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Journal of Chromatography B, 691 (1997) 277–286

JOURNAL OF CHROMATOGRAPHY B

Determination of agmatine in brain and plasma using high-performance liquid chromatography with fluorescence detection

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Received 1 May 1996; revised 23 August 1996; accepted 24 September 1996

Abstract

Decarboxylated arginine, agmatine, is a neurotransmitter candidate for imidazoline receptors. A method is described to measure agmatine in rat brain and human plasma by isocratic high-performance liquid chromatography (HPLC) with fluorescence detection and *o*-phthalaldehyde derivatization. Quantitation is based on the method of additions of internal agmatine spikes. This assay has sensitivity in the low picomole range and a detection limit of 100 fmol. The correlation coefficient for the agmatine standard curve was 0.999±0.001 S.D., and intra- and inter-assay C.V.s were less than 8%. The accuracy of our isocratic method compared favorably with a gradient HPLC protocol, originally developed for bacterial agmatine, which we modified for use with tissues. Agmatine concentrations in rat brain were proportioned similarly to the regional distribution of imidazoline-1 receptors. These methods can be used as reliable research tools in various biological samples.

Keywords: Agmatine; Arginine; Imidazoline receptors

1. Introduction

Agmatine is a primary amine formed by the decarboxylation of L-arginine by the enzyme arginine decarboxylase. Until recently, agmatine had only been detected in plants, bacteria, insects and invertebrates [1–4]. In an effort to isolate and establish the structure of an endogenous ligand for imidazoline receptors, Li et al. [5] discovered agmatine in bovine brain. Agmatine was shown to possess clonidine-displacing substance (CDS) activity for certain α_2- and non-adrenergic imidazoline-binding sites in rat brain [5]. Prior to this, CDS activity had only been partially purified from brain extracts [6]. Agmatine is now known to make up a significant portion, though not all, of the binding activity of brain CDS [7]. Accumulating evidence has indicated that agmatine is synthesized by arginine decarboxylase in the brain and is stored primarily in neurons [7]. Furthermore, agmatine immunoreactivity is enriched in synaptic vesicles prepared from whole brain, and agmatine is released from neurons [7]. Agmatine is also widely distributed throughout the body, but the lowest levels are reported in plasma [7].

Imidazoline receptors (IR_1 subtype) were first proposed to exist in brainstem to explain the non-

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PII S0378-4347(96)00458-6

adrenergic property of centrally-injected clonidine to lower blood pressure [8]. Subtypes of imidazoline receptors have been described in a number of tissues based on rank ordering of affinities of various imidazoline compounds [9]. Although not an imidazoline compound, agmatine possesses high affinity for a component of clonidine-selective IR_1 binding sites [5,10]. In addition, agmatine has micromolar affinity for nicotinic receptors [11]. Microinjection of agmatine into brainstem has been without effect on blood pressure [12]. However, evidences for unique roles for agmatine have been accumulating; including modulating the hypothalamic release of leutenizing hormone [13], being neuroprotective [14] and functioning as a stimulator of gastric secretion in the stomach [15]. A lowered production of agmatine has also been speculated to play a role in depressive illnesses [16].

A few analytical procedures have been devised for agmatine measurements. Li et al. [5] have measured agmatine in bovine brain using electrospray-HPLC mass spectroscopy. Unfortunately, due to the high cost of the required equipment, this method is not applicable for routine use. Another method [4] utilizing gradient HPLC, had been only applicable to measuring agmatine in bacterial cultures. Recently, Raasch et al. [17] used isocratic HPLC following solid–liquid extraction to measure the *o*-phthalaldehyde-2-mercaptoethanol (OPA-ME) derivative of agmatine in tissues. Unfortunately, their method lacked an internal standard and their method was further problematic owing to the instability of the OPA-ME derivative throughout the time-consuming extraction procedure. Other attempts to quantitate agmatine by immunological methods have been hampered by questions of cross-reactivity of antiserum with other amines [18]. Thus, there are currently no suitable methods for the routine measurement of agmatine in vertebrate tissues.

