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Short Communication

A reversed-phase ion-pair liquid chromatography method for the determination of 4-aminopyridine in Sprague–Dawley rats

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Introduction

4-Aminopyridine (4-AP) is a potassium channel blocker which has been used to treat several neurological conditions, including myasthenia gravis [1], and the Eaton–Lambert syndrome [2]. At high doses, it is a potent convulsant. Rats given 5 mg kg^{-1} of 4-AP demonstrated seizures and 'wet-dog' shakes which were potentiated by nifedipine [3]. In a rat model of CNS demyelination, all rats developed convulsions at doses between 5.6 and 7.2 mg kg^{-1} [4]. In the rat hippocampal slice preparation, 4-AP was noted to produce epileptiform activity, and to attenuate the anti-epileptic effect of carbamazepine [5].

We are interested in exploring the relationship between the pharmacodynamics of the convulsant activity of 4-AP and its pharmacokinetics in Sprague–Dawley rats. To measure 4-AP concentrations in rat plasma, a sensitive and specific ion-pair reversed-phase LC method employing liquid extraction was developed.

Materials and Methods

Reagents

All reagents used were of HPLC grade. Trifluoroacetic acid (TFA), 1-decane sulphonic acid (1-DSA), and 4-aminopyridine were obtained from Sigma (St Louis, MO). Propranolol HCl was obtained from Ayerst (Philadelphia, PA). Iso-disc® P-32 syringe filters were obtained from Supelco (Bellefonte, PA). Blank, non-sterile, mixed-sex rat plasma was obtained from Hilltop Lab Animals (Scottdale, PA).

Chromatographic equipment

The LC system consisted of a Model 6000A Solvent Delivery System set at a flow rate of 1.1 ml min^{-1}, a Lambda-Max variable wavelength detector set at 260 nm, and a WISP 710B auto-injector, all from Waters (Milford, MA). The integrator used was a Hewlett–Packard 3394A. The column was a reversed-phase cyanopropylmethylsilyl column with 5 μm size packing (Supelco, Bellefonte, PA). A Supelguard LC–CN guard column was used throughout.

The mobile phase consists of acetonitrile–1-DSA (0.5 mM)–KH_2PO_4 (0.025 M) (2:1:1, v/v/v). The pH* was then adjusted to 7.0 using 1 N NaOH. The mobile phase was then filtered through a 0.45 μm Nylon filter and de-gassed by vacuum and sonication before using.

Standards

Standards were prepared by spiking rat plasma with 4-AP at the following concentrations (ng ml^{-1}): 1980, 1485, 990.0, 495.0, 247.5, 123.8 and 61.9. A second set of plasma samples were prepared from a different stock solution in the following concentrations: 1110.0, 131.3 and 65.6 ng ml^{-1}. These samples

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served as unknowns' and were processed along with the standards during the 3 day validation. These 'unknowns' were prepared fresh each day of the validation. The internal standard used was propranolol. The working internal standard (WIS) was made up in deionized water to a concentration of 0.01 mg ml^{-1}.

Extraction method

In a 13 × 100 mm screw-top tube 200 μl of plasma standard or unknown was mixed with 200 μl of WIS and 200 μl of 1 N NaOH. To each tube was added 2 ml of ethyl acetate. The tubes were shaken horizontally for 20 min, then centrifuged for 10 min at 1500 rpm. The organic layer was transferred to a 12 × 75 mm tube and 200 μl of 0.5 M TFA in methanol was added to each sample or unknown to prevent any evaporation of 4-AP. The standards/unknowns were then dried over nitrogen in a 50°C waterbath for 10 min. The samples were then reconstituted with 100–200 μl of mobile phase. Each sample was filtered through a 0.2 μm Iso-disc® P-32 syringe filter into the auto-injector vial. The volume injected was 60 μl.

The efficiency of the extraction procedure was determined by extracting high and low plasma standards (five each) and comparing them to standards made up in methanol but not extracted.

