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High-performance liquid chromatographic assay for 1-aminocyclopropanecarboxylic acid from plasma and brain

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the analysis of 1-aminocyclopropanecarboxylic acid (ACPC) from plasma or brain tissue is described. Samples were deproteinized with perchloric acid, centrifuged, alkalinized with potassium hydroxide and recentrifuged. The supernatants were derivatized with *o*-phthaldialdehyde and injected onto a C_{18} 3-μm column (100 mm × 4 mm I.D.) pumped with 1 ml/min methanol–acetonitrile–0.1 *M* sodium phosphate buffer pH 6.0 (28:5:67, v/v). The retention times for ACPC and the internal standard were 15 and 31 min, respectively. The minimum detectable amount of ACPC was 0.08 nmol. The extraction recovery of ACPC (2.7–270 nmol) from spiked plasma or brain tissue ranged from 88 to 109%. The intra- and inter-day coefficients of variation for 27 nmol ACPC were 3.9 and 4.9%, respectively. This method was utilized to obtain preliminary pharmacokinetic parameters following ACPC administration to mice.

INTRODUCTION

1-Aminocyclopropanecarboxylic acid (ACPC) is an amino acid present in ripening fruit as an immediate precursor in the biosynthetic pathway of ethylene [1]. ACPC has been reported to bind with high affinity to the strychnine-insensitive glycine recognition site on the N-methyl-D-aspartate (NMDA) receptor complex in mammalian central nervous system [2,3]. ACPC mimics many of the pharmacological effects of both competitive NMDA antagonists (*e.g.* 2-aminophosphonoheptanoic acid; AP-7) and use-dependent cation channel blockers (*e.g.* dizocilpine, MK-801) when administered to rodents. Thus, ACPC has been reported to produce anticonvulsant [4,5], anxiolytic [6,7] and antidepressant [8,9] actions in animal models predictive of these pharmacological actions in man. Furthermore, ACPC was found to attenuate NMDA-mediated excitotoxicity in a fourteen-day embryonic chick retina model of acute neurotoxicity [10]. It was also established that chronic but not acute treatment of gerbils with ACPC significantly improved both survival and neurological outcome in a gerbil model of forebrain ischemia [11]. This latter observation, coupled with the spectrum of potential therapeutic uses of ACPC, suggests that a sensitive analytical assay is required for measurement of this compound from mammalian tissues.

While several analytical assays have been developed for the quantitation of ACPC from fruits [12–16], none is directly applicable to the analysis of ACPC from mammalian tissues. Many of these published methods are indirect, relying on the conversion of ACPC to ethylene by chemical or enzymatic means followed by the gas chromatographic analysis of ethylene [12–14]. Another method involves ion monitoring–isotope dilu-

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tion gas chromatography–mass spectrometry to detect a derivatized phthalimido ester of ACPC [15]. One method employed an ACPC phenylisothiocyanate derivatization procedure followed by high-performance liquid chromatographic (HPLC) detection [16]. This method utilized a large-scale extraction procedure to isolate ACPC from 10 g of apple pericarp. Currently, there is no available method for the microsample analysis of ACPC from mammalian matrices.

This paper describes a simple method for the extraction, derivatization, HPLC separation and quantitation of exogenously administered ACPC from mouse plasma and brain.

EXPERIMENTAL

Chemicals

ACPC was obtained from Research Organics (Cleveland, OH, USA). Cycloleucine, 2-mercaptoethanol, *o*-phthaldialdehyde (OPA) and various amino acids were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide and sodium phosphate dibasic heptahydrate were obtained from Malinckrodt (Paris, KY, USA) and sodium tetraborate from Allied Chemical (Morristown, NJ, USA). Perchloric (60–62%) and phosphoric (85%) acids were obtained from Baker (Phillipsburg, NJ, USA). Methanol and acetonitrile (HPLC grade) were purchased from Fischer Scientific (Fairlawn, NJ, USA). Adult, male (25–30 g) NIH Swiss mice were obtained from Harlan Sprague–Dawley (Frederick, MD, USA).

A stock OPA solution was prepared by dissolving 54 mg of OPA in 0.5 ml of methanol, 10 μl of 2-mercaptoethanol and 4.5 ml of 0.1 *M* borate buffer (pH 9.0). This stock solution was stable for at least three days when protected from ambient light. A working solution was prepared by diluting one part of the stock OPA solution with three parts of 0.1 *M* borate buffer (pH 9.0). This solution was prepared daily and protected from ambient light.