In the present study we have established a method for the extraction of agmatine from tissues followed by derivatization with OPA-ME and rapid injection onto an isocratic, HPLC system with fluorescence detection. We have used this method to determine the concentration of agmatine in human plasma and in rat brain. Quantitation is based on the method of additions of internal agmatine spikes. To confirm the utility of our method we modified a published gradient HPLC protocol for bacterial cultures [4] for use with human blood and rat brain, and compared it to the isocratic procedure. We report that the concentration of agmatine varies throughout several brain structures in a manner similar to that reported [19,20] for the regionalization of IR_1 binding sites.

2. Experimental

2.1. Chemicals and reagents

Agmatine sulphate salt, D-homocysteic acid, boric acid, potassium hydroxide, potassium dihydrogenphosphate, octyl sulfate sodium salt, potassium acetate, *o*-phthalaldehyde (OPA), 2-mercaptoethanol (ME), trifluoroacetic acid, trichloroacetic acid and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol, anhydrous ether and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Nitric acid, perchloric acid, hydrochloric acid and sodium hydroxide were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a pump and multisolvent delivery system (Waters Model 600; Marlborough, MA, USA), a Gilson Model 121 fluorescence detector (excitation wavelength of 350 nm and emission wavelength of 450 nm; Gilson Medical Electronics, Middleton, WI, USA), a chart recorder/integrator (Model SP 4270; Spectra-Physics, San Jose, CA, USA) and a 5 μm Biophase ODS, 250×4.6 mm I.D. column (part No. MF-6017, Bioanalytical Systems, West Lafayette, IN, USA). This was equipped with a syringe loading injector (Model 7161; Rheodyne, Berkeley, CA, USA) and a fixed 20-μl sample loop. Identical equipment was used to compare our modified gradient-HPLC [4] with the isocratic HPLC method which we also developed.

The gradient HPLC method of Patchett et al. [4] for bacteria, was modified in order to detect agmatine in plasma and brain. Potassium borate buffer (final 0.2 *M*, pH 9.4 at 20°C) was prepared by dissolving boric acid in water and adjusting the pH with a saturated solution of potassium hydroxide in a final volume of 250 ml. The buffer was passed

through a 0.22-μm filter (Gelman Sciences, Ann Arbor, MI, USA) and stored at 4°C. Eluent A (10× concentrated) was prepared by dissolving 13.61 g anhydrous potassium dihydrogenphosphate in 950 ml of water and adding potassium acetate to adjust the pH to 5.93 at 20°C in a final volume of 1 l. After filtering through a 0.45-μm membrane filter (Rainin, Woburn, MA, USA) this solution was stored at 4°C in the dark. Eluent A was diluted ten-fold with water and vacuum-degassed before use (final eluent A; 10 m*M* KH_2PO_4). Eluent B was a 4:3:3 (v/v/v) mixture of acetonitrile, methanol and water. Eluent B was also degassed before use. Homocysteic acid was used as an internal standard. The OPA-ME derivatizing reagent was prepared by dissolving 50 mg OPA in 1 ml of methanol, then adding 53 μl of ME and 9 ml of 0.2 *M* potassium borate buffer (pH 9.4) and was stored at 4°C for not more than three days before use. Stock solutions (20 m*M*) of agmatine and homocysteic acid were made up in 50 m*M* nitric acid and kept at 4°C.

The isocratic method of measuring agmatine also utilized derivatization with OPA-ME. The mobile phase consisted of a mixture of 46% 10 m*M* potassium dihydrogen phosphate containing 3 m*M* octylsulfate sodium salt in water (pH 5.93), 34% acetonitrile and 20% methanol. The mobile phase was degassed before use. Quantitation was based on the method of additions of internal agmatine spike height [21]. Other aspects of the HPLC were the same as the gradient method.

2.3. Sample preparation

2.3.1. Rat brain procedure

Brain samples were prepared by a modification of the method of Reed and Belleroche [22]. Seven-to eight-week old male Sprague–Dawley rats weighing 250–300 g (Harlan, Indianapolis, IN, USA) were used. Rats were killed by rapid decapitation and their brains were quickly excised on an ice-cold Petri-dish. Seven brain regions (brainstem, frontal cortex, hypothalamus, hippocampus, midbrain, striatum and cerebellum) were dissected according to the model of Glowinski and Iversen [23] and stored at −80°C until the time of processing and assay.

