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Determination of agmatine in biological samples by capillary electrophoresis with chemiluminescence detection

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Abstract

A fast and simple method based on capillary electrophoresis (CE) with chemiluminescence (CL) detection has been developed for the determination of agmatine, a recently identified neurotransmitter/modulator. The CE run time was approximately 2 min for each sample injected. CL detection employed a lab-built reaction flow cell and a photon counter. The CL reagents used were luminol and NaBrO. The optimized conditions for the CL detection were 5×10^{-4} M luminol added to the CE running buffer and 5.0×10^{-4} M NaBrO in 100 mM $NaCO_3$–NaOH buffer solution at pH 12.5 introduced post column. Detection limit for agmatine was 4.3×10^{-6} M (S/N = 3). The precision (R.S.D.) on peak height (at 1×10^{-5} M agmatine) and migration time were 3.7 and 2.5%, respectively. The present CE-CL method was evaluated with the determination of agmatine in tissue samples taken from rat brain, and rat and monkey stomachs. Samples were directly injected into the CE-CL system after the removal of proteins. A higher level of agmatine was detected in the stomach samples. Agmatine concentrations in the tissue samples taken from rat and monkey stomachs were similar at ∼1950 ng/g wet tissue.

Keywords: Capillary electrophoresis; Chemiluminescence detection; Agmatine; Tissue samples; Luminol

1. Introduction

Agmatine is formed by the enzymatic decarboxylation of L-arginine. It has been detected in human plasma, mammals, invertebrates, insect, bacteria, and plants [1–6]. Recent evidences indicate that agmatine is synthesized in the brain and stored primarily in neurons [7,8]. Studies have shown that agmatine exhibits clonidinedisplacing substance activity for certain α_2-and non-adrenergic imidazoline-binding sites in rat brain [1], modulates the hypothalamic release of leutenizing hormone [9], and functions as a stimulator of gastric secretion in the stomach [10].

In search for a better understanding of its physiological functions, the determination of agmatine in biological samples is necessitated. A few HPLC methods were developed [2,3,11]. Li et al. measured agmatine in bovine brain using HPLC–electrospray mass spectroscopy [1]. Recently, an HPLC method with highly sensitive laser induced fluorescence detection for the determination of agmatine in human plasma and rat tissues was reported [12].

Capillary electrophoresis (CE) offers many advantages including high separation efficiency, short run time, instrumentation simplicity, minimum operation cost, and compatibility with small sample volumes. Therefore, it is an attractive alternative to HPLC technique for analysis of biological samples. Chemiluminescence detection (CL) due to its simple optical system and low background nature has become a candidate for a new and sensitive CE detection scheme [13–15]. In this work, a CE-CL method was developed for the determination of agmatine in biological tissue samples, and the method was validated by analyzing rat brain tissue, rat stomach tissue, and monkey stomach tissue. The levels of agmatine in rat brain tissue and rat stomach tissue found were in accordance with the results reported previously [3], which were obtained by an HPLC procedure with fluorescence detection.

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2. Experimental

2.1. Chemicals and reagents

Luminol was purchased from Fluka (Buchs, Switzerland). Agmatine sulphate was obtained from Sigma Chemicals (St. Louis, MO, USA). All the other chemicals and organic solvents used in this work were of analytical grade. Agmatine was dissolved in 0.1 M HCl solutions. Milli-Q water was used throughout. All solutions were filtered through a 0.45 μm membrane filter (Costar, Cambridge, MA, USA).

The 0.1 M sodium borate stock solution was prepared by dissolving 3.814 g $Na_2B_4O_7{\cdot}10H_20$ in 100 ml water; 10 mM luminol solution was prepared by dissolving 0.0177 g luminol in 2 ml of 0.1 M NaOH solution, and diluting to 10 ml with water. The 0.1 M NaBrO stock solution was prepared by dissolving 0.2 ml Br_2 in 30 ml of 0.1 M NaOH solution, and used 2 days later. The running buffer was prepared by mixing 8.0 ml of 0.1 M sodium borate solution with 1.0 ml of 10 mM luminol solution and diluting to 20 ml with water. The pH value of the running buffer was adjusted to 9.3 with 0.1 M NaOH solution. The oxidizer solution was prepared by dissolving 125 μl of the NaBrO stock solution in 25 ml of 0.1 M $NaCO_3$–NaOH solution (pH 12.5).