Sample extraction and derivatization

Tissue samples (90 μl) from ACPC-treated mice or samples from untreated animals (previously spiked with ACPC for chromatographic calibration) were thawed on the day of the assay. All samples were spiked with 10 μl of the internal standard (cycloleucine as a 3.8 m*M* stock solution) prior to freezing. Samples were denatured by the addition of 300 μl of 6.2% perchloric acid, followed by 2 min of centrifugation at 14 000 *g*. The supernatants were transferred to clean microfuge tubes, placed on ice and alkalinized by adding 1 ml of 0.4 *M* potassium hydroxide. The samples were centrifuged for 2 min at 14 000 *g* to remove precipitated potassium perchlorate. Aliquots of the supernatants (800 μl) were derivatized with 400 μl of OPA working solution for 5 min. A 1-ml aliquot of the derivatized solution was injected for HPLC analysis.

Instrumentation and chromatographic conditions

The HPLC system consisted of two Waters Model 510 pumps, a Waters Model U6K universal liquid chromatograph injector with a 2-ml loop, and a Waters Model 490E programmable multiwavelength detector. The equipment was computer-programmed with a Waters MAXIMA 820 analytical workstation. The analytical column was an Alltech C_{18} (3 μm) cartridge (100 mm × 4.6 mm I.D.), fitted with an Alltech C_{18} (5 μm) guard column cartridge (10 mm × 4.6 mm I.D.).

The mobile phase A used to elute the analyte and internal standard was methanol–acetonitrile–0.1 *M* sodium phosphate buffer pH 6.0 (28:5:67, v/v) at a flow-rate of 1 ml/min. Under these conditions, ACPC and cycloleucine eluted at 15 and 31 min, respectively. After elution of the internal standard, it was necessary to wash the column free of avidly retained endogenous compounds. The MAXIMA software was programmed for a 2-min linear gradient elution to methanol–mobile phase A (40:60, v/v), 4 min at this new mobile phase composition, followed by a 2-min linear gradient elution back to the starting mobile phase A. The column was re-equilibrated with mobile phase A for 6 min before the next run. The elapsed time between injections was 51 min.

Assay characteristics

Calibration. Analytical calibration was performed by assaying 90-μl aliquots of blank plasma or brain homogenate spiked with ACPC (2.7–270 nmol) and cycloleucine (27 nmol). Four standards spiked with different amounts of ACPC were used for each calibration curve. Separate calibration curves were constructed for plasma and brain homogenate samples. An area ratio was calculated for each sample by dividing the area of the ACPC peak by the area of the cycloleucine peak. Linear least-squares regression analysis of the area ratio of spiked standards provided slope and intercept parameters which were then used to calculate the amount of ACPC present in unknown samples.

Extraction recovery. Blank samples of plasma or brain homogenate were spiked with a known quantity of ACPC or cycloleucine, extracted and derivatized as previously described. The peak area of the spiked sample was used to calculate the extraction recovery using a calibration curve generated from the peak areas of standards prepared in water.

Intra- and inter-day variability. Intra-day variability was determined by HPLC analysis of four or five samples spiked with a known amount ACPC or cycloleucine during a single day. Inter-day variability was determined by HPLC analysis of a sample spiked with a known amount of ACPC or cycloleucine on four or five different days.

Pilot pharmacokinetic study

Male NIH Swiss mice received an intraperitoneal ACPC dose of 200 mg/kg (198 mmol/kg). Mice were anesthetized with halothane at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6 and 8 h after dosing. Abdominal aortic blood was collected into a heparinized syringe and centrifuged for 2 min at 14 000 *g* to obtain plasma. Mice were then decapitated with sharp scissors, the forebrains were removed and frozen on solid carbon dioxide. The frozen forebrains were weighed and sonicated in two volumes of distilled, deionized water. Plasma or brain homogenates (90 μl) were spiked with 10 μl of the internal standard (cycloleucine as a 3.8 m*M* stock solution), frozen on solid carbon dioxide and stored at −20°C until assayed. Preliminary pharmacokinetic parameters were calculated by standard formulas of non-compartmental statistical moment analysis [17].

