# Determination of 5-aminoimidazole-4-carboxamide in human plasma by ion-pair extraction and LC–MS/MS

Xijing Chen *, Jing Jing, Weichao Ren, De-en Han, Ning Jing, Guangji Wang

*Center of Drug Metabolism & Pharmacokinetics, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China*

## Abstract

A liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was established for the determination of 5-aminoimidazole-4-carboxamide (AICA) in human plasma. The method included a solvent extraction of AICA as an ion pair with 1-pentanesulfonate ion and a separation on a Hypersil ODS2 column with the mobile phase of methanol–water (68:32, v/v). Determination was performed using an electrospray ionization source in positive ion mode (ESI$^+$). Multiple reaction monitoring (MRM) was utilized for the detection monitoring $m/z$ at $127 \rightarrow 110$ for AICA, and $172 \rightarrow 128$ for IS. The calibration curve was linear within a range from 20 to 2000 ng/mL and the limit of quantity for AICA in plasma was 20 ng/mL. RSD of intra-assay and inter-assay were no more than 5.90% and 5.65%.
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*Keywords:* Orazamide; AICA; Ion-pair extraction; LC–MS/MS

## 1. Introduction

Orazamide, which is composed of one molecule of 5-aminoimidazole-4-carboxamide (AICA), one molecule of orotic acid and two molecules of water, is used clinically for the treatment of hepatitis and cirrhosis [1–3].

The nucleoside of AICA (AICAR) is internalized and becomes phosphorylated by adenosine kinase to form AICAR mono-phosphate (AICA ribotide, ZMP), an intermediate in the late steps of *de novo* purine biosynthesis [4]. In hepatocytes, AICA can inhibit the fatty acid synthesis, sterol synthesis and gluconeogenesis [5–8]. The structures of orazamide and AICA are shown in Fig. 1.

In order to investigate the pharmacokinetics of AICA behavior after oral administration of orazamide, a sensitive determination method was expected. Detection of AICA in biological fluids by high-performance liquid chromatography (HPLC) with ultraviolet detection [9,10] has been reported, but it lacked sensitivity. In addition, a capillary electrophoretic method for screening of patients with AICA-ribosiduria has been reported [11]. The method is based on the direct ultra-violet detection of AICA and AICAr in untreated urine. However, an LC–MS/MS method suitable for the analysis of AICA has not been reported. In this paper, we reported a sensitive LC–MS/MS analytical method with novel sample preparation strategy for the determination of AICA in human plasma. The method has been successfully applied to study the pharmacokinetics of orazamide in healthy volunteers.

## 2. Experimental

### 2.1. Chemicals and reagents

Orazamide and metronidazole (internal standard) (>98 purity) were kindly donated by Guangdong Xianqiang Pharmaceutical Co., Ltd. (Guangzhou, China). The test preparation was orazamide capsule, which weighs 100 mg, containing 40 mg AICA per capsule (Guangdong Xianqiang Pharmaceutical Co., Ltd, Guangzhou, China). Ion-pair reagent 1-pentanesulfonate sodium salt was purchased from Shandong yuwang Co., Ltd. (Shandong, China). Methanol was of HPLC grade (Tedia CO., INC, USA). Ethyl acetate of analytical grade was purchased from Nanjing Chemical Reagent Co. (Nanjing, China). All other reagents were of analytical grade.

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* Corresponding author. Tel.: +86 25 83271286; fax: +86 25 83271335.
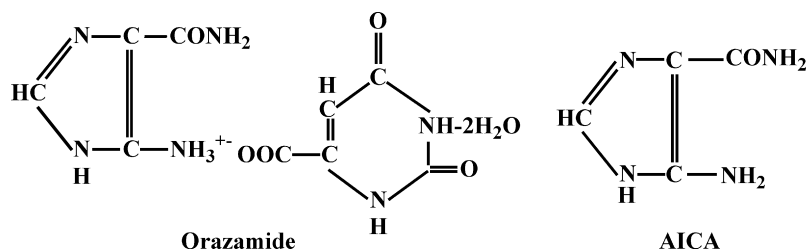  *E-mail address:* chenxj-lab@hotmail.com (X. Chen).

Fig. 1. Chemical structure of orazamide and 5-aminoimidazole-4-carboxmide (AICA).