Validation procedure

A 3 day validation procedure was performed to determine the precision and accuracy of the method. On each day three sets of standards and three sets of 'unknowns' were extracted and injected onto the system. A blank plasma sample was also extracted and injected each day. The first standard curve was subjected to weighted linear least-squares regression using a weighting of 1/conc., and the concentrations of the other standards and 'unknowns' were calculated using the resulting equation. Inter- and intra-day precision was expressed as mean, standard deviation, and relative standard deviation (RSD) for both standards and 'unknowns'. Inter- and intra-day accuracy was expressed as percentage deviation from the spiked value using the following equation:

$$\text{Predicted error} = ((C_{\text{mean obs.}} - C_{\text{spiked}})/C_{\text{spiked}}) \times 100\%,$$

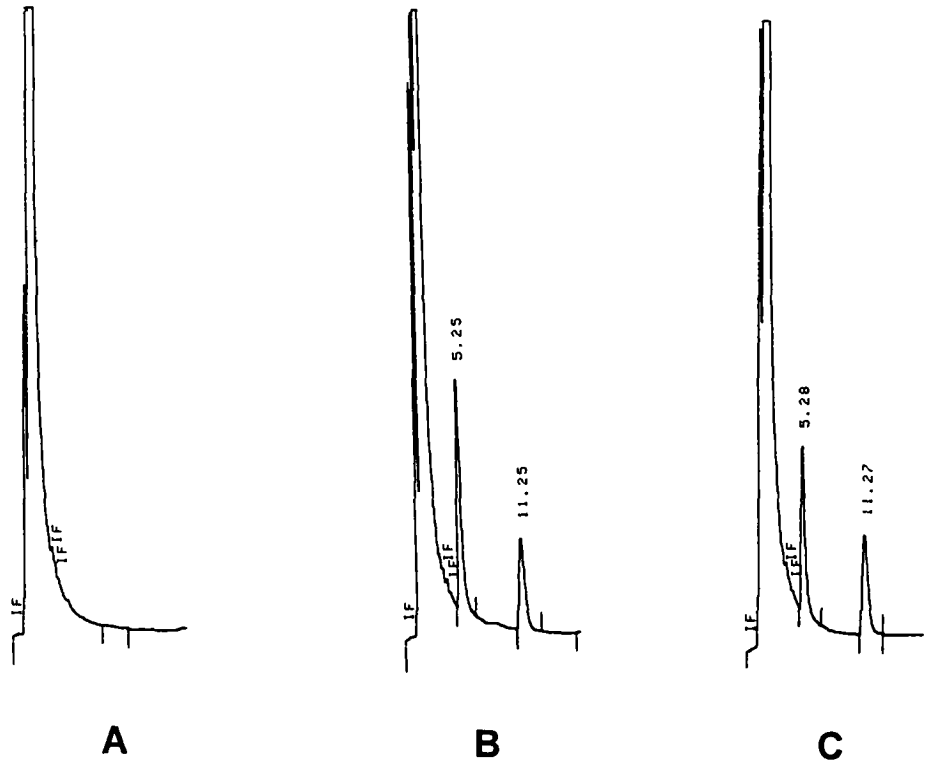

Figure 1
Typical chromatograms using the described method. (A) Blank rat plasma. (B) Rat plasma spiked with 4-AP. (C) Sample from rat given 4-AP 2 mg kg^{-1} iv. Retention time for 4-AP is at 5.25–5.28 min, internal standard peak is at 11.25–11.27 min.

where $C_{\text{mean obs.}}$ is the mean observed concentration for each standard or 'unknown' and C_{spiked} is the spiked concentration. Acceptable variability in the precision of the method was set at RSD ≤ 10% at the high end of the standard curve and ≤20% at the low end. Acceptable accuracy was a predicted error ≤10% from the spiked value.

Stability

To determine stability, plasma was spiked with 4-AP to give concentrations of 1000, 500, 250, 125 and 62.5 ng ml^{-1}. These samples were thawed, left on the lab bench at room temperature for 2–4 h and re-frozen six times over a period of 5 weeks. They were then extracted and compared with identical plasma solutions freshly spiked just prior to the analysis.

Results

Figure 1 shows typical chromatograms obtained using this method. The blank plasma is free of any intereferences with 4-AP or the internal standard. The 4-AP and the internal standard peaks are well-separated and are reasonably symmetric.

