Detection of endogenous agmatine content in rat brain and NG108-15 cells using high performance liquid chromatography

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Abstract: AIM To develop a method to determine endogenous agmatine (Agm) content in rat brains and cultured NG108-15 cells. **METHODS** High performance liquid chromatography (HPLC) with fluorescence detector was used to determine the endogenous Agm concentration in different regions (frontal cortex, thalamus, hippocampus, striatum and cerebellum) of rat brain and NG108-15 cells. **RESULTS** The concentration of endogenous Agm was 0.8－1.71 $\mu g \cdot g^{-1}$ wet tissues in different rat brain regions, and 0.044 $mg \cdot g^{-1}$ protein in NG108-15 cells, respectively. **CONCLUSION** This gradient HPLC procedure is applicable for routine detection of Agm in vertebrate tissues and cells.

Key words: agmatine; chromatography, high performance liquid; brain; cells, NG108-15

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Agmatine (Agm) is a primary amine formed by decarboxylation of *L*-arginine by an enzyme, arginine decarboxylase (*L*-ADC), which has long been known to be a constituent of bacteria, plants and many invertebrates, and believed to be a metabolic intermediate in the formation of polyamines in these organisms. Until 1994, it was proved to exist in mammalian tissue[1] and as an endogenous ligand of imidazoline receptors (I-R). Later the enzymes that degrade Agm, such as agmatinase and diamine oxidase, were also found to exist in mammalian brains[2,3]. Accumulating evidences have indicated that Agm is a new type of neurotransmitter, and the concentration in central nervous system (CNS) is at $nmol \cdot L^{-1}$ level. Agm can be stored in vesicles and released from the nervous cells under stimulation. Agm Possesses many physiological and pharmacological functions, such as the modulation of the release of luteinizing hormone and insulin through activation of I-R[4,5], and the modulation of opioid actions. The functions of endogenous Agm can be stopped by reabsorption and degradation by agmatinase[2,6].

Many methods for detection of Agm were established in recent years. Li, *et al*[1] measured Agm in bovine brain using electrospray-high performance liquid chromatography (HPLC) mass spectroscopy, but this method is difficult to be widespreadly applicated due to its high cost. Feng, *et al*[7] developed a HPLC method to determine endogenous Agm in rat brain and use *D*-homocysteic acid as internal standard, however, their method needs more than 80 min for one sample and is inconvenient for routine analysis. Detection of Agm with immunological methods were restricted by problems of cross-reactivity of antiserum with other amines. Thus until now there is not a convenient method for the routine measurement of Agm in mammalian tissues and cultured cells.

In the present study, we sought to establish a relatively convenient method for the isolation and determination of Agm from different rat brain regions and NG108-15 cells, in which a derivatization of *o*-phthaladehyde (OPA)-2-mercaptoethanol (ME) and a gradient HPLC system with fluorescence detector were used.

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1 MATERIALS AND METHODS

1.1 Chemicals

Agmatine sulphate, *L*-arginine, citrulline, *L*-ornithine, OPA, ME were purchased from Sigma (St. Louis, MO, USA). Boric acid, potassium hydroxide, hydrochloride acid, sodium hydroxide, potassium dihydrogen-phosphate, perchloric acid were purchased from Beijing Chemical Plant. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA).

1.2 Apparatus and chromatographic conditions

The HPLC system consisted of a binary pump system (HP1100), a Shimadzu RF530 fluorescence detector (λ_{ex} = 338 nm, λ_{em} = 425 nm), HPLC chemostation, and a 5 μm biophase ODS, 250 mm × 4.6 mm (inner diameter). stainless-steel column, which was equipped with a syringe loading injector and a fixed 20 μL sample loop.

The gradient HPLC method of Feng, *et al*[7] was modified to detect Agm in male Wistar rats [(200 ± 20)g ($\bar{x}$ ± s), Grade Ⅱ, Certificate No. 01-3039] brain and cultured cells. Stock A solution (10 × concentrated, 0.1 mol·L^{-1}) was prepared by dissolving 13.609 g anhydrous potassium dihydrogen-phosphate in 950 mL of water and adjust the pH to 4.05 at 20℃ in a final volume of 1 L. After filtering, the solution was stored at 4℃ in the dark. Eluent A was 1∶1∶8 (*V*/*V*/*V*) mixture of Stock A, methanol and water (final concentration of KH_2PO_4: 0.01 mol·L^{-1}). Eluent B was 5∶3 (*V*/*V*) mixture of acetonitrile and methanol. Both eluents A and B were vacuum-degassed for 30 min before use.

Potassium borate buffer (0.2 mol·L^{-1}) was prepared by dissolving 3.092 g boric acid in water and adjusting the pH to 9.40 with a saturated solution of potassium hydroxide in a final volume of 250 mL. The OPA-ME derivatization solution was prepared by dissolving 27 mg OPA in 1 mL of methanol, then adding 26.5 μL of ME and 9 mL of 0.2 mol·L^{-1} potassium borate buffer. The solution was freshly prepared before experiment daily.