## 2.2. Liquid chromatography conditions

HPLC–MS/MS analyses were performed using a Varian liquid chromatography system with two prostar pumps (Varrian, USA). AICA and IS were separated on a Hypersil ODS2 column (5 μm, 4.6 mm × 150 mm I.D., Elite Co., Dalian, China). The mobile phase consisted of methanol–water (68:32, v/v); and the flow-rate was 0.5 mL/min. Test samples were introduced using a 410 auto-sampler (Varrian, USA) with an effective volume of 20 μL.

## 2.3. Mass spectrometry conditions

The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI$^+$) with multiple reaction monitoring (MRM) (Varian LC–MS/MS-1200L; Varian, USA). The voltages of the needle, shield, capillary, the first multipole offset, second multipole offset, inter-multipole offset, and tube lens offset were auto-tuned using infusion injection. The detector voltage was set at 1.7 kV. Drying and nebulizing (N$_2$) gas pressures were 28.2 psi and 54.8 psi, respectively. Drying gas temperature was 380 °C. Collision energy was −13 V for AICA and −10 V for IS, separately. Collision cell pressure was 2.0 mTorr. The precursor and product ions selected to monitor AICA and metronidazole (IS) in MS/MS transitions were those at $m/z$ 127 > 110 for AICA and 172 > 128 for IS, respectively. The chromatograms were integrated using a Varian version 6.6 workstation. (Varrian, USA).

## 2.4. Preparation of work solutions

The stock solution of AICA was prepared at 200 μg/mL in water. A series of working solutions of AICA were prepared at concentrations of 0.2, 0.5, 1, 2, 5, 10 and 20 μg/mL by serially diluting the stock solution with water in separate 10 mL volumetric flasks. A solution containing 2 μg/mL IS was also prepared by further diluting the stock solution of 100 μg/mL IS with methanol. 1-Pentanesulfonate sodium salt (4 mM) was prepared in water as ion-pair regent. All the solutions were stored at −4 °C.

## 2.5. Sample preparation

1-Pentanesulfonate sodium salt (4 mM) in water (100 μL) was added into the plasma sample (0.2 mL) as ion-pair reagent,

after adding 20 μL of metronidazole (2 μg/mL, in methanol, IS) the plasma samples were then extracted with 4 mL ethyl acetate. The organic layer of 3 mL was removed after centrifugation and separation. Then the extracts (3 mL) were evaporated to dryness in vacuum at 50 °C. The residues were dissolved in 500 μL of 70% methanol and loaded 20 μL onto the analytical column using the previously described mobile phase by auto-sampler.

## 2.6. Preparation of calibration samples and quality control samples

The calibration curve samples were prepared in 0.2 mL of plasma, by adding aliquots of the stock solution of AICA to drug free plasma at concentrations of 20, 50, 100, 200, 500, 1000 and 2000 ng/mL. Then the plasma samples were treated according to the sample preparation procedure mentioned above. The peak-area ratios of AICA to IS were measured and plotted against the respective concentration of analyte.

The quality control samples (QCs) were prepared in 0.2 mL of plasma, by adding aliquots of the stock solution of AICA to drug free plasma at three different concentrations (50, 200 and 1000 ng/mL) to determine the accuracy precision and stability of the method. The QCs were dealt with in the same manner as the sample preparation.

## 2.7. Method validation

The method validation was conducted according to the SFDA guideline for bioanalytical methods validation for human studies [12]. Six blank human plasma samples and plasmas spiked with LOQ concentration of AICA were extracted by the sample preparation method described above to assay the specificity and matrix lot-to-lot reproducibility.

The accuracy was calculated as a percentage of inter-assay measured concentration to the nominal one. The intra-assay percentage relative standard deviation (%RSD) was calculated according to the determination of five QCs in one day. Otherwise, the inter-assay %RSD was calculated according to the determination of the QCs daily on five separate days.