2.2. CE-CL apparatus

The CE-CL separations were performed using a laboratory-built system described previously [16]. A high-voltage supply (0–30 kV, Beijing Cailu Science Instrument Company, Beijing, China) was used to drive the electrophoresis. A 50 cm × 75 μm i.d. uncoated fused-silica capillaries (Hebei Optical Fiber, China) was used for the separation. The polyimide coating on the 2.5 cm long end section of the capillary was burned and moved. After etching with HF for 1 h, this end was inserted into the reaction capillary, which was 530 μm i.d. (Hebei Optical Fiber, China). A four-way Plexiglas joint held the separation capillary and the reaction capillary in place. The CL solution was siphoned into a tee with a 50 cm × 530 μm i.d. fused-silica capillary at a height differential of 5 cm. The grounding electrode was put in one joint of the tee. The CL solution flowed down to the detection window, which was made by burning 1 cm of the polyimide of the reaction capillary and was placed in front of a photomultiplier tube (PMT, R374 equipped with a C1556-50 DA-type socket assembly, Hamamatsu, Shizuoka, Japan). The buffer reservoir at the high-voltage end was enclosed in a plexiglass box. CL emission was collected by the PMT, and recorded and processed with an IBM compatible computer using in-house written software. The CE conditions are described as following. A new capillary was preconditioned by flushing with 1 M NaOH for 30 min before the first use. Between two consecutive injections, the capillary was rinsed sequentially 0.1 M NaOH, water, and running buffer for 3 min each. Samples were injected into the capillary by hydrodynamic flow at a height differential of 20 cm for 10 s. Running voltage was 18 kV. The typical operating current was 80 μA. Electrophoresis running buffer was 40 mM borate buffer (pH 9.3) containing 5.0×10^{-4} M luminol. Oxidizer solution was 5.0×10^{-4} M NaBrO in 100 mM $NaCO_3$–NaOH buffer solution (pH 12.5).

2.3. Preparation of tissue samples

Rat brain, stomach, and monkey stomach tissues were stored at −80 °C till analysis. A potion of the tissue sample (brain tissue 200 mg; stomach tissue 100 mg) was cut into pieces as small as possible by scissors, and then ground with 0.1 M HCl solution (500 μl) on ice using a tissue grinder. The homogenate was transferred into a 1.0-ml centrifuge tube. The tube was sonicated for 5 min and centrifuged (2000 × *g* for 10 min). The supernatant was transferred into another 1.0-ml centrifuge tube and equal amount of ice-cold acetonitrile was added. The solution was vortexed and centrifuged (2000 × *g* for 10 min). The supernatant was transferred into another 1.0-ml centrifuge tube and dried with a N_2 stream. The residue was dissolved in 40 mM borate buffer of pH 9.3 (brain tissue 100 μl; stomach tissues 200 μl). The solution was vortexed and kept at 4 °C before injection.

3. Results and discussion

3.1. Chemiluminescence detection of agmatine

Agmatine, a polyamine, was found either to inhibit or to enhance the CL reaction between luminol and NaBrO depending on the experimental conditions (e.g. the concentrations of luminol and NaBrO). To maximize the sensitivity of CL detection, the effects of luminol and NaBrO concentrations, and the pH value of the CL solution were investigated. In these experiments, a 5×10^{-5} M agmatine solution was injected into the CE-CL system. The CL background and the agmatine peak height were recorded. Keeping NaBrO concentration at 5×10^{-4} M and pH value of the oxidizer solution at 12.5, luminol concentration in a range from 1×10^{-4} to 3×10^{-3} M was tested. As can be seen from Fig. 1, agmatine enhanced the CL emission producing a positive peak when luminol concentration was $<2.25 \times 10^{-3}$ M. The CL background increased tremendously as luminol concentration increased. To keep the baseline CL emission at minimum

