solution the pH of the mobile phase had to be about neutral. The very limited solubility at pH<7 suggests that, in order for pamoic acid to be soluble, both carboxylic acid groups must be ionised. However, at neutral pH pamoic acid only exhibited little retention on the C_{18} column, and therefore the ion-pair-forming agent cetyltrimethylammonium hydrogen sulphate was added to the mobile phase to enhance retention.

Column life-time was prolonged by use of a saturation column. Because of the well-known influence of temperature on the solubility of silica [12] the saturation column was placed in the column oven.

3.2. Extraction

The very limited solubility of pamoic acid in aqueous solution at pH<7 has led to the conclusion that acidic extraction (i.e. extraction of the unionised form) is not practically possible.

Initial experiments showed that simple liquid–liquid extraction of pamoic acid from serum into various organic solvents (heptane, dichloromethane,

ethyl acetate) was not applicable due to the low solubility in most solvents.

Ion-pair liquid/liquid extraction of serum with dichloromethane after addition of a quaternary ammonium compound (e.g., tetrabutylammonium acetate) was found to give high recoveries of pamoic acid (>70%). This method was not ideal, however, because of the potential toxicity of dichloromethane and practical difficulties due to the high density of dichloromethane. Alternatively, ion-pair solid-phase extraction was tried.

3.3. Ion-pair solid-phase extraction (SPE)

We found that simple solid-phase extraction using C_{18} packed disc columns was not possible, because of the very low affinity of pamoic acid in serum at pH 7.4 for the C_{18} column material. The recovery was only about 1%. The reason for the low recovery was loss of pamoic acid during sample loading. It was not possible to acidify the serum in order to enhance the affinity because of the very low solubility of pamoic acid in solution at pH<7.

3.3.1. Influence of TBA, methanol and sodium hydroxide on recovery

From Table 1 it can be seen that neither the addition of methanol nor sodium hydroxide alone or together influenced the recovery from serum (experiments 1–4). With the addition of TBA alone recovery was about 16% (experiment 5). Simultaneous addition of methanol and TBA strongly enhanced recovery to about 69% (experiment 6). Simultaneous addition of sodium hydroxide and TBA also strongly enhanced recovery to about 75% (experiment 7). Simultaneous addition of both methanol and sodium hydroxide and TBA further enhanced recovery to about 95% (experiment 8). TBA was thought to enhance lipophilicity and thereby retention by forming ion-pairs with pamoic acid. Methanol was added to induce disruption of the drug–protein interaction. Sodium hydroxide was thought to enhance ion-pair formation.

3.3.2. Influence of volume of methanol added on recovery

From Fig. 2 it can be seen that the recovery vs. added volume of methanol can be described as a bell-shaped curve for both dog and rat serum. A larger volume of methanol results in a higher recovery up to a certain amount of methanol added. Optimal recovery was achieved by adding 600 μl methanol corresponding to about 30 vol.% methanol in the sample. Recovery decreases as the added volume of methanol exceeds 600 μl. The reason for this is loss of pamoic acid during sample loading, because of the rather high content of organic solvent in the sample. Achieving the highest possible recovery is a compromise between, on the one hand, adding a sufficient amount of methanol and, on the other, not adding too much methanol and thereby reducing the recovery of pamoic acid because of breakthrough of pamoic acid during sample loading.

The influence of the added amount of methanol on recovery was much more pronounced for rat serum than for dog serum. The reason for this is unknown, however this observation stresses that, in general, it is very important to use serum from the actual species as early as possible in the course of the development of the analytical method.

As described earlier the sample tubes were corked prior to placement in the ASPEC™ sample processor. This was done in order to prevent evaporation of methanol from the samples before extraction while standing in the sample processor, leading to decreasing recovery during assaying a series of samples. The problem with decreasing recovery during a run with uncorked sample tubes was, as expected from the above findings, more pronounced for rat serum than for dog serum.

3.4. Validation

3.4.1. Selectivity

Typical HPLC chromatograms from the analysis of extracted dog serum samples are shown in Fig. 3. The extracts from both dog and rat serum samples were generally very clean. Endogenous substances did not interfere with the analysis.

3.4.2. Linearity

Linearity was tested by analysing serum calibration standards containing known (spiked) amounts of pamoic acid at concentrations of 2, 10, 20, 40, 100

