ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 403–409

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

High performance liquid chromatographic analysis of a novel aminoalkylpyridine anticonvulsant 2-(4-chlorophenyl)amino-2-(4-pyridyl)ethane

Ge Lin [a,*], Ivo P. Nnane [a,b], Lyaquatali A. Damani [b], Kai K. Tse [b], Albert H.L. Chow [b], Pankaja K. Kadaba [c]

[a] *Department of Pharmacology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong*
[b] *Department of Pharmacy, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong*
[c] *College of Pharmacy, Chandler Medical Centre, University of Kentucky, Lexington, KY 40536-0082, USA*

Received 11 November 1997; received in revised form 10 February 1998; accepted 21 February 1998

Abstract

A simple, rapid and specific high performance liquid chromatographic (HPLC) method for the quantitation of 2-(4-chlorophenyl)amino-2-(4-pyridyl)ethane (AAP-Cl) and identification of its putative metabolite, 2-(4-chlorophenyl)amino-2-(4-pyridyl)ethanol (β-AA) in rat blood and urine has been developed. AAP-Cl, β-AA and an appropriate internal standard were extracted from rat biofluids by a solid phase extraction technique using C18 cartridges prior to the HPLC analysis. The extractibility was 92% for AAP-Cl and 98% for β-AA. The HPLC analysis employed a symmetrical or standard reversed-phase HPLC column (Apex ODS, 5 μm, 25 cm × 0.46 cm) for blood or urine analysis, a mobile phase of water–methanol–acetonitrile (40:30:30) containing 20 μl 100 ml^{-1} diethylamine at a flow rate of 1 ml min^{-1}, and UV detection at 254 nm. The limit of detection was 100 ng ml^{-1} for both analytes in both blood and urine. The calibration curves for AAP-Cl in rat biofluids were shown to be linear in both low and high concentration ranges (blood: 0–1 and 1–10 μg ml^{-1}; urine: 0–10 and 10–100 μg ml^{-1}) with intra- and inter-day coefficients of variation of no more than 18% for blood and 14% for urine. The method developed was successfully applied to a preliminary analysis of intact AAP-Cl in both blood and urine obtained from rats dosed with AAP-Cl.

Keywords: Aminoalkylpyridine anticonvulsant; HPLC analysis; AAP-Cl

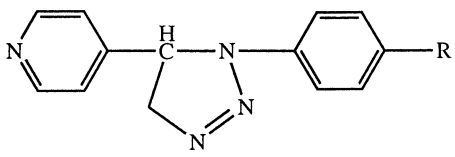
1. Introduction

Epilepsy is one of the leading neurological disorders with an estimated 20–40 million people worldwide suffering from some forms of this disease. The currently available antiepileptic drugs, irrespective of their mechanism of action, are unable to control seizures in as many as 20% of epileptic cases [1]. Thus, there is a need to develop new antiepileptic agents with reduced toxicity and enhanced efficacy.

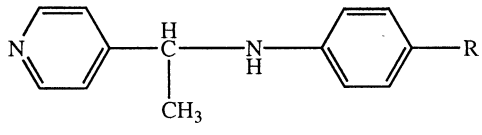
* Corresponding author. Tel.: + 852 26096824; fax: + 852 26035139; e-mail: linge@cuhk.edu.hk

0731-7085/98/$ - see front matter
PII S0731-7085(98)00097-1

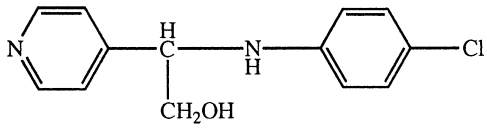
Studies by Kadaba [2,3] have led to the discovery of the triazoline heterocycles as a potentially unique family of anticonvulsant agents. Neurochemical studies suggest that 1-(4-chlorophenyl) amino-5-(4-pyridyl)-1,2,3-triazoline (TAZ-Cl, Fig. 1), a representative member of the triazoline heterocycles, may exert its anticonvulsant activity in a unique 'dual action' mechanism, while its active metabolite, 2-(4-chlorophenyl)-amino-2-(4-pyridyl)ethanol, the primary β-amino alcohol (β-AA, Fig. 1), appears to block postsynaptic excitatory amino acid (EAA) receptors, the parent triazoline impairs presynaptic release of L-glutamate and L-aspartate, the transmitters for the EAA receptors [4–6].