For the gradient HPLC method [4], tissue samples were weighed and homogenized using a sonicator for 10 s in ice (setting 5; Sonifier Cell Disruptor, Model W185; Plainview, L.I., NY, USA) in 4 ml of ice-cold 10% (w/v) trichloroacetic acid per 100 mg tissue (wet weight). Homocysteic acid (4 μg) was added to serve as an internal standard. For the isocratic HPLC method, tissue samples were divided into six aliquots. Samples were homogenized as mentioned above, and 0, 0.5, 1.0, 2.0, 4.0 or 5.0 μ*M* (final concentration) of agmatine were added to serve as an internal standard. Sample homogenates for either the isocratic HPLC or gradient HPLC were then left on ice for 1 h and then centrifuged at 20 000 *g* for 25 min. The supernatant was washed 5 times using an equal volume of diethyl-ether and the aqueous phase was saved. Any remaining ether was evaporated with nitrogen at room temperature for 5 min. A volume of 15 μl of sample plus 15 μl of the OPA-ME derivatizing reagent was mixed for 2 min at room temperature. Immediately thereafter, 20 μl was injected into the HPLC system.

2.3.2. Plasma procedure

For the gradient HPLC method, heparinized blood was obtained from 4 healthy subjects, consisting of 3 males, ages 36.0±7.9 S.D. and 1 female, age 23 years. For the isocratic HPLC method, heparinized blood was obtained from 8 different healthy subjects, consisting of 6 males, ages 38.0±9.5 S.D. and 2 females, ages 33.5±3.5 S.D.. Subjects underwent a physical examination and psychiatric interview to ascertain physical health and normalcy prior to being enrolled in the study. This diagnostic interview included questionaires of prior illnesses, medication use, menstrual cycle record, smoking history and a life events questionaire. None of the subjects had a personal or family history amongst first-degree relatives of psychiatric illness, and none indicated a history of drug abuse. All subjects signed informed consent forms and they were paid for their participation.

At the time of blood drawings, subjects were required to verify that they had not consumed more than the equivalent of one glass of wine within the past three days, that they had turned the lights off at 11:00 p.m. to sleep the night before, and that they had refrained from breakfast (except juice), caffeine, vitamin pills, cigarettes and strenuous physical exertion on the morning of the blood drawing. Female's blood was not collected during the late luteal phase or during the menses of their cycles. Subjects arrived

for blood giving between 8:00 and 9:00 a.m. A 20-gauge angiocatheter was inserted into an arm vein, kept patent with heparin, and subjects were required to recline for 20 min prior to and during the blood collection. The first 3 ml of blood were discarded to avoid tissue thromboplastin. Ten ml of blood was then drawn into a green top vacutainer (Becton Dickinson, Cockeysville, MD, USA).

For the gradient HPLC method, blood was kept on ice for a short time until being centrifuged at 2000 *g* at 4°C for 15 min. Then, the plasma was immediately deproteinized with ice cold trifluoracetic acid (0.05 ml trifluoroacetic acid per 1 ml plasma) and left on ice for 1 h before centrifuging again at 10 000 *g* for 15 min at 4°C. The deproteinized plasma supernatant was collected and stored at −80°C until the time of assay. On the experimental day, 1 ml of plasma supernatant was mixed with 1 ml of 10% trichloroacetic acid and 2 μg of homocysteic acid, and then left on ice for 1 h before centrifuging at 20 000 *g* for 25 min at 4°C. The subsequent washes with diethylether and the derivatization of the samples were performed exactly as was done with the brain samples.

For the isocratic HPLC method, heparinized blood was obtained in a similar manner from healthy subjects. After being chilled on ice at 4°C, blood samples were centrifuged at 5000 *g* at 4°C for 15 min. The plasma was stored at −80°C prior to use. On each experimental day the samples were thawed and divided into 4 aliquots of 250 μl and 0, 0.5, 1.0 or 2.0 μ*M* (final concentration) of agmatine were added to serve as internal standards. Samples were then deproteinated by addition of 175 μl of 1.0 *M* perchloric acid and 75 μl of 0.1 *M* hydrochloric acid in methanol. The samples were left on ice for 1 h and then centrifuged at 6000 *g* for 10 min at 4°C. The 325 μl of supernatant was neutralized with 20 μl of 5 *M* NaOH. The sample (15 μl) was mixed with derivatization agent (15 μl) and then immediately injected (20 μl) into the column as before.

