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Short communication

Gas chromatographic–mass spectrometric determination of urinary 1-aminocyclopropanecarboxylic acid in mice using a deuterated internal standard

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Abstract

A gas chromatographic–mass spectrometric method is described for the determination of 1-aminocyclopropanecarboxylic acid in mouse urine using the 2,2,3,3-[$^2H_4$] isotopolog as an internal standard. Samples (0.1 ml) were extracted using an exchange resin, then derivatized with pentafluoropropanol and pentafluoropropionic anhydride at 100°C for 25 min. Gas chromatography was performed on a (5% phenyl)methylpolysiloxane column and detection was by selected-ion monitoring of M − $CO_2CH_2CF_2CF_3$ fragment ions. The method provided high response linearity (mean $r = 0.999$) and precision ($< 5\%$ coefficient of variation). After orally dosing mice with 1-aminocyclopropanecarboxylic acid (300 mg/kg), 46 and 10% of the dose was excreted unchanged in the 0–24 h and 24–48 h urines, respectively.

1. Introduction

Initial biological studies of 1-aminocyclopropanecarboxylic acid (ACPC) centered on its role as the immediate metabolic precursor of ethylene [1], a plant hormone which functions to cue the ripening of fruit. Recent pharmacological evaluation of this amino acid in animal models indicates therapeutic potentials as an anticonvulsant [2,3], antidepressant [4,5], anxiolytic [5–7], and neuroprotectant (in cerebral ischemia) [8]. These central effects of ACPC may be mediated through partial agonism at a glycine modulatory site associated with the N-methyl-D-aspartic acid receptor complex [9–11].

Methods utilizing high-performance liquid chromatography (HPLC) [12–14] and gas chromatography–mass spectrometry (GC–MS) with electron-impact (EI) ionization [12] have been applied to ACPC determinations from plant matrices. In addition, an HPLC method for ACPC analysis from plasma and brain [15] and a GC–MS chemical-ionization method for cerebrospinal fluid and brain ACPC determinations

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[16] have been reported. The present study details a GC–MS-EI method for urine ACPC analysis which benefits from (1) the analytical control offered by a deuterated internal standard [12,14], (2) rapid GC sample throughput, (3) the technical simplicity of EI relative to chemical ionization, and (4) sample derivatization (see Fig. 1) which does not involve multiple steps [12,14] nor pose product stability concerns [15]. This method was validated and applied to a pilot study of the percent of orally administered ACPC eliminated unchanged in the urine of mice.

2. Experimental

2.1. Chemicals

ACPC (batch no. 071 b/2) was obtained from Senn Chemicals (Dielsdorf, Switzerland). 2,2,3,3-[$^2H_4$]ACPC was generously donated by J.K. Heald and R. Horgan (Department of Botany and Microbiology, University College of Wales, Dyfed, UK) and by R.W. Woodard (College of Pharmacy, University of Michigan, MI, USA) but is available from Sigma (St. Louis, MO, USA). Pentafluoropropionic anhydride and pentafluoropropanol were obtained from Aldrich (Milwaukee, WI, USA). Strongly acidic cation-exchange resin (Amberlite 1R-118H) was from Sigma. Acetic acid and ammonium hydroxide were from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol was from Pharmco (Bayonne, NJ, USA). Acetonitrile was from Baxter (Muskegon, MI, USA). All chemicals were used without further purification.

2.2. Sample collection and extraction

Two female C57BL/6J mice (weighing 18.7 g and 22.0 g, respectively) were administered ACPC perorally at a dose of 300 mg/kg in a volume of 0.02 ml/g body weight. The animals were then placed in a single metabolic cage where food and water were available ad libitum. Pooled urine samples (and any drinking water leakage) were collected 0–24 h and 24–48 h after dosing; the volumes were measured, then the samples were frozen (−30°C) until analysis. Solid-phase extraction columns were prepared from pasteur pipets (6 ml) plugged with glass wool, packed with 0.8 g of the cation-exchange resin, and conditioned by rinsing with water (3 ml), 1 *M* acetic acid (9 ml), water (6 ml), ethanol (3 ml), and water (3 ml). After applying the urine samples (0.1 ml) which were spiked with [$^2H_4$]ACPC (15 μg in 0.5 ml of water), the columns were sequentially washed with water (3 ml) and ethanol (3 ml). The samples were then eluted using 5 ml of 28% ammonium hydroxide–ethanol (1:3, v/v) and collected in 2 dram screw-cap vials fitted with PTFE cap liners. The eluates were evaporated to dryness at 55°C in a sand bath under a stream of nitrogen. The samples were derivatized using pentafluoropropanol (20 μl) and pentafluoropropionic anhydride (50 μl) at 100°C for 25 min, then evaporated to dryness under nitrogen at room temperature. Aliquots (0.1 ml) of blank human urine, determined to be substitutable for mouse urine, were fortified with a range of ACPC concentrations (see Table 1) and run in parallel with the unknowns.