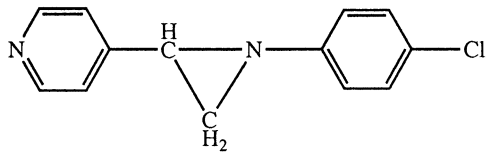
Triazoline
TAZ-Cl (R = Cl)



AAPs
AAP-Cl (R = Cl)



ß-AA



AZI-Cl

Fig. 1. Structures of TAZ-Cl, AAP-Cl, β-AA and AZI-Cl.

The identification of the pharmacophore [4] of the unique triazoline anticonvulsants has led to the development of aminoalkylpyridines (AAPs) as a new generation of potential anticonvulsants. In AAPs, the hydroxylmethyl group of the β-AA is replaced by a methyl group (Fig. 1), thus, they are likely to undergo biotransformation to generate pharmacologically active β-amino alcohol metabolites similar to those produced by triazolines in the body [7]. 2-(4-Chlorophenyl)-amino-2-(4-pyridyl)ethane (AAP-Cl, Fig. 1), a representative member of this new class of potential anticonvulsant agents, has been demonstrated to be orally active and more potent than the triazolines for seizure protection in both mice and rats [7]. It has a long duration of action and no apparent signs of motor toxicity [7]. Furthermore, AAPs do not show the inherent instability as exhibited by triazolines in neutral and acidic medium [8].

Since the AAPs are a novel class of potential anticonvulsant agents, no data on their analytical methods have been reported. This paper reports the development of an high performance liquid chromatographic (HPLC) assay that simultaneously analyzes AAP-Cl and its putative metabolite β-AA in rat biofluids. A preliminary application of the developed HPLC method to the quantitation of AAP-Cl in both blood and urine samples obtained from rats dosed with AAP-Cl is also described.

2. Experimental

2.1. Chemicals

AAP-Cl, β-AA and the internal standard for HPLC analysis, 1-(4-chlorophenyl)-2-(4-pyridyl)-aziridine (AZI-Cl, Fig. 1), were synthesized as previously described [7]. Methanol, acetonitrile and diethylamine (HPLC grade) were purchased from Riedel-de Häen (Seelze, Germany). All other chemicals were of analytical grade and obtained

from BDH (Dorset, UK). Delory and King's carbonate and bicarbonate buffer (pH 10) was freshly prepared by mixing 21.3 ml anhydrous sodium carbonate (0.2 M) with 3.8 ml sodium bicarbonate (0.2 M) in 100 ml distilled water. C18 cartridges for solid phase extraction were purchased from International Sorbent Technology (Hengoad, UK).

2.2. HPLC conditions

The HPLC system consisted of an ISCO liquid chromatograph (model 23150) with a gradient programmer (model 2361) (ISCO, Lincoln, USA). A symmetrical ODS 5 μm (25 cm × 0.46 cm) column (Jones Chromatography, Hengoed, UK) was employed for blood samples, while a standard Apex ODS 5 μm (25 cm × 0.46 cm) column (Jones Chromatography) was used for urine. The mobile phase consisted of methanol–acetonitrile–water (30:30:40 v/v/v) and diethylamine (20 μl 100 ml^{-1}) eluted at 1 ml min^{-1}. The mobile phase was filtered and degassed prior to use. Eluents were detected by an ISCO V® variable wavelength UV–vis detector at 254 nm. Data manipulation was conducted by ISCO ChemResearch 150 software. The HPLC analysis was performed at ambient temperature.