QCs were stored at −20 °C to assess freeze-cold stability (0, 3, 5, 7 and 14 days); Freeze–thaw stability was checked through three freeze–thaw cycles (−20 °C to room temperature). The
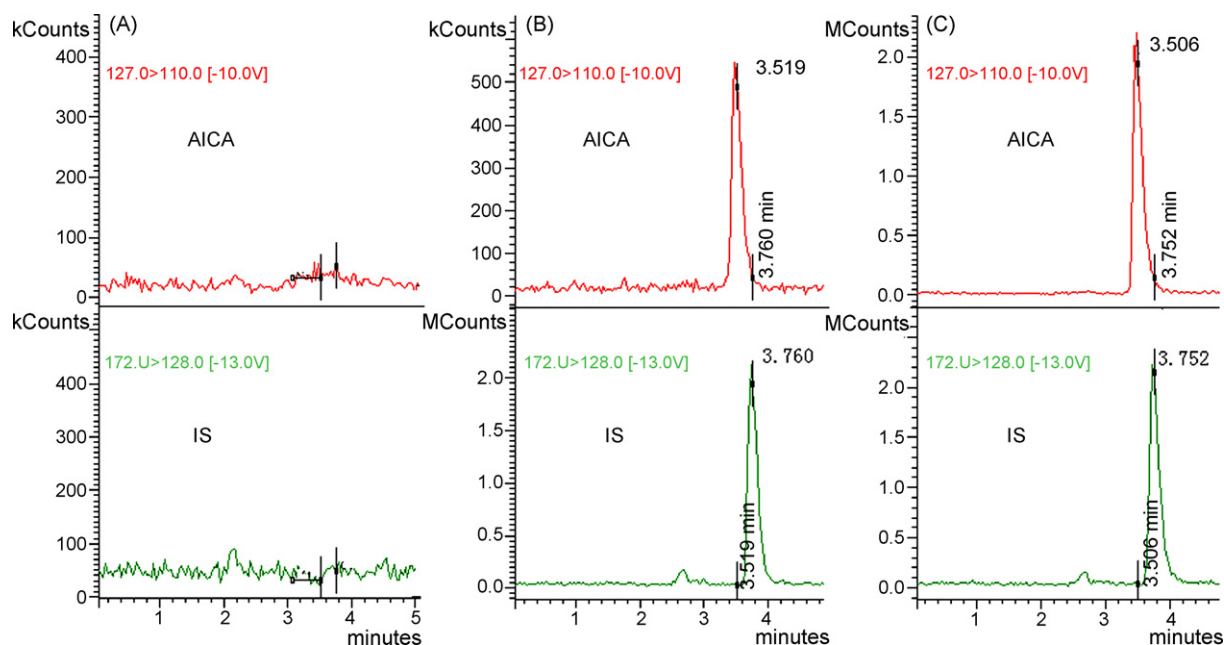
Fig. 2. MRM chromatograms of: (A) blank human plasma, (B) human plasma spiked with AICA (200 ng/mL) and IS, (C) plasma obtained from a volunteer after 1.0 h. AICA channel: $m/z$ 127 → 110; IS channel: $m/z$ 172 → 128.

stability of AICA in extracts was also examined after 0, 4, 8, 16 and 32 h of storage at room temperature.

### 2.8. Drug administration and sampling

Each of 20 healthy volunteers was orally administered of two capsules of orazamide, which contained 80 mg of AICA. Blood samples (3 mL) were collected by venepuncture at 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0 h after administration. Plasma samples were obtained by centrifugation of blood samples at 4000 rpm for 5 min and stored at −40 °C until analysis.

## 3. Results

### 3.1. Chromatography

The LC–MS/MS method described above provides a good separation of AICA and IS from the other indigenous plasma constituents and maintained fine peak shapes. Fig. 2 shows the chromatograms of blank plasma (A), plasma spiked with AICA (200 ng/mL) and IS (B), and plasma obtained from a volunteer after 1.0 h (C). The retention times were about 3.5 min for AICA and 3.7 min for IS, respectively.

### 3.2. Linearity and LOQ

Linear least-squares regression analysis of the calibration graph demonstrated linearity in the range of 20–2000 ng/mL. A typical standard curve ($r = 0.9996$) could be described by the equation As/Ais = 0.3565C − 0.0024. Representative chromatograms of extracted plasma samples spiked with LOQ (20 ng/mL) and ULOQ (2000 ng/mL) of AICA and IS were shown in Fig. 3.