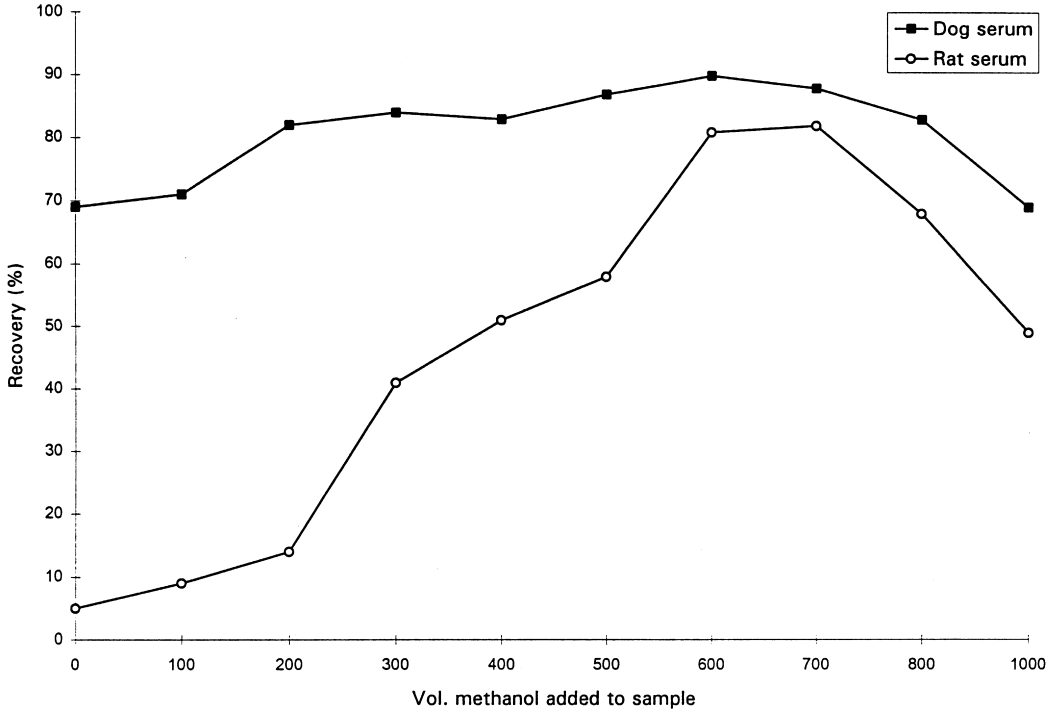

Fig. 2. Recovery of pamoic acid from 500 μl dog and rat sera versus volume of methanol added to the sample.

and 200 ng/ml. The calibration curves were found to be linear in the concentration range 2–200 ng/ml (r^2>0.990) and passed through the origin on all working days for both dog and rat serum. The back-calculated results of the above linearity study indicated good precision and accuracy (Table 2).

3.4.3. Accuracy and precision

A summary of accuracy and precision data for the test samples at all four concentrations for dog and rat sera are given in Table 3. At the 2 ng/ml serum sample level for dog samples the accuracy was 113% and the coefficient of variation was 21%. At the 5 ng/ml serum sample level the accuracy was 99.1 and 97.8% for dog and rat serum samples, respectively. The corresponding variations were 12 and 10%. At the remaining concentrations in dog and rat serum samples the accuracy is estimated to be between 96.3 and 105%. The corresponding variations were between 8.1 and 4.9%.

3.4.4. Limit of quantification

The criteria used to estimate the limit of quantification (LOQ) were a maximal coefficient of variation of 20% and a mean deviation from the nominal concentration also less than 20%. The LOQ was therefore set at 5 ng/ml for both rat and dog sera.

4. Conclusion

An analytical method using ion-pair solid-phase extraction (IP-SPE) followed by ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) for quantitative determination of pamoic acid in rat and dog sera has been developed. The present method is, to my knowledge, the first bioanalytical method described for the quantitative determination of pamoic acid at the low nanogram per milliliter serum level.

The overall performance of the HPLC method was found to be satisfactory for the purpose of determin-