2.4. Chromatographic procedure

The gradient HPLC schedule is given in Table 1. A flow-rate of 1.0 ml/min was maintained throughout. Following each gradient HPLC procedure the columns were re-equilibrated with 80% eluent A and 20% eluent B for 20–30 min until a stable baseline was re-established, before injecting the next sample. Each sample was injected twice and peak heights were measured with a digital caliper (Brown and Sharpe, North Kingstown, RI, USA). The external standard curve was prepared fresh on each experimental day. For the isocratic HPLC method the flow-rate was also maintained at 1.0 ml/min throughout. The isocratic chromatography procedure took less than 15 min per sample.

Table 1
Chromatographic conditions for gradient HPLC of agmatine

Time (min)	A (%)	B (%)
0	80	20
5	73	27
15	50	50
25	30	70
35	25	75
45	20	80
50	40	60
55	60	40
60	80	20

2.5. Accuracy and precision

Aliquots of plasma were spiked with 50, 100 and 200 ng/ml of agmatine and concentrations of agmatine were determined against an external standard curve. The accuracy of the method was calculated based on the difference between the mean calculated and added concentrations of agmatine. The precision of the method was determined by calculating the within- and inter-day coefficients of variation (C.V.). The limit of detection of agmatine was defined as the amount of agmatine standard that resulted in a peak of approximately 2 times the noise level.

2.6. Quantitation and statistical analysis

Using the gradient HPLC method, the concentration of agmatine in each sample was calculated by measuring peak heights relative to an internal standard (homocysteic acid), and by comparing these peak heights with those obtained from an external standard curve for each substance. For the isocratic HPLC method the concentration of agmatine in each

sample was calculated by linear regression analysis of peak heights to the addition of varying concentrations of agmatine to samples (see Ref. [21]). Results are expressed as mean±S.E.M. Significance tests were performed using the Newman-Keul's test. A p-value (two-tailed) of less than 0.05 was considered significant.

3. Results and discussion

In this report we describe the development of gradient- and isocratic HPLC procedures for the quantitation of agmatine in rat brain and human plasma. The chemical structure of agmatine is shown in Fig. 1.

Representative chromatograms from our modified gradient HPLC procedure are shown in Fig. 2; an OPA-ME blank (A), a derivatized standard containing 4.0 ng agmatine (B), a derivatized extract of rat hypothalamus (C) and a derivatized human plasma extract (D). The gradient HPLC elution times of homocysteic acid and agmatine were about 12 and 46 min, respectively. The internal standard of homocysteic acid (designated peak-1) and agmatine (designated peak-2) were usually well separated from other substances in brain tissues and plasma. However, in some samples there were slow eluting peaks that interfered with the agmatine peak using the gradient procedure. Because small interfering peaks ran close to agmatine in some lots of OPA-ME, we always ran an OPA-ME blank with each batch of samples. If there were co-eluting peaks in the blank, we subtracted them from the sample peaks. Different ratios of eluent A and B were also attempted. If the ratio of eluent A was lowered we could not separate homocysteic acid. If the ratio of eluent A was increased, the overall elution time became longer (i.e., ≥70 min). Extending the time of higher percent B (80%) to 20 min, resulted in a cleaner profile, but the overall experiment took in excess of 80 min before all the peaks eluted. Finally, based on the method of Raasch et al. [17] we also added octyl sulfate to the mobile phase (3 mM throughout), but the addition of this ion-pairing agent did not lead to a better resolution of agmatine in the gradient.

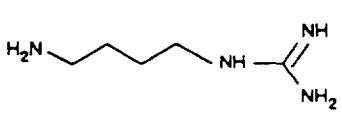

Fig. 1. Structure of agmatine.

The gradient HPLC method yielded a correlation coefficient (r^2) for the agmatine external standard curve of 0.997±0.004 S.D. (n=8). The average percent recovery of the internal standard (homocysteine acid) was 70.28%. The lower limit of detection of agmatine in the external standard was 250 fmol. The intra- and inter-assay C.V.s were less than 10%.