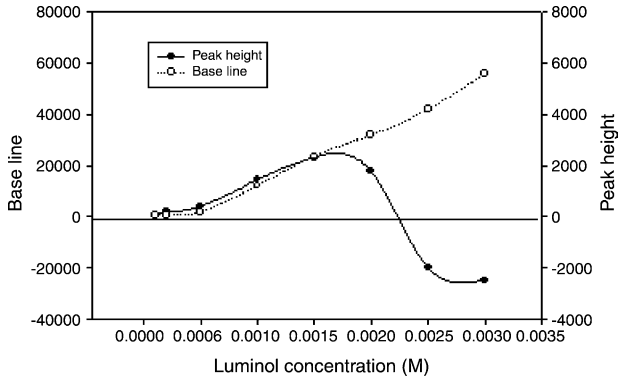

Fig. 1. Effects of luminol concentration on CL detection of agmatine. Electrophoresis electrolyte was 40 mM borate (pH 9.3) containing luminol at different concentrations. Oxidizer solution was 100 mM $NaCO_3$–NaOH solution (pH 12.5) containing 5.0×10^{-4} M NaBrO. Capillary was 75 μm i.d. × 50 cm effective length. Voltage applied was 18 kV. Concentration of agmatine = 5×10^{-5} M.

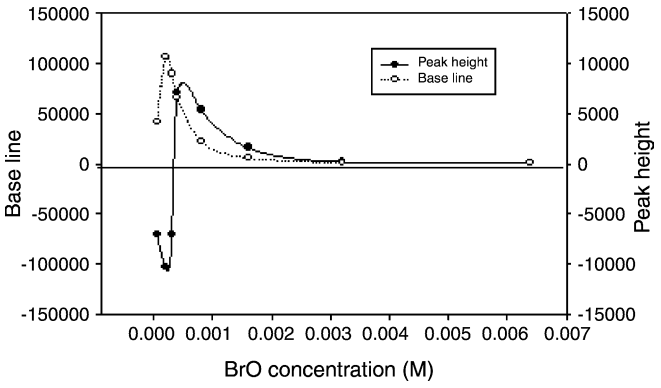

Fig. 2. Effects of NaBrO concentration on CL detection of agmatine. Electrophoresis running buffer was 40 mM borate buffer (pH 9.3) containing 5.0×10^{-4} M luminol. Oxidizer solution was 100 mM $NaCO_3$–NaOH solution (pH 12.5) containing NaBrO at different concentrations. Other conditions were as in Fig. 1.

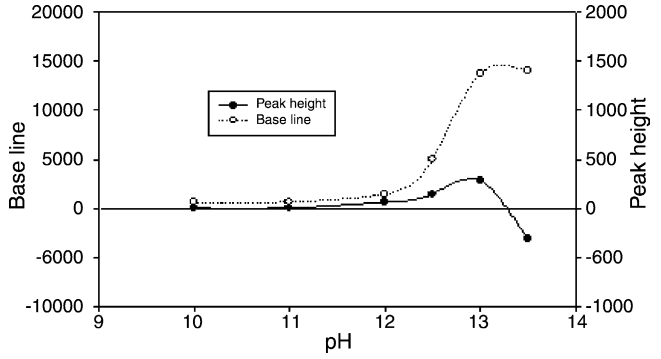

Fig. 3. pH effects on the CL detection of agmatine. Electrophoresis running buffer was 40 mM borate buffer (pH 9.3) containing 5.0×10^{-4} M luminol. Oxidizer solution was 5.0×10^{-4} M NaBrO in 100 mM $NaCO_3$–NaOH solution at varying pH. Other conditions were as in Fig. 1.