All standard curves fitted a linear model over the range of concentrations used, with a mean slope of 8.28×10^{-4} (SE = 4.65×10^{-5}, $n = 3$) and a mean intercept of 1.2×10^{-2} (SE = 4.48×10^{-3}, $n = 3$). Predicted concentrations were within 10% of spiked concentrations. The coefficients of determination ranged from 0.9973 to 0.9992.

Table 1 shows the intra-day precision and accuracy for the 3 day validation. Both standards and 'unknowns' are quantified well, with RSDs ranging from 0.4 to 8.2%. The method is also accurate, with predicted errors well within 10%. Table 2 shows the inter-day precision and accuracy for the 3 day validation. Again, the method is shown to be both precise and accurate. The lowest standard (61.9 ng ml^{-1}) had a % RSD of 6.1 and a predicted error of 3.8%; this is taken as the limit of quantification.

Figure 2 shows the results of the stability study. No degradation was noted after the spiked plasma samples had been thawed and refrozen over a period of 5 weeks. Spiked standards were generally used within 1 month of preparation.

The extraction efficiency for the method ranged from 79.6% (±2.4%) at a concen-

Table 1
Intra-day precision and accuracy for both standards and unknowns

Conc. (ng ml^{-1})	Day	Mean conc.* (ng ml^{-1})	SD	RSD (%)	Error (%)
Standards					
1980.0	1	2012.8	61.4	3.0	1.7
	2	2047.1	40.4	2.0	3.4
	3	2081.8	63.2	3.0	5.1
1485.0	1	1517.4	32.8	2.2	2.2
	2	1528.6	26.6	1.7	2.9
	3	1527.2	18.7	1.2	2.8
990.0	1	957.9	31.5	3.3	−3.2
	2	1000.1	42.2	4.2	1.0
	3	1019.6	47.1	4.6	3.0
495.0	1	471.4	2.3	0.5	−4.8
	2	472.8	15.6	3.3	−4.5
	3	467.0	13.1	4.6	−5.7
247.5	1	252.7	4.7	1.9	2.1
	2	237.3	5.7	2.4	−4.1
	3	242.5	2.6	1.1	−2.0
123.8	1	130.3	3.5	2.7	5.3
	2	126.8	2.5	2.0	2.5
	3	124.8	2.4	1.9	0.9
61.9	1	66.5	5.2	7.9	7.6
	2	63.2	3.6	5.7	2.1
	3	62.4	0.3	0.4	0.8
Unknowns					
1110.0	1	1169.6	23.1	2.0	5.4
	2	1219.8	10.3	0.8	9.9
	3	1100.3	15.4	1.4	0.9
131.3	1	130.9	0.5	0.4	−0.3
	2	131.2	4.5	3.4	−0.1
	3	126.0	4.5	3.6	−4.0
65.6	1	69.3	4.7	6.8	5.6
	2	69.8	3.9	5.7	6.4
	3	70.6	5.8	8.2	7.7

*$n = 3$ for all.

Table 2
Inter-day precision and accuracy for standards and unknowns

Conc. (ng ml^{-1})	Mean conc.* (ng ml^{-1})	SD	RSD (%)	Error (%)
Standards				
1980.0	2047.2	48.2	2.4	3.4
1485.0	1516.9	24.3	1.6	2.1
990.0	992.5	44.7	4.5	0.3
495.0	470.4	10.6	2.2	−5.0
247.5	244.2	7.8	3.2	−1.3
123.8	127.3	3.4	2.7	2.9
61.9	64.2†	3.9	6.1	3.8
Unknowns				
1110.0	1163.2	54.0	4.6	4.8
131.3	129.1	3.9	3.0	−1.6
65.6	69.9	4.3	6.1	6.5

*$n = 9$, †$n = 8$.

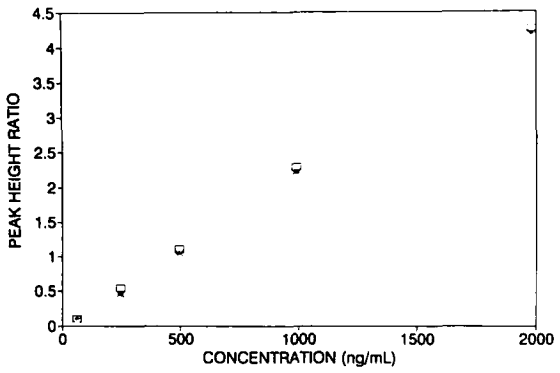

Figure 2
Results of stability study. No degradation is seen after repeated freezing and thawing over 5 weeks. *, Fresh standards; □, thawed/frozen.