Concentrations of agmatine as measured by gradient HPLC procedure are shown in Table 2. Rat brain region varied in concentrations of agmatine from 0.331 to 1.105 μg/g. Agmatine concentrations were highest in the brainstem and lowest in the cerebellum (p<0.01). Li et al. [5] found the concentration of agmatine in whole bovine brain to be 1.5 to 3.0 nmol per gram of tissue (0.2 to 0.4 μg/g) by mass spectroscopy method. Therefore, our results are similar to those found by Li et al. [5]. Conversely, Raasch et al [17] reported the concentration of agmatine in rat brain was 2.4 ng/g, which is much lower. This difference may be due to differences in sample preparation (see below).

Unfortunately, our modificatory gradient HPLC method to quantitate agmatine was inconvenient for routine analyses use due to closely eluting peaks and to the length of each assay. Recently, Raasch et al. [17] indicated that the detection of agmatine in tissue samples after derivatization with OPA-ME required pre-HPLC solid–liquid extraction because otherwise primary amines in the homogenate would overcrowd agmatine in an isocratic HPLC elution profile. Unfortunately, their method lacked an internal standard and the OPA-ME derivative of agmatine in their samples was found to be unstable during the solid–liquid extraction step before HPLC. From this standpoint we next sought to extract the sample before derivatization and then immediately perform isocratic HPLC without solid–liquid extraction.

An isocratic HPLC method was developed that allows a clear separation of agmatine from other peaks (Figs. 3 and 4). Our method has a sensitivity to 100 fmol and a single analysis required less than