2.3. Protocol

Male Sprague-Dawley rats (body weight 250–300 g) were supplied by the Animal House at The Chinese University of Hong Kong. The rats were divided into three groups with five in each group. The first group of rats were cannulated in the jugular vein and carotid artery aseptically under light ether anaesthesia. The animals were allowed to recover overnight before administration with a single intravenous dose of AAP-Cl (20 mg kg^{-1} in 50% β-cyclodextrin) via the jugular vein. Blood samples (0.25 ml) were collected from the carotid artery cannula before dosing and at selected intervals for up to 5 h after administration. An equal volume of heparinised saline (0.25 ml) was given to the rats following each blood collection. Blood was collected into a test tube containing heparinised saline (100 I.U., 15 μl) and Delory and King's buffer (0.75 ml) was added immediately.

The rats in the second group were dosed with AAP-Cl (100 mg kg^{-1} in 50% β-cyclodextrin) intraperitoneally, and placed in individual metabolic cages. Food and water were allowed ad libitum. Urine samples (0–24 and 24–48 h) were collected from the individual dosed rat. The third group of rats, which served as the control group, was treated with 50% β-cyclodextrin only. Blank urine samples were collected, and subsequently the animals were sacrificed and blank blood was obtained by cardiac puncture. The collected urine and blood samples were stored at 20 and 4°C, respectively, prior to analysis.

2.4. Preparation of calibration samples

AAP-Cl and AZI-Cl stock solutions were prepared in methanol (1 mg ml^{-1}) and stored at 4°C. Diluted solutions of 10 and 100 μg ml^{-1} in methanol were prepared from this stock for use as calibration standards. The samples were further diluted to cover both low and high concentration ranges (blood: 0–1 and 1–10 μg ml^{-1}; urine: 0–10 and 10–100 μg ml^{-1}). Briefly, aliquots of the stock solutions of AAP-Cl and AZI-Cl (internal standard) were spiked into the blank rat blood (0.25 ml) or urine (0.5 ml) samples, and mixed with appropriate amounts of Delory and King's buffer (pH 10) to make up to a total volume of 1 ml. Quantities of the internal standard used were equivalent to the highest concentrations of the analyte in each calibration range. Each sample was then subjected to a solid phase extraction by being loaded onto a C18 cartridge pre-washed with 2 ml methanol followed by 2 ml distilled water. The cartridge was washed with distilled water (2 ml) and then eluted with methanol (2 ml). The methanol eluate was evaporated to dryness, and the residue was reconstituted with 100 μl of the HPLC mobile phase before chromatographic analysis (5 μl for each injection). Peak area ratios (APP-Cl:internal standard) were plotted against concentrations of AAP-Cl to yield the blood and urine calibration curves.

Table 1
Intra- and inter-day variability for the assay of AAP-Cl spiked in rat blood and urine

Concentration (μm ml^{-1})	Intra-day			Inter-day		
	Found	C.V. (%)	Accuracy (%)	Found	C.V. (%)	Accuracy (%)
Spiked blood ($n=3$)						
0.2	0.18 ± 0.006	3.3	10.0	0.23 ± 0.04	17.4	15.0
0.8	0.67 ± 0.03	3.7	16.2	0.87 ± 0.09	10.6	8.8
2.0	1.73 ± 0.17	9.8	13.5	2.22 ± 0.35	15.8	11.0
4.0	3.92 ± 0.48	12.2	2.0	4.34 ± 0.56	12.9	8.5
Spiked urine ($n=3$)						
2.0	2.10 ± 0.03	1.7	5.0	2.28 ± 0.32	14.0	14.0
8.0	7.74 ± 0.14	1.8	3.2	8.10 ± 0.56	6.9	1.3
25	22.55 ± 1.19	5.3	9.8	24.2 ± 1.78	7.4	3.2
75	81.15 ± 4.44	5.5	8.2	75.3 ± 1.64	2.2	0.4

CV (%) = (S.D./mean) × 100; accuracy (%) = ((mean measured concentration − spiked concentration)/spiked concentration) × 100.