### 3.3. Accuracy, precision and stability

The accuracy and precision of the method were found to be well within the acceptable limits (Table 1). No significant loss of AICA was observed in 32 h at room temperature and no degradation was observed after three cycles of freezing and thawing and after two weeks of freeze-cold (Table 2).

### 3.4. Application of the method in pharmacokinetics

The LC–MS/MS method described above has been applied to the pharmacokinetics study of AICA in human plasma. The concentration versus time profile of AICA in human plasma after single oral dose 80 mg AICA is shown in Fig. 4. The main pharmacokinetic parameters $C_{max}$, $t_{1/2}$, $t_{max}$,

Table 1
Recovery and precision for the determination of AICA in human plasma ($n = 5$)

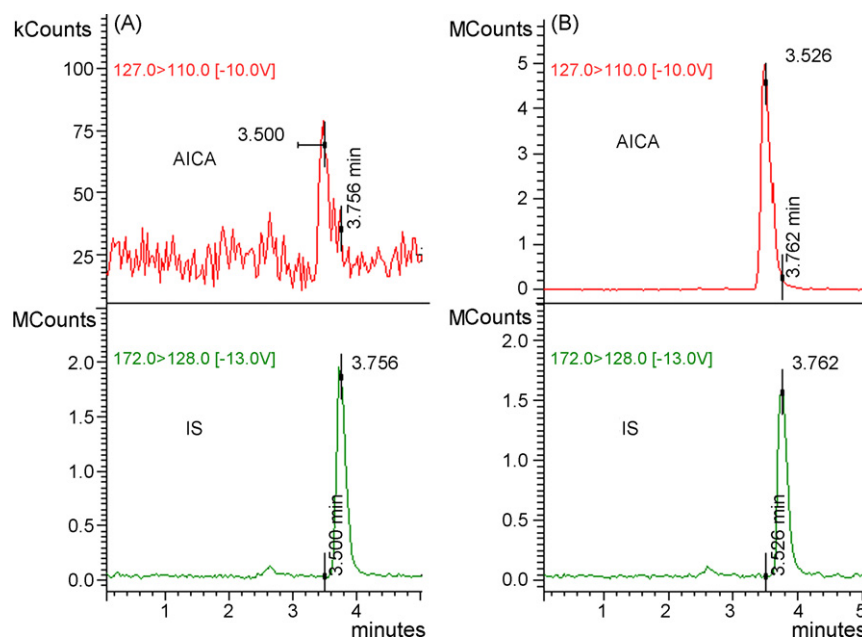| Added concentration (ng/mL) | Intra-assay measured concentration Mean ± SD (ng/mL) | Recovery (% nominal) Mean ± SD | RSD% | Inter-assay measured concentration Mean ± SD (ng/mL) | RSD% |
|---|---|---|---|---|---|
| 50 | 48.4 ± 2.8 | 96.96 ± 5.53 | 5.70 | 49.6 ± 1.2 | 2.46 |
| 200 | 195.6 ± 11.6 | 97.72 ± 5.72 | 5.90 | 191.6 ± 10.8 | 5.65 |
| 1000 | 972.8 ± 32 | 97.28 ± 3.23 | 3.30 | 972.4 ± 32.8 | 3.37 |

Fig. 3. MRM chromatograms of (A) human plasma spiked with AICA at LOQ (20 ng/mL) and IS, (B) human plasma spiked with AICA at ULOQ (2000 ng/mL) and IS in calibration curve. AICA channel: $m/z\ 127 \to 110$; IS channel: $m/z\ 172 \to 128$.

Table 2
Stability of AICA after storage under indicated condition

| Nominal Concentration (ng/mL) | Room ($n = 5$) | | Freeze-cold ($n = 5$) | | Freeze-thaw ($n = 3$) | |
|---|---|---|---|---|---|---|
| | Measured concentration (mean ± SD)[a] | RSD (%) | Measured concentration (mean ± SD)[b] | RSD (%) | Measured concentration (mean ± SD)[c] | RSD (%) |
| 50 | 50.0 ± 5.6 | 11.3 | 46.8 ± 3.6 | 7.9 | 46.0 ± 2.0 | 4.2 |
| 200 | 226.0 ± 15.5 | 6.9 | 181.6 ± 6.4 | 3.5 | 177.6 ± 2.8 | 1.6 |
| 1000 | 1049.6 ± 85.2 | 8.1 | 984.4 ± 17.6 | 1.8 | 980.4 ± 10.8 | 1.1 |