RESULTS AND DISCUSSION

Chromatograms from representative plasma and brain samples are shown in Fig. 1. Using the described mobile phase, ACPC and cycloleucine eluted at 15 and 31 min, respectively, and were resolved from other known amino acids (data not shown). Plasma and brain samples spiked with ACPC were chemically stable for up to a month when stored at −20°C.

A derivatization time of 5 min was found to be optimum for an essentially complete reaction of OPA with ACPC or cycloleucine. Derivatization

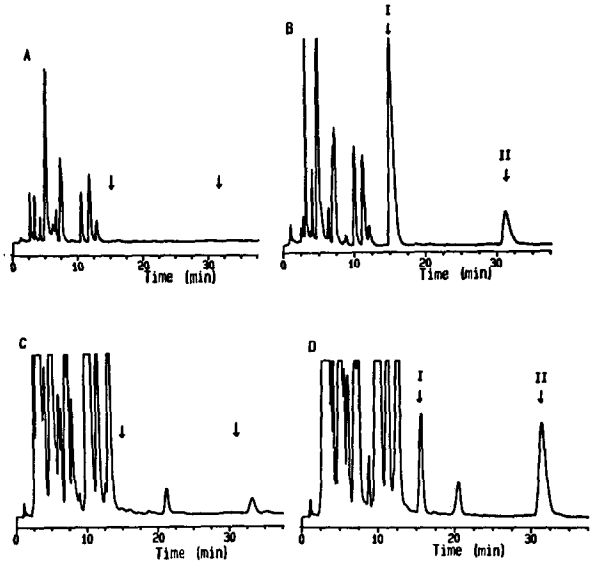

Fig. 1. Representative chromatograms of (A) blank mouse plasma, (B) plasma collected 1 h after ACPC administration to a mouse, (C) blank mouse brain and (D) brain extract collected 1 h after ACPC administration to a mouse. The concentrations of ACPC in (B) and (D) were 0.14 mg/ml (1.38 mmol/l) and 0.89 μg/mg of protein (8.8 μmol/g of protein), respectively. Arrows identify the peak retention times of ACPC (peak I; 15 min) and the internal standard, cycloleucine (peak II; 31 min). Blank mouse brain (C) contained a minor endogenous peak which co-eluted with cycloleucine. See Results and discussion for further details.

times of less than 3 min resulted in an incomplete reaction of OPA with cycloleucine, while intervals greater than 10 min resulted in the decomposition of OPA-derivatized ACPC. Consequently, samples were derivatized for 5 min and immediately assayed. The stability of OPA-derivatized ACPC constrains the use of automated autosampling injection equipment.

Calibration of the assay was linear over the range 2.7–270 nmol of ACPC for both spiked plasma and brain homogenate. The extraction recovery of 2.7–270 nmol of ACPC from plasma and brain homogenates was approximately 100%, and both intra- and inter-day coefficients of variation for this measure were typically $\leq 11\%$. Similar results were observed for cycloleucine (Table I). For the lowest mass of ACPC analyzed (2.7 nmol), the calculated amount was within 10% of the actual amount. The minimum detectable mass of ACPC injected onto the analytical column was 0.08 nmol, equivalent to 18.7 μM (1.89 μg/ml) for a 90-μl plasma sample at a signal-to-noise ratio of at least 3:1.

Criteria for selection of cycloleucine as an internal standard were based on its chemical and chromatographical similarities to ACPC as well as low cost and ready availability. Moreover, the internal standard should also be chromatographically resolvable from known amino acids, not be an endogenous constituent of mammalian tissues, and be useful for both plasma and brain samples. While blank plasma did not contain endogenous materials interfering with the detection of both ACPC and cycloleucine (Fig. 1A and B), analysis of brain tissue revealed a minor interference eluting as a shoulder of the internal standard (Fig. 1C and D). The amount of endogenous interfering material did not vary remarkably among brain samples, contributing approximately 10–12% to the spiked cycloleucine (27 nmol) area. The amount of interfering material was unaffected by ACPC administration. Nonetheless, the present assay could be further refined by selection of an alternative internal standard for brain tissue.