2.5. Preparation of test samples

2.5.1. Blood

Aliquots (0.25 ml) of blank blood and blood obtained from the rats dosed with AAP-Cl were mixed with 0.75 ml Delory and King's buffer (pH 10) containing an appropriate quantity of the internal standard, and then extracted and analyzed as described for the calibration samples.

2.5.2. Urine

Both test and blank urine samples were mixed with an aliquot (0.50 ml) of Delory and King's buffer (pH 10) containing an appropriate amount of the internal standard, and then subjected to solid extraction prior to HPLC analysis as described above.

2.6. Validation studies

2.6.1. Stability of APP-Cl in rat blood and urine

To determine the stability of AAP-Cl in rat blood and urine, an aliquot (10 μg) of AAP-Cl was spiked into the blood (0.25 ml) or urine (0.5 ml) sample, to which was added an aliquot (0.75 ml for blood and 0.5 ml for urine) of Delory and King's buffer (pH 10). The samples were then stored at 4°C, and analysed at various intervals over a period of 12 weeks using the extraction and analytical procedures as described above.

2.6.2. Recovery

Known amounts of AAP-Cl and β-AA with either low (0.2 μg ml^{-1} for blood and 2.0 μg ml^{-1} for urine) or high levels (4.0 μg ml^{-1} for blood and 50 μg ml^{-1} for urine) were spiked into the biological samples. The extraction recoveries of both AAP-Cl and β-AA were determined by comparing the HPLC peak area of each analyte obtained after solid phase extraction from the spiked samples with that obtained from the appropriate standard solution without extraction.

2.6.3. Accuracy and precision in the quantitation of AAP-Cl

The measurements of intra- and inter-day variability were utilized to determine the precision and accuracy of the developed assay. Known quantities of AAP-Cl (0.2, 0.8, 2.0 and 4.0 μg for blood and 2.0, 8.0, 25.0 and 75.0 μg for urine) were added to blood (0.25 ml) or urine (0.5 ml), mixed with an aliquot (0.75 ml for blood or 0.5 ml for urine) of Delory and King's buffer (pH 10) containing appropriate concentrations of the internal standards, and the resulting samples were analyzed on three separate days. The coefficient of variation (CV) was taken as a measure of assay precision and the percentage difference between amounts determined and amounts spiked as a measure of accuracy (Table 1).

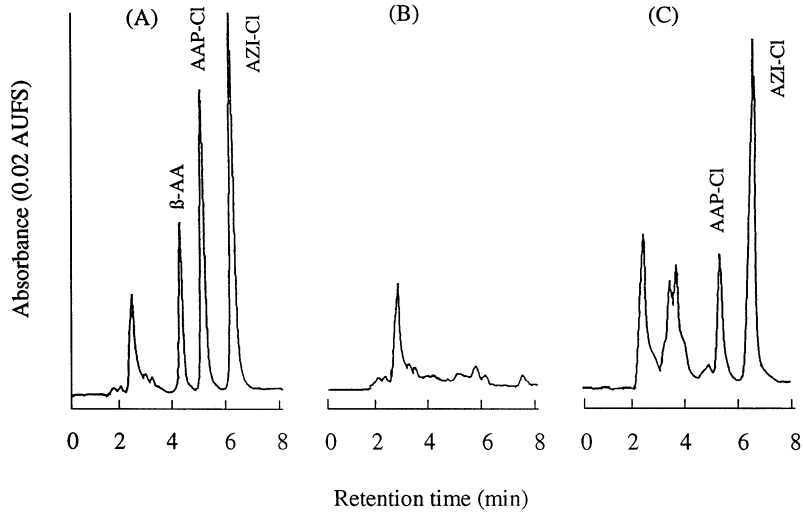


Fig. 2. HPLC chromatograms of the extracts of (A) blank blood with addition of the standards, (B) blank rat blood and (C) 10 min blood sample of a rat administered with AAP-Cl (C). AUFS, absorbance units full scale.