[a] Mean ± standard deviation, average of concentration in 0, 4, 8, 16, 32 h.
[b] Mean ± standard deviation, average of concentration in 0, 3, 5, 7, 14 days.
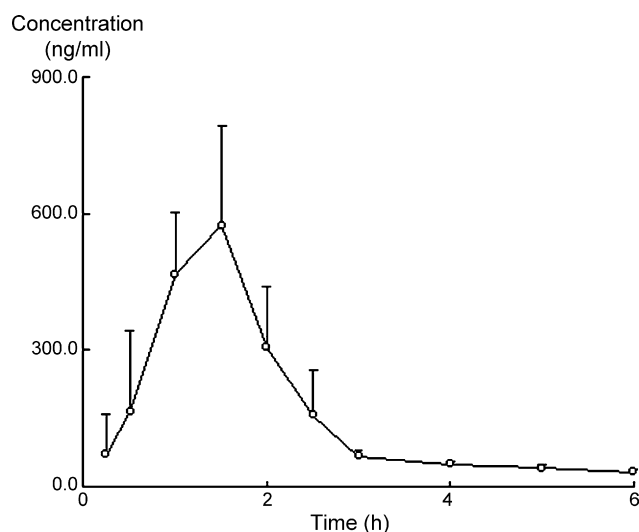[c] Mean ± standard deviation, average concentration of 1, 2 and 3 freeze–thaw cycles.



Fig. 4. Mean plasma concentration–time profile of AICA after single oral dose of 80 mg of AICA to 20 healthy volunteers.

$AUC_{0\sim t}$ are $627.6 \pm 182$ ng/mL, $1.98 \pm 0.62$ h, $1.33 \pm 0.34$ h, $951.2 \pm 318$ h ng/mL, respectively.

## 4. Discussions

AICA, which is a basic compound, is difficult to be dissolved in any organic solvent, including ethanol, aether, chloroform, etc. In the early stage of our study, a solid phase extraction of AICA from human plasma was established, but the sample preparation process is complicated and with lower recovery compared to the ion-pair extraction method which was mentioned above in this paper. Therefore, the use of an ion-pair reagent in the extraction enhanced the recovery of the compound.

In conclusion, when 0.2 mL plasma was used for extraction, the assay had a lower limit of quantification (20 ng/mL) for AICA. In addition, this assay has been successfully applied to measure the concentration of AICA in plasma in healthy volunteers who has been treated with single oral doses of orazamide capsules containing 80 mg of AICA.

## References

[1] E. Roda, C. Sama, D. Festi, Clin. Ter. 89 (1979) 489.

[2] F. Chanoine, Acta Gastroenterol. Belg. 32 (1969) 383.

[3] A.E. Tammaro, O. Bonaccorso, Clin. Ter. 75 (1975) 141.

[4] B.S. Jhun, Q. Jin, Y.T. Oh, S.S. Kimm, Y. Kong, Y.H. Cho, J. Ha, H.H. Baik, I. Kang, Biochem. Biophys. Res. Commun. 318 (2004) 327.

[5] M.F. Vincent, P.J. Marangos, H.E. Gruber, G. Van den Berghe, Diabetes 40 (1991) 1259.

[6] J.M. Corton, J.G. Gillespie, S.A. Hawley, D.G. Hardie, Eur. J. Biochem. 229 (1995) 558.

[7] N. Henin, M.F. Vincent, H.E. Gruber, G. Van den Berghe, FASEB J. 9 (1995) 541.

[8] P.A. Lochhead, I.P. Salt, K.S. Walker, D.G. Hardie, C. Sutherl, Diabetes 49 (2000) 896.

[9] D. Fiore, A.J. Jackson, M.S. Didolkar, V.R. Dandu, Antimicrob. Agents Chemother. 27 (1985) 977.

[10] R. Dixon, J. Fujitaki, L. Chrastil, J. Lee, Res. Commun. Chem. Pathol. Pharmacol. 65 (1989) 405.

[11] P. Hornik, P. Vyskocilova, D. Friedecky, T. Adam, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 843 (2006) 15.

[12] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001.