for optimal detection repeatability, a luminal concentration of 5×10^{-4} M was chosen for further studies. Fig. 2 shows the effects of NaBrO concentration. A concentration range from 1×10^{-4} to 6.4 $\times 10^{-3}$ M was tested. The CL background first increased and then decreased with the increase in NaBrO concentration, remaining constant when the concentration was greater than 2×10^{-3} M. The CL signal produced by agmatine was either positive or negative depending on NaBrO concentration. An oxidizer solution containing 5×10^{-4} M NaBrO was selected for further experiments. The pH effects were studied in a pH range of 10.0–13.5. The results are illustrated in Fig. 3. The CL background increased sharply when the pH value became higher than 12.3 and a negative peak from agmatine was observed when the pH value was greater than 13.2. Therefore, the CL oxidizer solution was buffered at 12.5 for a maximum enhanced CL signal.

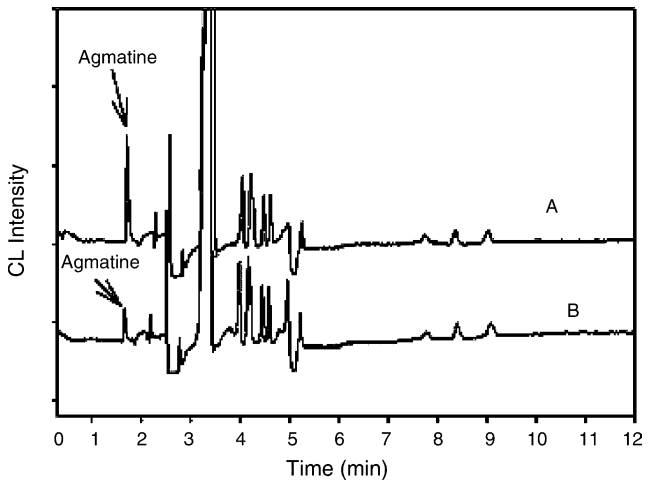

Fig. 4. Electropherograms obtained from separating mixture solutions containing agmatine at 1.0×10^{-5} M (A) or 3.0×10^{-5} M (B) and dopamine, adrenaline, and 20 protein amino acids (2.5×10^{-5} M each). Electrophoresis running buffer was 40 mM borate buffer (pH 9.3) containing 5.0×10^{-4} M luminol. Oxidizer solution was 5.0×10^{-4} M NaBrO in 100 mM $NaCO_3$–NaOH buffer solution (pH 12.5). Capillary was 75 μm i.d. × 50 cm effective length. Voltage applied was 18 kV.

3.2. Optimization of CE conditions

Many endogenous compounds such as amino acids and other biogenic amines are present in biological samples and interfere with the CL detection of agmatine. In order to achieve an efficient separation of agmatine from these compounds, CE conditions such as the concentration of borate buffer, pH of the electrolyte solution, and the applied voltage were investigated. After careful studies, the separation conditions were selected as following: 75 μm i.d. × 50 cm long capillary, 18 kV voltages, and a running buffer containing 40 mM borate at pH 9.3. Under the optimized conditions, endogenous compounds such as dopamine, adrenaline, and 20 protein amino acids were well separated from agmatine. As can be seen from Fig. 4, agmatine peak heights from these mixture solutions were in line with those obtained from authentic agmatine solutions. An advantage of this CE separation was that agmatine was eluted out before all of these endogenous compounds in less than 2 min after injection, which shortened significantly the run time of sample analysis.

3.3. Analytical figures of merit

The CE-CL method was evaluated in terms of the reproducibility of migration times, peak heights/peak areas, response linearity, and the limit of detection. The reproducibility was investigated by injecting a 2.0×10^{-5} agmatine solution 11

Table 1
Reproducibility of migration time, peak area, and peak height

	Migration time		Peak area		Peak height	
	Average (min)	R.S.D. ($n = 11$, %)	Average (μV s)	R.S.D. ($n = 11$, %)	Average (μV)	R.S.D. ($n = 11$, %)
Agmatine	1.68	2.5	388.4	5.9	446.7	3.7