2.6.4. Limits of detection

Aliquots of APP-Cl and β-AA were spiked into the blank blood or urine samples to afford concentrations ranging from 10 to 1000 ng ml^{-1}. The samples were extracted and analyzed using the procedures described above. The limit of detection for each analyte was determined when the signal of the testing peak to noise ratio was higher than 5.

3. Results and discussion

The solid phase extraction allowed the separation of the analytes from endogenous interfering substances. This preparation procedure simplified the liquid–liquid extraction for the analysis of triazoline anticonvulsants as previously developed by us [9]. The recoveries of AAP-Cl and β-AA from both blood and urine samples were 92 ± 3.0 and $98 \pm 2.0\%$, respectively. Satisfactory separation of AAP-Cl, β-AA and the internal standard was achieved by using a symmetrical (blood) or standard (urine) Apex ODS 5 μm HPLC column with the mobile phase of water–methanol–acetonitrile (40:30:30 v/v/v) containing 20 μl 100 ml^{-1} diethylamine. Representative chromatograms for the analysis of blood and urine samples spiked with standards are shown in Fig. 2A and Fig. 3A, respectively, all of which exhibited sharp and symmetrical peaks for the analytes. The limit of detection for both analytes was 100 ng ml^{-1} in both urine and blood.

For the quantitation of AAP-Cl in biofluids, linear calibration curves were obtained at low and high concentration ranges of AAP-Cl in both blood and urine samples. The linear regression equations for AAP-Cl over the stated concentration ranges were determined to be: blood (0–1 μg ml^{-1}), $y = 2.4x + 0.007$ ($r^2 = 0.999$, mean CV 6.6%); blood (1–10 μg ml^{-1}), $y = 0.25x + 0.02$ ($r^2 = 0.999$, mean CV 2.2%); urine (0–10 μg ml^{-1}), $y = 0.21x + 0.09$ ($r^2 = 0.997$, mean CV 5.4%); urine (10–100 μg ml^{-1}), $y = 0.25x + 0.02$ ($r^2 = 0.997$, mean CV 1.6%). The assays were reproducible at both low and high concentrations with overall intra- and inter-day variations less than 18 or 14% (Table 1), respectively. The accuracy of the assay was acceptable with a range of 84–98% for blood and 86–98% for urine samples (Table 1). Moreover, there was no significant change in the concentration of AAP-Cl after prolonged storage at 4°C, indicating that AAP-Cl is highly stable in both blood and urine and the developed HPLC assay can be performed within at least 3 months.

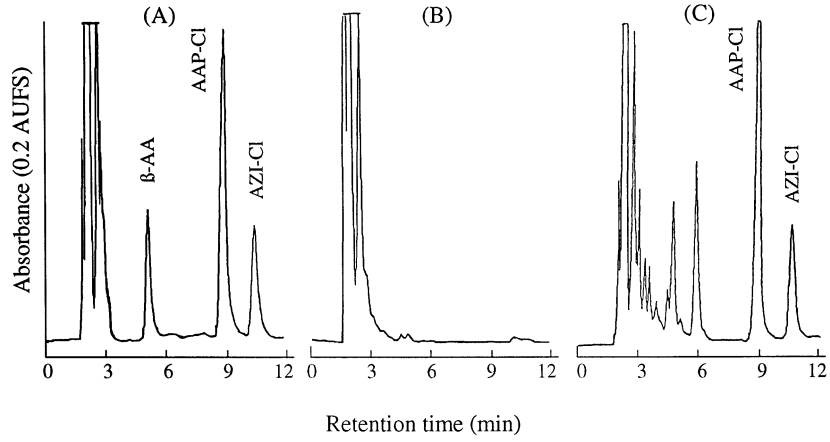


Fig. 3. HPLC chromatograms of extracts of (A) blank urine with addition of the standards, (B) blank rat urine and (C) 0–24 h urine from a rat administered with AAP-Cl (C). AUFS, absorbance units full scale.