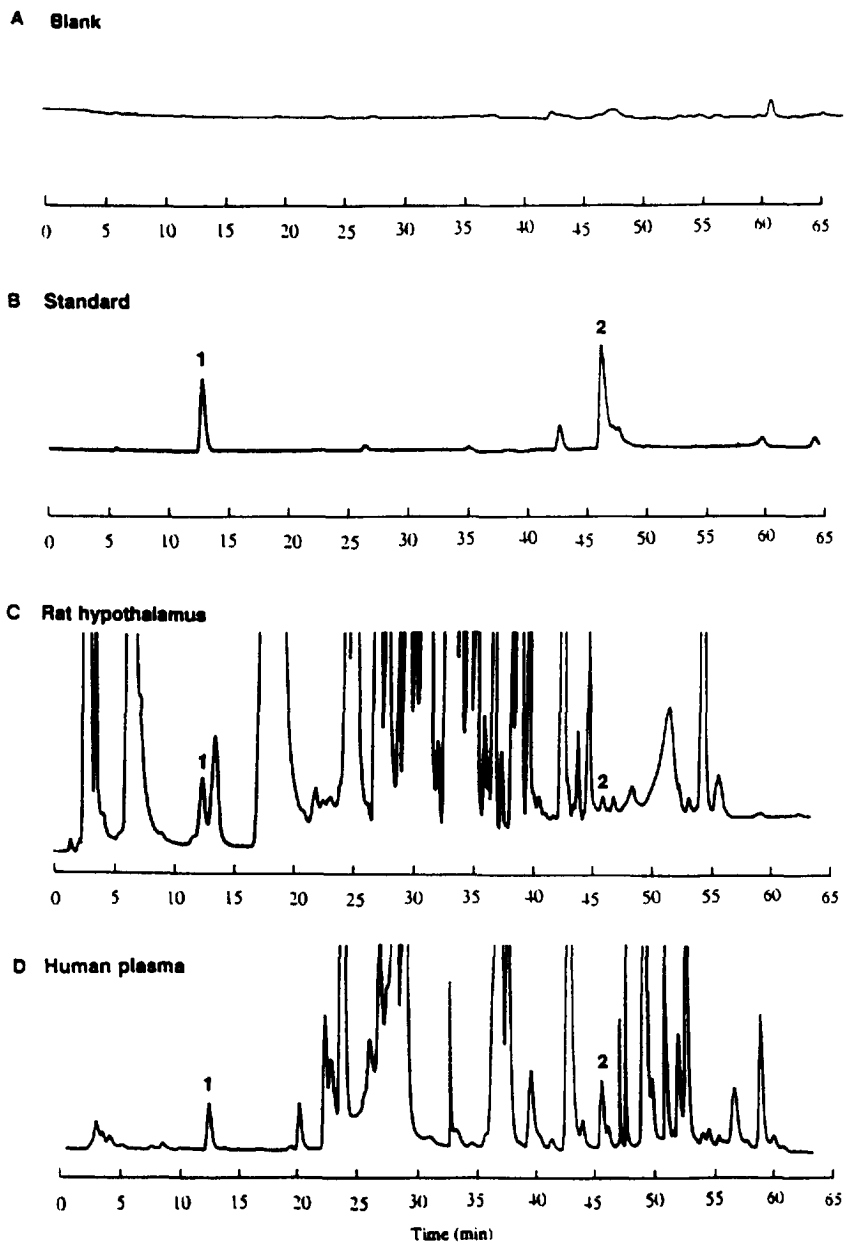

Fig. 2. Gradient HPLC chromatograms were obtained from the OPA-ME blank (A), a mixture of external standards (B; agmatine 4.3 ng; homocysteic acid 1.2 ng), a preparation of rat hypothalamus (C; agmatine 0.95 μg/g), human plasma (D; agmatine 51.6 ng/ml). Peak-1 is homocysteic acid spiked at the start of each tissue preparation, or injected as an external standard. Peak-2 is agmatine. Column, 5 μm Biophase ODS (250×4.6 mm I.D.); flow-rate, 1.0 ml/min. For eluent and gradient conditions, see Experimental Sections 2.2–2.4 and Table 1, respectively.

15 min (elution time of agmatine was 8–9 min). Three concentrations of spiked agmatine were chosen for the test of recovery. The average recovery of added agmatine was 84.1±3.1%, 95.5±2.8% and 98.8±1.5% for 50, 100 and 200 ng/ml concentrations spiked into human plasma, respectively

Table 2
Concentration of agmatine in rat brain and human plasma

Brain region	Gradient HPLC concentration (μg/g) (mean±S.E.M., $n=5$)	Isocratic HPLC concentration (μg/g) (mean±S.E.M., $n=4$)
Pons/medulla	1.105±0.149	1.272±0.044
Frontal cortex	0.760±0.194	0.891±0.079
Midbrain	0.873±0.159	–
Hypothalamus	0.844±0.189	–
Hippocampus	0.611±0.027	–
Cerebellum	0.331±0.080[a]	–
Human plasma (ng/ml)	46.84±3.73[b]	52.83±8.21[c]

The samples were not split samples for either method.
[a] $p<0.01$ (Values of pons/medulla are significantly higher than that of the cerebellum).
[b] $n=4$; [c] $n=8$.