The HPLC assay for ACPC reported here is sufficiently sensitive, reproducible and precise for application to preliminary pharmacokinetic studies. Thus, the ACPC plasma concentration–time profile was suitably described by a monoexpo-

TABLE I

ASSAY INTRA- AND INTER-DAY VARIABILITY AND EXTRACTION RECOVERY OF 1-AMINOCYCLOPROPANECARBOXYLIC ACID AND CYCLOLEUCINE FROM BIOLOGICAL MATRICES

Extraction recovery data are the mean ± standard deviation of four or five values. Coefficients of variation were calculated from the mean and standard deviation of four or five values.

Matrix	Spiked amount (nmol)	Extraction recovery (%)	Coefficient of variation (%)	
			Intra-day	Inter-day
1-Aminocyclopropanecarboxylic acid				
Plasma	270	97.0 ± 2.3	1.6	5.3
	27	100.4 ± 1.8	2.6	3.8
	2.7	97.8 ± 5.2	4.8	11.0
Brain	270	107.5 ± 2.7	6.6	5.7
	27	97.8 ± 1.7	7.5	3.3
	2.7	99.2 ± 2.4	8.7	5.2
Cycloleucine				
Plasma	270	98.8 ± 1.8	1.9	4.2
	27	105.9 ± 1.0	3.1	2.2
Brain	270	113.6 ± 2.7	6.4	5.5
	27	112.0 ± 1.7	6.5	2.9

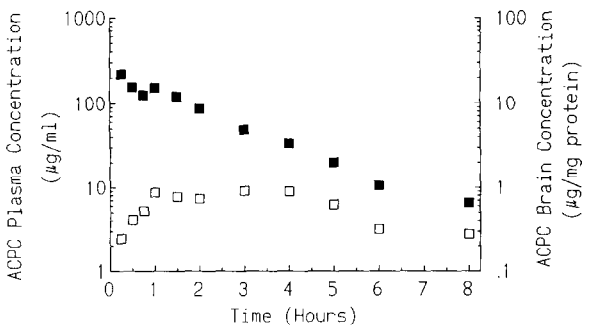

Fig. 2. ACPC concentration–time courses (■, plasma; □, brain) following intraperitoneal injection of 200 mg/kg ACPC to mice. Abdominal aortic blood and brain tissue were collected at the indicated times under halothane anesthesia. The anesthetic was administered immediately prior to sampling. Each point represents the mean of two mice.

nential decay process following intraperitoneal administration (Fig. 2). Statistical moment analysis was used to estimate preliminary pharmacokinetic parameters from the plasma concentration–time data of Fig. 2. The half-life of ACPC in mice was 1.49 h (calculated from a terminal slope of 0.464 h^{-1}), with a mean residence time of 2.04 h. The plasma clearance of ACPC was 7.34 ml/min/kg or 0.22 ml/min per 25 g mouse. Comparison of the plasma clearance of ACPC (7.34 ml/min/kg) to estimates of liver blood flow in the mouse (~105 ml/min/kg [18]) suggests that ACPC could be a low hepatic clearance compound. Comparison of the plasma clearance of ACPC (0.22 ml/min per 25 g mouse) to estimates of glomerular filtration rate in the mouse (~0.21–0.38 ml/min per 25 g mouse, obtained from interspecies allometric scaling relationships [19,20]) suggests that ACPC also could be eliminated by renal filtration. Studies on the metabolism and elimination of ACPC are needed to determine whether the observed ACPC clearance is primarily by hepatic or renal mechanisms. The steady state volume of distribution for ACPC was 878 ml/kg. Since ACPC is a cyclic homologue of glycine, it is not surprising that its estimated volume of distribution is similar to that of compounds which distribute into total body water (~700 ml/kg [21,22]).

The detection of significant concentrations of ACPC in mouse brain homogenates demonstrates that ACPC partitions *in vivo* across the blood–brain barrier, in spite of its zwitterionic nature. This finding, taken together with recent data demonstrating that ACPC is not actively taken up by the glycine transport system in a synaptosomal preparation [9], suggests that ACPC crosses the blood–brain barrier via passive diffusion.

In conclusion, a direct, precise and sensitive HPLC method was developed for the analysis of ACPC from mammalian tissues. This method is useful for understanding the pharmacokinetic and pharmacodynamic relationships governing the pharmacological effects of ACPC following acute and chronic administration.

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