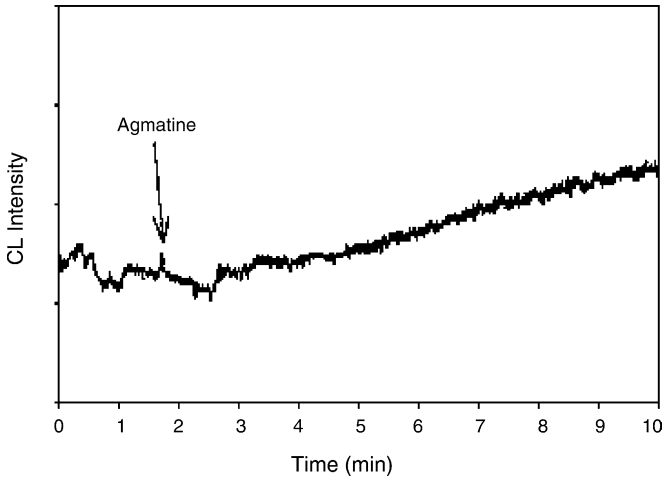

Fig. 5. Electropherogram obtained from separating a diluted agmatine solution containing 2.5×10^{-6} M agmatine (very close to the detection limit). CE and CL conditions were as in Fig. 4.

times and recording the migration times and peak heights/areas. The results obtained are summarized in Table 1. As can be seen, good reproducibility was obtained. The precision (R.S.D. in peak height) was 3.7%. To test the CL response linearity, a series of agmatine standard solutions were tested to determine the linearity between the agmatine concentration and CL intensity. Linear regression analysis of the results yielded the following equations:

$$H = 5.562C + 3.070 \qquad r = 0.9996$$

where H is peak height (μV) and C is the concentration of the agmatine in μM. The calibration curve was linear over the concentration range of from 3.5×10^{-6} to 2.2×10^{-4} M. Although these results were from agmatine standard solutions, a similar calibration curve was obtained from the use of rat brain tissue extracts. To investigate the detection limit of this CE-CL method, diluted agmatine solutions were analyzed. Fig. 5 shows the electropherogram obtained from an analysis of a sample containing 2.5×10^{-6} M agmatine. From this electropherogram, the limit of detection (signal/noise = 3) for agmatine in water was estimated to be 2.0×10^{-6} M.

3.4. Analysis of biological samples

Brain and stomach tissue samples dissected from two adult rats and two monkeys were analyzed. A typical electropherogram obtained from a rat brain sample (~2 nl injected) is shown in Fig. 6A. The peak corresponding to agmatine was well identified. To verify the peak identification, the sample was spiked