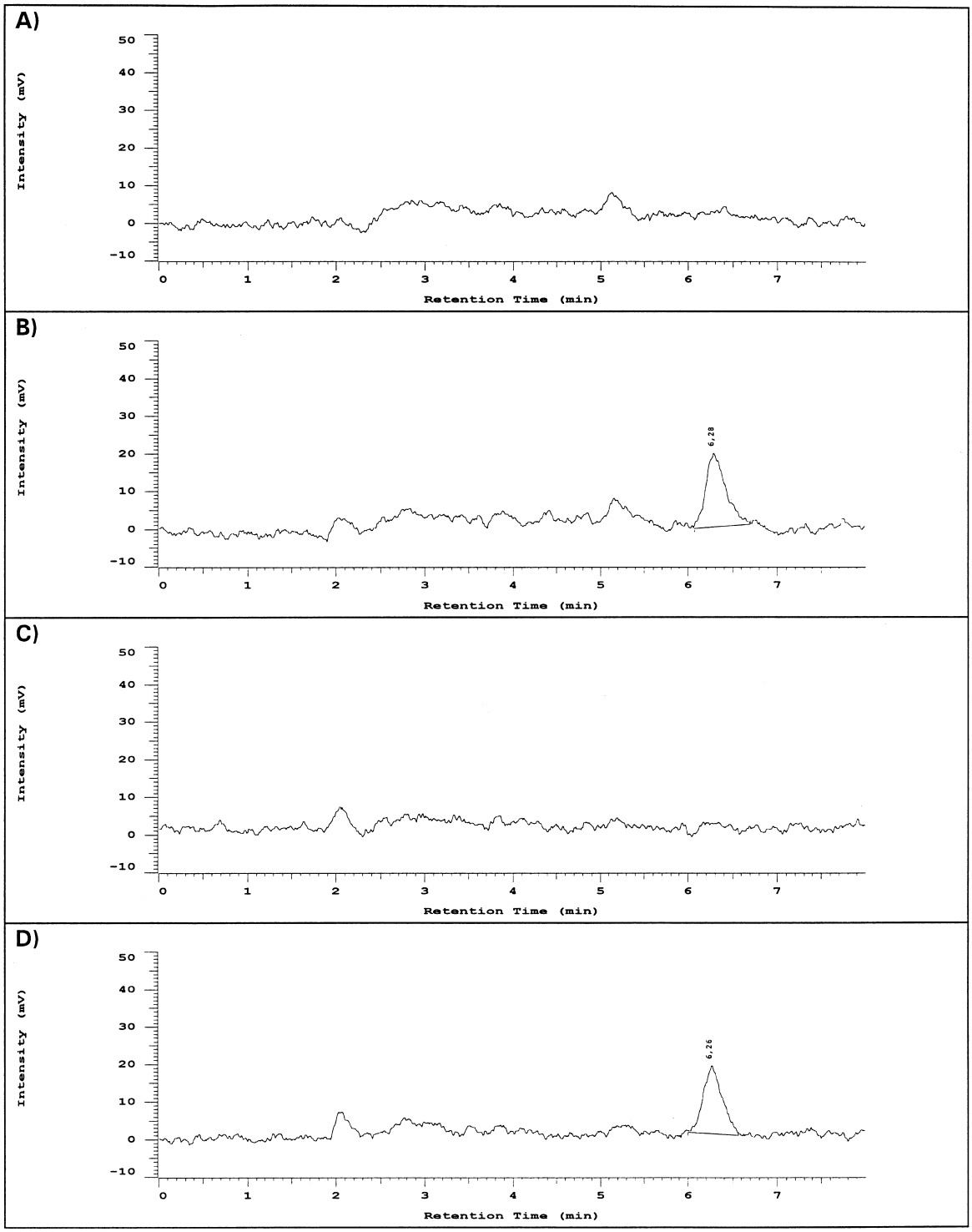

Fig. 3. Typical chromatograms from the analysis of serum test samples spiked with pamoic acid (retention time about 6.3 min). The concentration of pamoic acid was (A,B) 0 and 5 ng/ml dog serum and (C,D) 0 and 5 ng/ml rat serum.

Table 2
Linearity, accuracy and precision over the analytical range 2–200 ng/ml

Day	Species	Correlation coefficient (r^2)	Pamoic acid concentration (ng/ml)					
			2	10	20	40	100	200
1	Dog	0.9961	1.82	9.68	18.7	39.5	98.2	211
			1.74	9.78	17.7	39.1	92.2	194
	Rat	0.9989	1.78	9.84	18.9	41.4	100	205
			1.66	9.66	18.9	39.8	99.1	195
2	Dog	0.9979	1.40	10.1	19.1	42.7	97.4	194
			2.84	9.38	19.0	42.8	96.8	207
	Rat	0.9945	1.74	9.50	18.8	41.2	94.6	188
			1.58	9.14	19.8	41.8	102	212
3	Dog	0.9991	2.18	9.28	18.3	35.4	100	201
			2.34	8.80	18.0	41.7	96.1	202
	Rat	0.9983	1.94	9.72	18.5	38.5	107	195
			1.82	9.60	19.2	38.6	99.7	202
Mean	Dog		2.05	9.50	18.5	40.2	96.8	201
	Rat		1.75	9.58	19.0	40.2	100	199
SD	Dog		0.51	0.44	0.56	2.81	2.61	6.93
	Rat		0.13	0.24	0.45	1.43	4.16	8.60
CV (%)	Dog		25	4.7	3.0	7.0	2.7	3.4
	Rat		7.2	2.5	2.3	3.6	4.1	4.3
Accuracy (%)	Dog		103	95	95	101	97	101
	Rat		88	96	92	101	100	100

Table 3
Quantitative determination of pamoic acid in dog and rat sera. Accuracy and precision

Concentration (ng/ml)	Dog serum			Rat serum		
	Accuracy (%)	Precision		Accuracy (%)	Precision	
		SD	CV (%)		SD	CV (%)
2	113	0.46	21	–	–	–
5	99.1	0.58	12	97.8	0.51	11
10	–	–	–	96.3	0.71	7.4
40	102	2.06	5.0	105	2.06	4.9
160	104	9.64	5.8	104	13.3	8.1

ing the concentrations of pamoic acid in serum samples from pharmacokinetic measurements of pamoic acid in dog and rat.

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