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HPLC/MS/MS for quantification of two types of neurotransmitters in rat brain and application: Myocardial ischemia and protection of Sheng-Mai-San

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ABSTRACT

Sheng-Mai-San (SMS), a traditional Chinese multiherbal formula, is widely used in clinic for the treatment of myocardial ischemia (MI) in China. Recently, it has been shown that the change of neurotransmitters in central nervous system is closely related to the pathogenesis of MI, whether SMS might affect the neurotransmitters at central nervous system in MI patients has not been studied yet. In this study, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous determination of two types of neurotransmitters (neuropeptides and monoaminergic neurotransmitters) in rat brain tissue was developed. The instrument was operated under the multiple reaction monitoring (MRM) mode using electrospray ionization (ESI) in the positive ion mode. A good linear relationship with coefficients $\geq$0.99 was achieved over the concentration ranges of 10–1000 ng mL^{-1} for 5-hydroxytryptamine (5-HT) and norepinephrine (NE); 2–250 ng mL^{-1} for methionine-enkephalin (M-ENK) and leucine-enkephalin (L-ENK). Quantification limit for 5-HT and NE was 4.0 ng mL^{-1}; and 2.0 ng mL^{-1} for M-ENK and L-ENK. The intra- and inter-day precision was less than 15% and accuracy was within ±15%. The analysis revealed significant reductions at the levels of 5-HT ($p < 0.01$), NE ($p < 0.01$), M-ENK ($p < 0.05$) and L-ENK ($p < 0.01$) in the MI group compared to the control group. These findings demonstrate that myocardial ischemia reduces the concentrations of 5-HT, NE, M-ENK and L-ENK in rat brains, while SMS shows protective effects on MI associated with reversing these four neurotransmitters to sham levels.

1. Introduction

Myocardial ischemia (MI) is characterized by an imbalance between myocardial oxygen supply and demand, complex neural pathophysiology, and substantial morbidity and mortality [1]. Recent clinical and experimental evidence has showed that the change of neuropeptides and monoaminergic neurotransmitters in central nervous system might be critical mediator in the pathogenesis of myocardial ischemia. It has been shown that brain catecholamines were closely associated with the regulation of cardiovascular function [2] and both 5-HT and catecholamine mediate the sympathetic excitation [3]. Meanwhile, the release of monoaminergic neuronal transmitters in central nervous system was increased in heart failure patients; and local ischemia has been shown to induce the release of monoamines into synapses and the surrounding extracellular region [4]. In addition, enkephalins act as functional, physiological, and humoral transmitters between the central nervous system and the immune system [5]; they were reported to modulate the immune responses of myocardial ischemia, reduce the force of contraction [6] and regulate the sympathetic nervous system activity [7]. Therefore, the determination of changes in basal levels of neuropeptides and monoaminergic neurotransmitters in the brain could provide evidence to explore the mechanism of MI related to the central nervous system.

Currently many bioanalytical techniques have been developed for the analysis of monoaminergic neurotransmitters and neuropeptides. GC [8,9], CE [10,22], LC–UV [11,12] and LC-electrochemical [13,14] fluorescent detection [14–17] have been reported to determine monoaminergic neurotransmitter and neuropeptide levels. However, most of these methods require derivatization of the analytes, the use of ion-pairing reagents, and large quantities of samples. LC with tandem mass spectrometry (LC–MS/MS) method for the measurement of monoaminergic neurotransmitters and neuropeptides individually in biological fluids or tissues has been reported [18–22], primarily due to the inherent selectivity and sensitivity of MS/MS detection. But there is no report about the simultaneous detection of two types of neurotransmitters in brain tissue. Thus, the determination of monoaminergic neurotransmitters and neuropeptides in a single run will be more efficient and require less samples compared with previous methods.

Sheng-Mai-San (SMS) is an important prescription in traditional Chinese medicine (TCM), consisting of three herbs of Radix Ginseng, Radix Ophiopogonis and Fructus Schisandrae. It was firstly described by an eminent Chinese physician Dongheng Li in Yuan Dynasty. Currently, it is widely used clinically for the treatment

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of ischemic heart disease in China. Recent research has showed that the total saponins in SMS are indispensable for the efficacy of the treatment of myocardial ischemia [23]. However, there is little research concerning the effect of SMS on the secretion of neurotransmitters in brain at MI disease model. In order to evaluate the effect of SMS on the secretion of brain neurotransmitters at myocardial ischemia model, a LC–MS/MS assay method was developed and validated for the determination of 5-hydroxytryptamine (5-HT), norepinephrine (NE), methionine-enkephalin (M-ENK) and leucine-enkephalin (L-ENK) in brain tissue.

2. Materials and methods

2.1. Materials and animals

Norepinephrine (NE), 5-hydroxytryptamine (5-HT), leucine-enkephalin (L-ENK), methionine-enkephalin (M-ENK), isoprenalin, 3,4-dihydroxybenzylamine (DHBA, IS1) and [D-Ala2, D-Leu5]-enkephalin (DADLE, IS2) were purchased from Sigma (St. Louis, MO, USA). Chemical structures of test neurotransmitters and internal standards are given in Fig. 1.

Propranolol hydrochloride tablets were purchased from Xingpasi Pharmaceutical Co., Ltd. (Shanghai, China).

HPLC-grade acetonitrile was obtained from Tedia (Fairfield, OH, USA); AR-grade formic acid, chloroform, isopropanol, TFA and methanol were obtained from Jiangsu Hanbon (Jiangsu, China); water was purified by a Milli-Q academic water purification system (Milford, MA, USA).