The developed HPLC assay was subsequently applied in a preliminary pharmacokinetic and metabolic study of AAP-Cl in rats. A representative HPLC chromatogram of a blood sample obtained from a rat dosed with AAP-Cl is shown in Fig. 2C. Blood samples collected at different time intervals after intravenous administration were determined for AAP-Cl and β-AA. Fig. 4 shows the blood concentration–time profile of AAP-Cl with an elimination half life ($t_{1/2}$) of 3.0 ± 0.4 h. Unlike the case with the triazoline anticonvulsants, there was no detectable amount of the putative β-AA metabolite present in the blood up to 5 h after administration of AAP-Cl. Analysis of urinary samples revealed that 1–2% of the unchanged AAP-Cl was excreted in the urine after i.p. administration, and most of them excreted within 0–24 h after administration with only traces detected in the 24–48 h urine. As with the blood samples, no β-AA could be detected. However, there appeared to be metabolites present in both blood and urine samples, as indicated by the extra peaks (Fig. 2C and Fig. 3C which were not exhibited in the HPLC chromatograms of the blank samples (Fig. 2B and Fig. 3B). A more detailed investigation into these metabolites of AAP-Cl is being conducted and will be reported separately. Since our preliminary metabolic studies in rats failed to show the presence of β-AA (the postulated active metabolite of AAP-Cl), HPLC quantitation of this putative metabolite was not necessary and thus was not reported here.

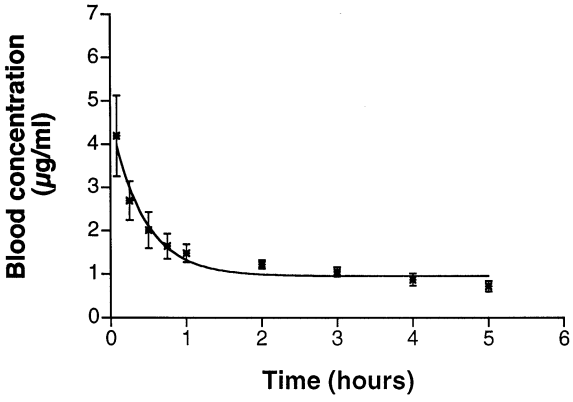


Fig. 4. Blood concentration–time profile of APP-Cl following a single intravenous dose (20 mg kg^{-1}) of AAP-Cl in rats ($n = 5$).

In conclusion, a simple, sensitive and specific HPLC assay has been developed for the novel aminoalkylpyridine anticonvulsant, AAP-Cl, in rat blood and urine. The assay is reproducible and has been fully validated. This HPLC method allows quantitation of AAP-Cl in rat blood and urine, and identification of its puta-

tive metabolite β-AA. This developed HPLC assay is currently being used to study the metabolic and pharmacokinetic profiles of AAP-Cl in various laboratory animals.

Acknowledgements

Financial support from the Research Grant Council (RGC) of Hong Kong (direct allocation to LAD and GL, Grant No. MD 94035) and a postdoctoral research fellowship to IPN from the Chinese University of Hong Kong are gratefully acknowledged.

References

[1] M.A. Rogawski, R.J. Porter, Pharmacol. Rev. 42 (1990) 223–286.
[2] P.K. Kadaba, J. Pharm. Sci. 73 (1984) 850–852.
[3] P.K. Kadaba, J. Med. Chem. 31 (1988) 196–203.
[4] P.K. Kadaba, Drugs Future 15 (1990) 1013–1204.
[5] P.K. Kadaba, J.T. Slevin, Epilepsia 29 (1988) 330–337.
[6] M.A.F. Mamelijnck, P.J. Stevenson, P.K. Kadaba, L.A. Damani, J. Pharm. Sci. 81 (1992) 100–105.
[7] T.R. Deshmukh, P.K. Kadaba, Med. Chem. Res. 3 (1993) 223–232.
[8] M.A.F. Hamelijnck, P.J. Stevenson, P.K. Kadaba, L.A. Damani, J. Pharm. Sci. 81 (4) (1992) 392–396.
[9] P.J. Stevenson, M.A.F. Hamelijnck, P.K. Kadaba, L.A. Damani, J. Chromatogr. 563 (1991) 419–426.