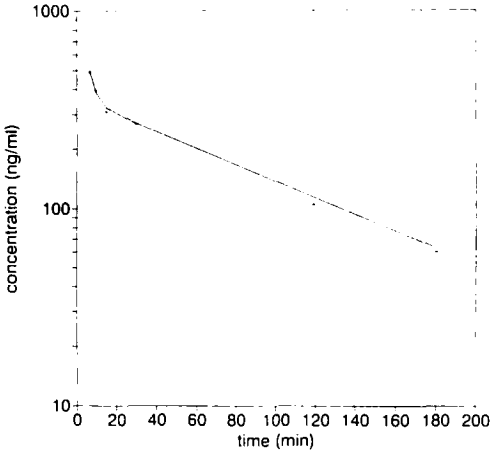

Figure 3
Typical plasma concentration vs time curve obtained from a rat given 2 mg kg^{-1} iv. Observed data points are denoted by asterisks; the line is the best-fit line obtained from least-squares regression.

tration of 1980.0 ng ml^{-1} to 80.7% (±9.1%) at a concentration of 61.9 ng ml^{-1}. The extraction efficiency for the internal standard was 84.4% (±3.1%).

Several anticonvulsant drugs and an antispasmodic were checked for interference with 4-AP or the internal standard, since they may be used in future EEG studies. Solutions of baclofen, carbamazepine, diazepam, phenobarbital, and phenytoin in methanol at 10 μg ml^{-1} were injected onto the system. No peaks were seen at the retention times of either 4-AP or the internal standard.

Male Sprague–Dawley rats (300–400 g, n = 7) were obtained from Hilltop Lab Animals (Scottsdale, PA) and were injected intravenously with 2 mg kg^{-1} of 4-AP dissolved in heparinized saline. Samples at 5, 10, 15, 30, 60, 120 and 180 min were taken via a jugular vein cannula. The blood was spun down and the plasma analyzed by the described method. A typical plasma concentration vs time curve is shown in Fig. 3.

Discussion

A precise, accurate and sensitive HPLC method for the determination of 4-AP in rat plasma is described. The extraction method is straightforward and gives reproducible recovery. The method is reasonably rugged and requires no special equipment or techniques.

Comparison of our method with previous methods found in the literature is difficult, primarily because such an extensive validation is not described. Ray *et al.* [6] described a method for the determination of 4-AP in the stomach contents of horses, but gives little information about the validation of their assay. Uges and Bouma [7] described a reversed-phase method and claimed a sensitivity of 5 ng ml^{-1}, but provided insufficient data to support this claim, reporting only the mean correlation coefficient from standard curves prepared on different days. Shinohara and Miller [8] developed a reversed-phase ion pair method and claims a quantification limit of 1 ng ml^{-1}, but intra- and inter-day studies were done only on serum containing 98.4 ng ml^{-1} of 4-AP. Finally, Lamiable and Millart [9] used a reversed-phase method using protein precipitation as a sample clean-up method. Data for within-run precision was obtained by analysing serum containing 5 mg l^{-1} 4-AP; such a high level is unlikely to be obtained *in vivo*. They claim a limit of detection of 50 ng ml^{-1}, but there is no indication that this is the limit of quantification.

In conclusion, we have developed a sensitive and specific HPLC assay for measuring 4-AP in rat plasma. Our method is simple and rugged, and has been extensively validated.

Conclusions

A reversed-phase ion-pair LC method for the analysis of 4-aminopyridine (4-AP) in Sprague–Dawley rats has been described. Sample preparation was by liquid extraction. UV detection was performed at 260 nm. The method was precise, with inter-day RSD ranging from 0.3 to 3.8%, and intra-day RSD

ranging from 0.5 to 7.9%. Accuracy for the method was excellent, with predicted errors less than 8%. The limit of quantification is 61.9 ng ml^{-1}.

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