2.3. Instrumental analysis

All analysis utilized a Finnigan Model 9610 GC and a Model 4000 MS interfaced to an IBM-AT computer using a Teknivent Vector/One data system and software (St. Louis, MO, USA). The injector was adapted to capillary bore using a 17.8-cm conversion sleeve and a reducing union (Supelco, Bellefonte, PA, USA). The MS was calibrated with perfluorotributylamine (FC-43). Detection was by selected-ion monitoring with EI ionization at 60–70 eV and 280–300 μA. The electron multiplier was operated at 1825 V. The data system acquired two channels of ion current: that of the derivatized ACPC α-cleavage fragment *m/z* 202 (M − $CO_2CH_2CF_2CF_3$, see Fig. 1) and that of the corresponding tetradeuterated fragment *m/z* 206. The data system scan-rate was every 0.1 s with a sweep width of 0.1 a.m.u., integrating each acquisition sample for 4 ms.

Each sample was reconstituted with acetoni-

trile (300 μl) immediately before injection. A 0.5-μl Hamilton syringe was used to inject 0.1 μl by the splitless mode onto a (5% phenyl)-methylpolysiloxane fused-silica column, 30 m × 0.32 mm I.D., 0.25 μm film thickness (DB-5, J and W Scientific, Folsom, CA, USA). The filament was powered approximately 1.1 min after sample injection. The column oven was maintained at 105°C and the injection port and interface oven at 220°C. The helium carrier gas linear velocity was 50 cm/s. Under these conditions, ACPC and [$^2H_4$]ACPC eluted 1.52 and 1.50 min after injection, respectively (see Fig. 2).

2.4. Validation and calculations

The accuracy and precision were assessed by back-calculating individual standard data points using the slope and intercept of the associated standard curves from 5 separate runs. Recovery was determined by spiking duplicate blank (internally standardized) samples with ACPC (150 μg/ml) after elution into the derivatization vials, then comparing the GC peak-area ratios against the associated calibration plot. The concentrations of ACPC in the unknowns were calculated from the slope and intercept of the associated standard curve, plotted as GC peak-area ratio (ACPC/[$^2H_4$]ACPC) versus known ACPC concentration. The dose and total volumes collected were then used to establish the percentages eliminated unchanged.

3. Results and discussion

The amphoteric nature of ACPC obviated a liquid–liquid extraction from the urine samples. Accordingly, a cation-exchange resin was used to extract the urine by a modification of the amino acid separation method of Okeke et al. [17]. This procedure provided 69 and 72% recovery from duplicate ACPC samples. During development of the method attempts were made to use cycloleucine [15,16] or azetidine-2-carboxylic acid as internal standards. However, neither compound provided the linearity of response offered by the incorporation of [$^2H_4$]ACPC. The convenient one-step derivatization of both the amine and carboxylic acid functionalities (Fig. 1)