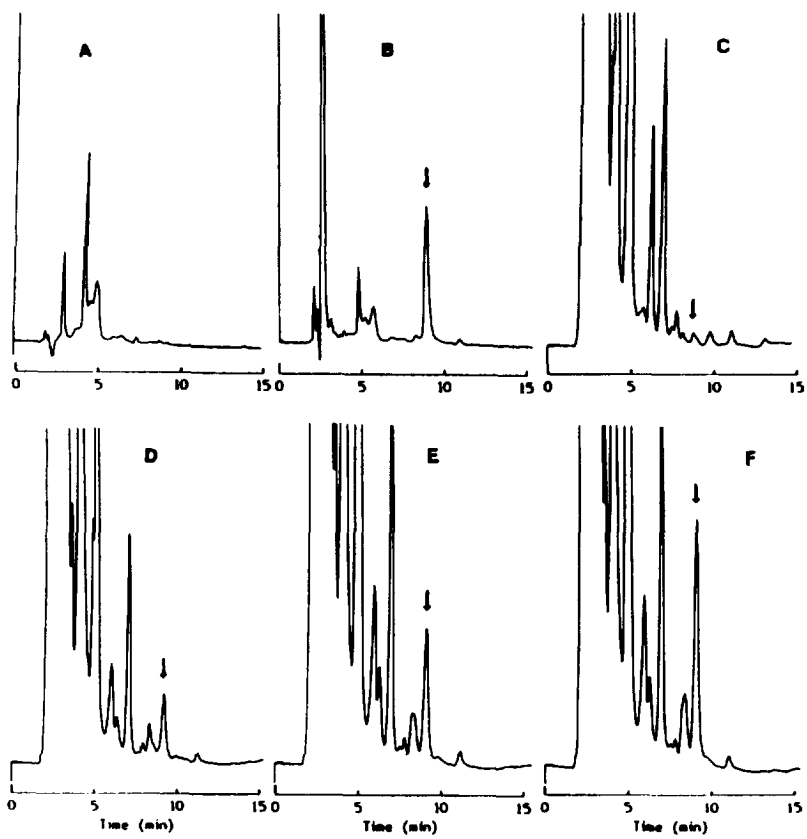

Fig. 3. Isocratic HPLC chromatograms showing method of agmatine additions in rat brain. Samples were (A) OPA-ME blank; (B) agmatine external standard (5.0 ng); (C) brain extract without added agmatine; (D) same brain homogenate spiked with 2.28 ng agmatine before sample extraction; (E) same brain homogenate spiked with 4.56 ng agmatine and (F) same brain homogenate spiked with 9.13 ng agmatine. Sample preparation, OPA-ME derivatization and chromatographic conditions are as described in Experimental Section 2.2, 2.3.1 and 2.4.

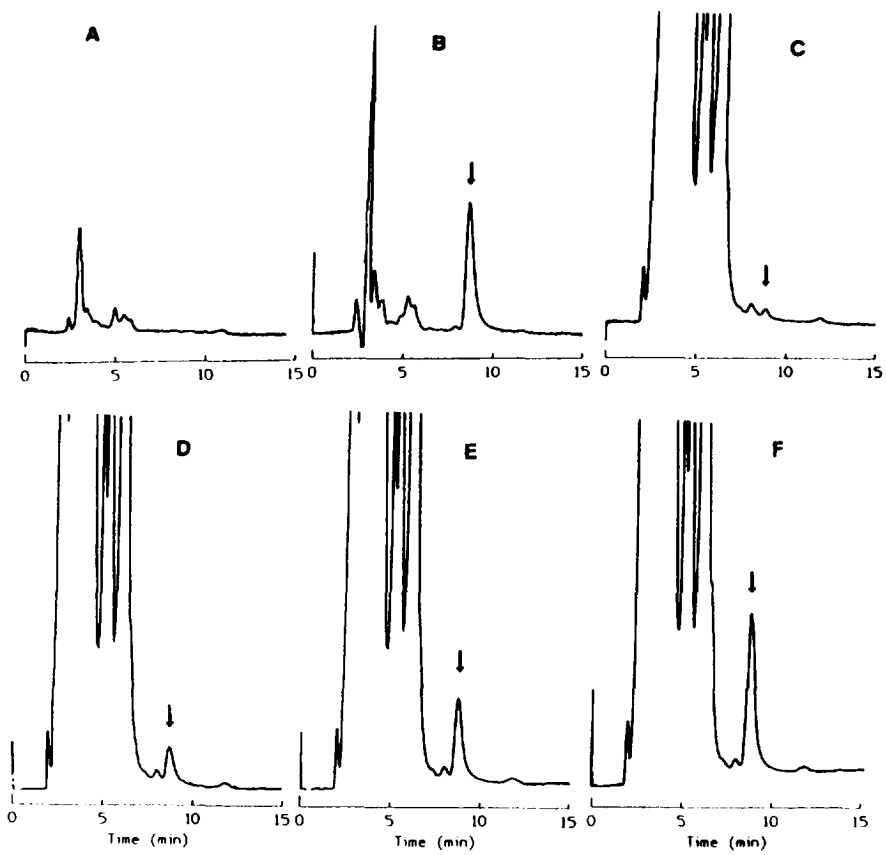

Fig. 4. Isocratic HPLC chromatograms showing method of agmatine additions in human plasma. Samples were (A) OPA-ME blank; (B) agmatine external standard (4.1 ng); (C) plasma extract without added agmatine; (D) same plasma spiked with 1.024 ng agmatine before sample extraction; (E) same plasma spiked with 2.048 ng agmatine and (F) same plasma spiked with 4.096 ng agmatine. Sample preparation, OPA-ME derivatization and chromatographic conditions are as described in Experimental Section 2.2, 2.3.2 and 2.4.