The gradient HPLC schedule was listed in Tab 1. Following each gradient HPLC procedure, the columns were re-equilibrated with 80% eluent A and 20% eluent B for 5 – 10 min until a stable baseline was re-established, before injecting the next sample. Each sample was injected and determined twice.

Tab 1. Chromatographic conditions for gradient high performance liquid chromatography (HPLC) to determine the concentration of agmatine

Retention time/min	A/%	B/%	Flow rate/mL·min^{-1}
0.0	80	20	1.0
2.3	80	20	1.0
3.8	73	27	1.0
14.3	73	27	1.0
17.3	0	100	1.0
19.3	80	20	1.0
20.8	80	20	2.0
21.8	50	50	2.0
23.8	80	20	2.0
26.8	0	100	2.0
29.8	0	100	2.0
30.3	80	20	1.0

To make sure that the substance eluted in the tissue preparation is Agm, the eluent of tissue preparation was collected at the same elution time with external standard, the substance structure in the eluent was analyzed by mass spectrometry. Agm, citrulline, *L*-ornithine and *L*-arginine in the same sample were analyzed to be sure that these four substances can be separated.

1.3 Sample preparation

Rats were sacrificed by rapid decapitation and the brains were quickly excised in ice-cold PBS (0.2 mol·L^{-1}, pH 7.2). Five brain regions (frontal cortex, thalamus, hippocampus, striatum and cerebellum) were dissected. Tissue samples were weighed and homogenized using a glass homogenizer for 30 s in ice bath in 1 mL of perchloric acid (0.6 mol·L^{-1}) and hydrochloric acid

(0.1 mol·L^{-1}) per 100 mg tissue (wet weight). The samples were left on ice for 1 h to precipitate the protein and then centrifuged for 10 min (12 000 × *g*, 4℃), the supernatant was neutralized with 5 mol·L^{-1} NaOH. Supernatant 20 μL and OPA-ME derivatizing reagent 20 μL were mixed for 2 min at room temperature, and 20 μL derivatized sample was then injected into the HPLC system.

NG108-15 cells were cultured using Dubecco's modified Eagle's medium (DMEM) with 0.1 mmol·L^{-1} hypoxanthine, 17 μmol·L^{-1} thymidine, 10 μmol·L^{-1} aminopterin, 2 mmol·L^{-1} glutamine, 10% new-born bovine serum and 100 U·L^{-1} penicillin, 100 μg·L^{-1} streptomycin. Culturing medium was decanted and washed 2 times with PBS (pH 7.2), then the cells were collected with a policeman, homogenized by a glass homogenizer for 30 s in ice bath in 1 mL of perchloric acid (0.6 mol·L^{-1}) and hydrochloric acid (0.1 mol·L^{-1}) per 5×10^6 cells. The sample was left on ice for 1 h and was then centrifuged for 10 min (12 000 × *g*, 4℃). The protein was detected with Coumassie bright blue method, then the supernatant was neutralized with 5 mol·L^{-1} NaOH, 20 μL supernatant was mixed with 20 μL OPA-ME derivatizing reagent for 2 min at room temperature, then 20 μL mixture was injected into the HPLC system.

1.4 Quantitation and statistical analysis

The accuracy of the method was calculated based on the difference between the mean calculated and added concentrations of Agm. The precision of the method was determined by calculating the intra- and inter-day coefficients of variation (*CV*). External standard of different concentrations was injected intermittently to evaluate the stability of the HPLC method. The limit of detection of Agm was defined as the amount of external standard that resulted in a peak of approximately 2 times the noise level. The concentrations of Agm in samples were calculated with reference to the peak heights of external standards. Agm concentrations were expressed as $\bar{x} \pm s$, significance was taken for $P < 0.05$.

2 RESULTS AND DISCUSSION

2.1 Accuracy and precision of HPLC methods

Representative chromatograms are shown in Fig 1, a derivatized standard containing 1 ng Agm (A) and a derivatized extract of cortex cerebral without added Agm (B). Agm was well separated from other substances in brain tissue and the gradient HPLC elution time of Agm was about 25.20 min. The structure of the substance with the same retention time as external standard in rat brain was analyzed by mass spectrometer and proved to be Agm (data not shown).