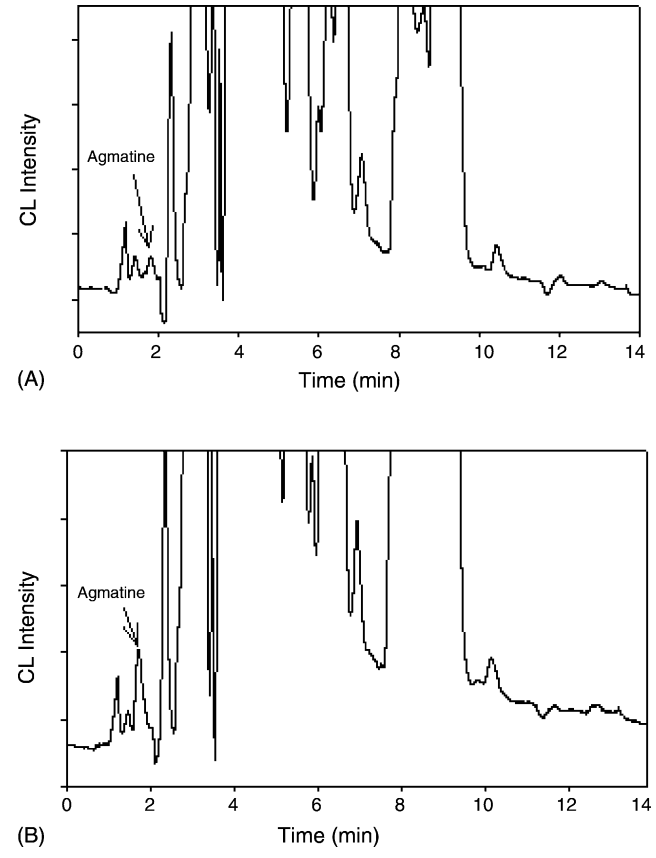

Fig. 6. Electropherograms obtained from the separation of a rat brain sample (A) and the sample spiked with agmatine at 2.0×10^{-5} M (B). CE and CL conditions were as in Fig. 4. Note that the CE run could be stopped and the capillary flushed with running buffer after agmatine was eluted at ~2 min. These 14-min electrophoregrams intend to show that many compounds in the sample matrix were separated and detected by the CE-CL system.

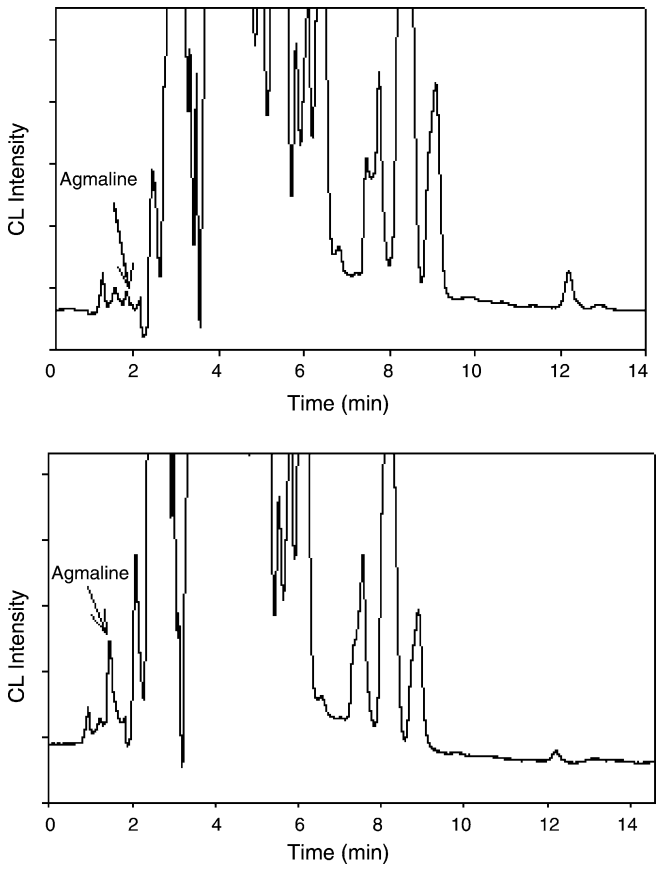

Fig. 7. Electropherogram obtained from the separation of a rat stomach sample (A) and the sample spiked with agmatine at 2.0×10^{-5} M (B). CE and CL conditions were as in Fig. 4.