(Table 3). The average recovery of added agmatine (100 ng/ml) was 91.2±7.6% for rat brain. Chromatograms of agmatine are shown in Figs. 3 and 4. OPA-ME blank (A), external agmatine standard (B), brain sample without spike (C) and brain sample spiked with agmatine (D–F), are shown in Fig. 3. The chromatograms of human plasma without and with added agmatine are shown in Fig. 4.

Table 3
The recovery of added agmatine in human plasma (n=8) for isocratic-HPLC method

Added concentration (ng/ml)	Recovery (%) (mean±S.E.M.)	C.V.[a] (%)
50	84.1±3.1	9.9
100	95.5±2.8	7.9
200	98.7±1.5	4.4

[a] C.V.=(S.D./mean)·100.

Agmatine eluted from our isocratic HPLC with the same sharp peak and retention time regardless of whether it was an external standard, plasma endogenous substance or plasma spiked with agmatine standards. However, an additional small peak was detected just before the elution of agmatine in rat brain (Fig. 3). This may be a chromatographic artefact owing to the fact that brain samples were not at neutral pH (see Section 2.3.1). Alternatively, this peak could be an agmatine salt. This was not a significant problem in terms of quantitation since

<9% of the spiked agmatine in rat brain could possibly be accounted for in that minor peak.

The fluorometric detection of agmatine using isocratic HPLC was linear over the range tested (0–11.4 ng, $r=0.999$) and the accuracy was comparable to that obtained for calibration standards. Complete standard curves of this type were obtained on each experimental day. The concentrations of agmatine in rat brain and human plasma measured by the method of additions were nearly identical to those determined by gradient HPLC using homocysteic acid as internal standard (Table 2).

One shortcoming of our isocratic HPLC procedure was that a suitable internal standard could not be found under this condition. We overcame this problem by adding different concentrations of agmatine spike as internal standard [21]. Quantitation is based on the method of additions of internal agmatine spikes. The within-day precision of our assay was determined at three concentrations by replicate assays of external standard and of samples spiked with agmatine (50, 100 and 200 ng/ml). The day-to-day assay variation was assessed at these three concentrations over a period of three consecutive days with three replicates at each concentration. The within-day coefficients of variation were less than 6% for external standards and were less than 7% for plasma samples. The day-to-day coefficients of variation were less than 3% for external standards and were less than 8% for samples (Table 4). These values indicate good reproducibility of the method.

This study establishes that agmatine is present in human plasma and in various rat brain regions. The distribution of agmatine among rat brain regions is similar to that reported for imidazoline receptors [19,20] and is therefore consistent with a potential role for agmatine as a neurotransmitter for brain imidazoline receptors. Our two HPLC techniques provide reliable methods for analysis of agmatine in various biological samples.

Acknowledgments

This research was supported by a grant from the National Institute of Mental Health MH49248. We

Table 4
Precision and accuracy of the isocratic-HPLC method

Theoretical concentration (ng/ml)	Concentration found (mean±S.D., ng/ml)	C.V. (%) ($n=4$)	Accuracy (% of mean deviation)
External standard			
Within-day			
50	48.23±2.89	5.99	−3.54
100	96.12±5.41	5.64	−3.88
200	201.43±9.46	4.70	+0.72
Day-to-day			
50	49.38±0.99	2.00	−1.24
100	98.48±2.39	2.42	−1.52
200	203.08±2.46	1.21	+1.54
Nominal plasma sample added agmatine[a]			
Within-day			
50	55.21±3.82	6.92	+10.42
100	100.04±6.16	6.16	+0.04
200	198.20±4.57	2.30	−0.90
Day-to-day			
50	52.25±4.21	8.06	+4.50
100	95.56±6.42	6.72	−4.44
200	195.91±8.74	4.46	−2.04

[a] Minus the amount of endogenous agmatine ($n=4$).

thank Dr. He Zhu for assistance in preparing the plasma samples.

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