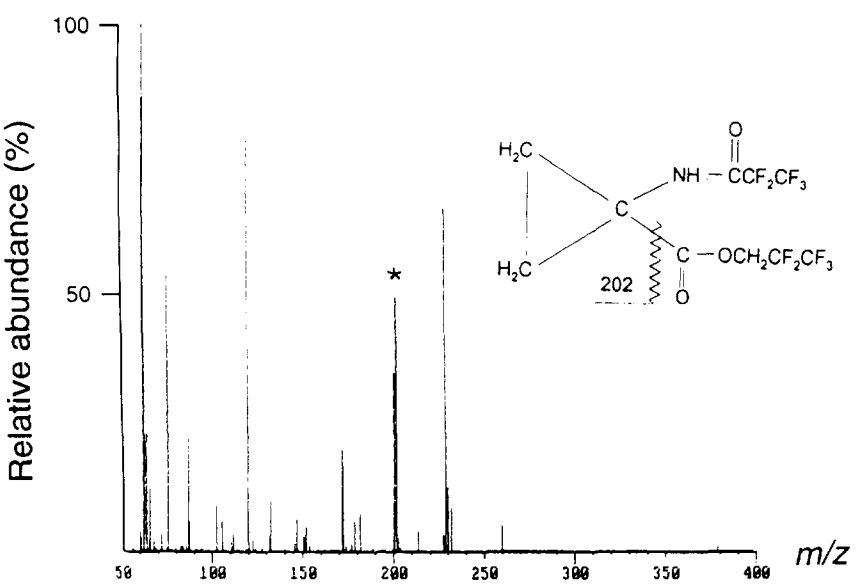

Fig. 1. Structure and the EI mass spectrum of derivatized ACPC indicating (*) the α-cleavage fragment ion (*m/z* 202) selected for monitoring by GC–MS. The 2,2,3,3-[$^2H_4$] isotopolog served as the internal standard with the corresponding *m/z* 206 ion monitored.

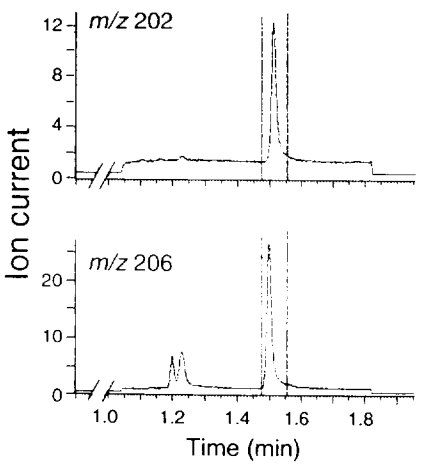

Fig. 2. Representative selected-ion chromatogram of derivatized ACPC (top) and the deuterated internal standard (bottom) extracted from urine (0.1 ml) containing 50 μg/ml ACPC. The vertical lines flanking the peaks represent the integration boundaries used in the peak-area determinations.

generated a stable and volatile GC substrate (Fig. 2). At a temperature of 100°C, complete derivatization of ACPC required 25 min. The esterification was incomplete using shorter reaction times. The fragment ion *m/z* 202 (49% relative abundance) of ACPC was selected for monitoring. The higher abundance ion *m/z* 229 (65%, $M - HOCH_2CF_2CF_3$) was not selected due to the presence of a low abundance (0.8%) ion *m/z* 233 in the mass spectrum of ACPC which contributed to the *apparent* ion current of the corresponding [$^2H_4$] species.

The quality of this analytical method is indicated in Table 1 which shows the results of five sets of calibration standards analyzed on five separate days. One of these five sets included a 450 μg/ml sample to allow for high concentrations in the mouse urine unknowns. Precision ranged from 0.07 to 4.60% (coefficient of variation) and all calibration plot correlation coefficients exceeded 0.998 (mean 0.999). A chromatogram from a 50 μg/ml urine ACPC sample is shown in Fig. 2 and represents a direct injection of less than 17 ng of ACPC (see Experimental).

The ACPC dose of 300 mg/kg administered to the mice in the present study was chosen based on doses that have been used for pharmacodynamic evaluation of this agent in rodents [2–6,8]. As speculated by Miller et al. [15], renal elimination of ACPC was found to be a significant route; 46 and 10% of the oral ACPC doses were accounted for as the parent compound in urine during 0–24 h and 24–48 h after administration, respectively.

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Table 1
Accuracy and precision

Concentration of ACPC (μg/ml of urine[a])		*n*	Coefficient of variation (%)
Added	Detected (mean[b])		
50	47.3	5	1.83
100	96.4	5	4.25
150	148.6	5	4.60
300	302.0	5	0.07
450	451.1	1	

[a] Using 0.1-ml sample volumes.
[b] Evaluated by back-calculating each individual calibration standard data point against the associated calibration plot. All *r* values exceeded 0.998 (mean 0.999).

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