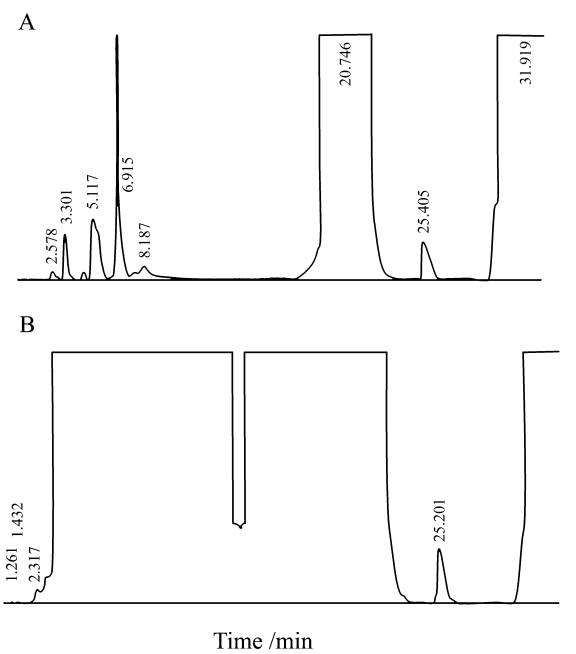

Fig 1. Gradient HPLC chromatograms of external standard of Agm (A, 4 ng) and a tissue preparation of rat cerebral cortex (B). The peak at 25.40 min is Agm. For eluent and gradient conditions, see Materials and Methods.

Detection of amines by derivatization with OPA is a well-established procedure and many substances in tissue homogenate can conjugate to OPA during the derivatization step. Current procedure is able to eliminate most of these labeled molecules and avoid chromatograms overcrowded with unidentifiable peaks. In the chromatogram we can see retention times of many analogs of Agm

such as citrulline, *L*-ornithine and *L*-arginine were 18.79, 18.87 and 19.97 min, respectively. There is only the peak of Agm between 22.20 and 28.00 min, indicated a good separation of Agm from other peaks.

The gradient HPLC method yielded a correlation coefficient for the Agm external standard curve of 0.9998 ($n = 6$, 0 – 19.6 ng), the accuracy was comparable to that obtained for calibration standards. The lower limit of detection of Agm in the external standard was 4 pmol. Three concentrations of spiked Agm were added for the test of recovery. The average recovery of added Agm (100 $\mu g \cdot L^{-1}$) was (93.3 ± 3.6)% for brain samples. The intra- and inter-day *CV* were less than 7%, indicate a good reproducibility in this method. We also investigated whether freezing samples and the Agm standards at −70℃ for up to 30 d after dissection of the brain samples would affect the stability of Agm. No degradation was observed during storage.

2.2 Quantitation of agmatine in rat brain and NG108-15 cells

Concentrations of Agm were (1.0 ± 0.6) $\mu g \cdot g^{-1}$ in cerebral cortex, (1.71 ± 0.42) $\mu g \cdot g^{-1}$ in thalamus, (1.4 ± 0.5) $\mu g \cdot g^{-1}$ in hippocampus, (1.2 ± 0.6) $\mu g \cdot g^{-1}$ in striatum and (0.8 ± 0.4) $\mu g \cdot g^{-1}$ in cerebellum, respectively (wet brain tissue, $n = 10$, $P < 0.05$). Agm concentration was highest in thalamus and lowest in cerebellum which is identical to the result reported by Feng, *et al*[7]. Since Agm was postulated to be the endogenous ligand of I-R and can exert pharmacological effect through inhibition of NOS activity[8], it is possible that I-R density and NOS activity may also be higher in thalamus like Agm. However, different from this hypothesis, I-R density was found to be higher in cerebellum[9] and NOS activity was also lower in thalamus but higher in cerebellum[10]. Furthermore, the concentration of Agm in different brain regions in Feng's report is lower than we have detected, this discrepancy may originated from the different process of sample preparation and neutralization process. We also detected the concentration of Agm in NG108-15 cells. The results showed that Agm concentrations of NG108-15 cells [(0.044 ± 0.016) $mg \cdot g^{-1}$ protein].

In summary, our method allows for the detection of Agm in rat brains and cultured NG108-15 cells. The overall recovery rate was 93.3% and the detection limit was about 4 pmol. The internal standard which was used in Feng's procedure, *D*-homocysteic acid, could not be separated from other substances in our procedure, therefore, quantitation of Agm was based on the method of additions of internal Agm spikes. Therefore, we still need to find an internal standard to minimize the loss of Agm during recovery to improve the procedure.

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高效液相色谱法测定大鼠脑和 NG108-15 细胞内胍丁胺含量

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摘要：目的　建立测定大鼠不同脑区和 NG108-15 细胞内源性胍丁胺含量的方法。**方法**　用高效液相色谱荧光检测法测定大鼠不同脑区（包括皮层、海马、丘脑、纹状体和小脑）及 NG108-15 细胞内胍丁胺含量。**结果**　正常大鼠不同脑区胍丁胺的含量范围为 0.8～1.71 $\mu g \cdot g^{-1}$湿脑组织；NG108-15 细胞内含量为 0.044 $mg \cdot g^{-1}$蛋白。**结论**　此法适于常规测定动物脑组织及培养细胞内的胍丁胺含量。

关键词：胍丁胺；色谱法，高效液相；脑；细胞，NG108-15

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