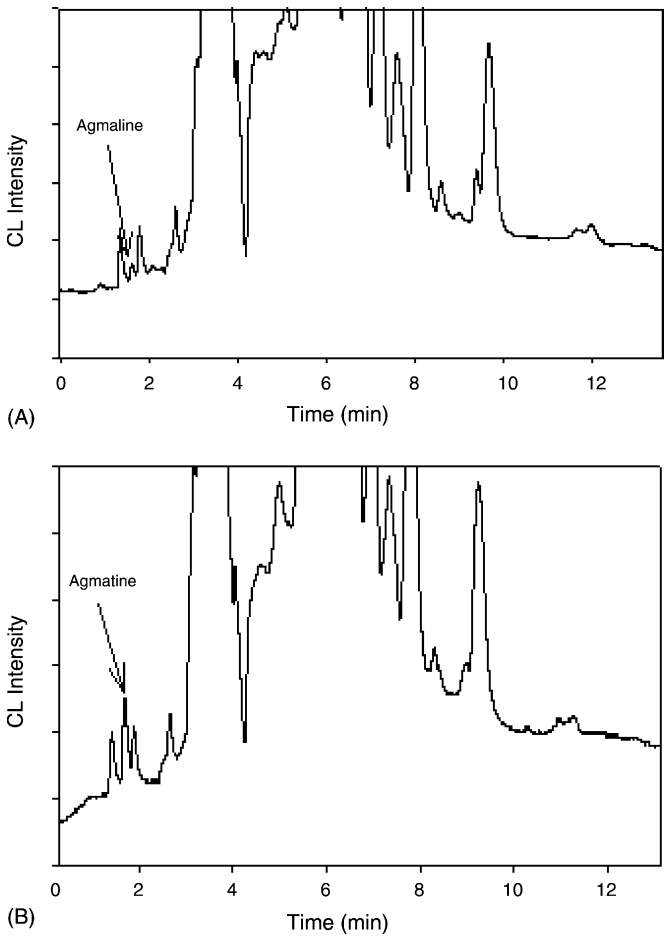

Fig. 8. Electropherogram obtained from the separation of a monkey stomach sample (A) and the sample spiked with agmatine at 2.0×10^{-5} M (B). CE and CL conditions were as in Fig. 4.

with agmatine at 20 μM and separated again. The electropherogram obtained is shown in Fig. 6B. By comparing the two CE traces shown in Fig. 6A and B, it can be seen that only agmatine peak increased in size without other major changes in the electropherograms. Electropherograms from separating rat and monkey stomach samples are shown in Figs. 7A and 8A. Agmatine was detected in both tissue samples. The agmatine peaks were also verified by spiking agmatine at 20 μM and re-run the sample. The electropherograms obtained are shown in Figs. 7B and 8B. The analytical results from analyzing all the tissue samples are summarized in Table 2. Agmatine was detected at a significantly higher level in rat stomach (1945.6 ng/g wet tissue) than in the brain (464.1 ng/g wet tissue). These results are in accordance with those obtained by a procedure based on HPLC/fluorescence detection [3]. Compared with the HPLC/fluorescence methods reported previously, the present CE-CL method is much faster and needs no pre-column derivatization. In addition, the sensitivity of the CL detection is comparable with that of fluorescence detection. Agmatine level in monkey stomach was determined for the first time in this work. It was found to be similar to that in rat stomach. Recoveries of agmatine from these tissue matrixes were studied. Agmatine was added to these tissues samples at 5 μg/g wet tissue. The recoveries were found to be in the range of 92–104% (Table 3).

Table 2
Analytical results of agmatine in rat and monkey tissues

Sample	Agmatine content (ng/g wet tissue)
Rat brain-1	507.60 ± 26.1
Rat brain-2	420.60 ± 22.0
Rat stomach-1	1760.1 ± 70.2
Rat stomach-2	2131.1 ± 76.7
Monkey stomach-1	1915.3 ± 64.6
Monkey stomach-2	2053.2 ± 73.9

Mean ± S.D. of four measurements.

Table 3
Recovery of agmatine from rat and monkey tissue samples

Sample	Concentration of agmatine (ng/g)			
	In the sample	Added	Total found	Recovery (%)[a]
Rat brain-2	420.60	5000.0	5050.0	92.5 ± 3.6
Rat stomach-1	1760.1	5000.0	6965.1	104 ± 2.7
Monkey stomach-1	1915.3	5000.0	6650.3	94.7 ± 4.1

[a] Recovery = (total found − found in the sample)/added (%). Mean ± S.D., $n = 3$.

4. Conclusions

Agmatine in biological samples was efficiently separated from the endogenous compounds by CE within 2 min. Chemiluminescence detection based on luminol–NaBrO reaction was simple and sensitive enough for agmatine determination following the CE separation. The CE-CL method was proven useful for the determination of agmatine in tissue samples including rat brain, and rat and monkey stomachs. The major advantages of the present method were its short run time and simple instrumentation. After agmatine being eluted out within 2 min after sample injection, the capillary could be rinsed with running buffer and a new run be started.

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