Male Wistar rats (200–250 g) were purchased from Yangzhou University (Yangzhou, China). The animals were housed in cages with free access to food and tap water under standard conditions of humidity (50 ± 10%), temperature (25 ± 2 °C) and light (12 h light/12 h dark cycle). All animals were handled with humane care throughout the experiment.

2.2. Preparing sample of SMS

The sample of SMS was prepared by combining Radix Ginseng (20 g), Radix Ophiopogonis (60 g), Fructus Schisandrae (30 g). Then the mixture was decocted to boil with 1100 mL, 880 mL and 660 mL water for 60 min, respectively. The extracted solutions were combined and evaporated by a Buchi rotavapor (Flawil, Switzerland) to approximate 220 mL. The sample can be administrated orally.

2.3. HPLC–MS/MS conditions

HPLC–MS/MS analysis was performed with a Waters Alliance HPLC system (Waters, Milford, MA, USA), both a PDA 2996 photo diode array detector (Waters, Milford, MA, USA), a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) and a Z-spray electrospray ionization (ESI) source operated in both negative and positive mode. MassLynx™ software (version 4.0, Waters, Milford, MA, USA) was used to control the instruments, and for data acquisition and processing.

An Alltima C18 column (250 mm × 4.6 mm, i.d. 5 μm, Alltech Company, Deerfield, IL, USA) used for all chromatographic separations. Column temperature was maintained at 30 °C. The samples were separated using a gradient mobile phase consisting of CH_3CN (A) and H_2O–HCOOH (B) (100:0.05, v/v). A gradient programmer was used as follows: 0–10 min, 4–10% A; 10–15 min linear increase to 100% A. The flow rate was 1 mL/min and 20 μL of sample solution was injected in each run. HPLC effluent was introduced directly to the electrospray source operated in positive ionization mode connected to a triple quadrupole mass spectrometer.

Nitrogen was used as nebulizer gas at 50 L/h flow rate and as desolvation gas at 500 L/h. Ion source block and desolvation temperatures were set respectively at 120 °C and 400 °C. Capillary voltage was 3 kV. Total ion current chromatograms were obtained by mass spectrometer in multiple monitoring modes, the MRM m/z transitions monitored were: 177.11 → 160.06 for 5-HT; 170.15 → 152.07 for NE; 574.04 → 120.1 for M-ENK; 556.07 → 120.06 for L-ENK; 140.1 → 123.04 for IS1 and 570.18 → 177.15 for IS2, respectively, see Fig. 2.

2.4. Sample preparation

2.4.1. M-ENK and L-ENK sample preparation

A method from literature was modified and used in sample preparation [22]. The rats were decapitated, and the hypothalamus were dissected and frozen on dry ice. Each frozen hypothalamus tissue sample was weighed quickly and homogenized in 2 mL cold 1 M acetic acid. The homogenate (800 μL) mixed with 20 μL of the IS2 (200 ng mL^{-1}) and then incubated for 4 h at 4 °C. After centrifugation at 11,000 rpm for 10 min at 4 °C, the supernatant was subject to the solid-phase extraction (SPE). The analytes were extracted from the hypothalamus tissue using SPE cartridges with C18 sorbent (500 mg/3 mL Supelclean™ ENVI-18 SPE Tube; Supleco Inc.). The SPE column was activated and equilibrated using 2 mL of methanol and 2 mL of 0.04% aqueous TFA before the sample was applied. After rinsing the column with 0.5 mL of CH_3CN/0.04% aqueous TFA (5:95, v/v), the analytes were eluted with 2 mL of CH_3CN/0.04% aqueous TFA (45:55, v/v), which was collected as eluent A.

2.4.2. NE and 5-HT sample preparation

A method from literature was modified and used in sample preparation [21]. Brain tissues (not including hypothalamus) were isolated and frozen on dry ice after rats were decapitated. Each frozen brain tissue sample was weighed quickly and homogenized in cold methanol (1:4, w/v). An aliquot of 1 mL homogenate was put in plastic tubes and mixed with 20 μL of the IS1 (1 μg mL^{-1}). The mixtures were vortexed for 3 min and then centrifuged at 11,000 rpm for 10 min. The supernatant was evaporated to dryness under vacuum at 45 °C. The residue was reconstituted with 200 μL water by being vortexed. Then 300 μL of chloroform–isopropanol (10:3, v/v) was added and mixed for 2 min by vortex. The mixture was centrifuged at 3000 rpm for 10 min to get the water layer B.

The eluent A and the water layer B were mixed and evaporated under nitrogen at room temperature. Then residue was reconstituted in 200 μL water and the solution was centrifuged at 11,000 rpm for 10 min. The supernatant was analyzed using LC–MS/MS.

2.5. Preparation of calibration standards and quality control samples

The primary reference standards and quality controls (QC) stock solutions of four reference substances (5-HT, NE, M-ENK and L-ENK) were prepared, respectively. The stock solutions were prepared by dissolving in water at 1 mg mL^{-1}. These solutions were stored at −20 °C until use. The stock solution of DHBA (IS1) and DADLE (IS2) were prepared in water at a concentration of 1 mg mL^{-1} and were stored at −20 °C.

Calibration standards were prepared by spiking untreated rat brains and hypothalamus homogenate with the stock standard solution, which was further diluted with the matched matrix to give seven calibration standards in the concentration range of 10, 20, 50, 100, 200, 500 and 1000 ng mL^{-1} for NE and 5-HT and six calibration standards in the range of 2, 5, 10, 25, 50, 100 and 250 ng mL^{-1} for M-ENK and L-ENK. Similar to calibration standards, QC samples were prepared in replicates at four concentration levels represent-

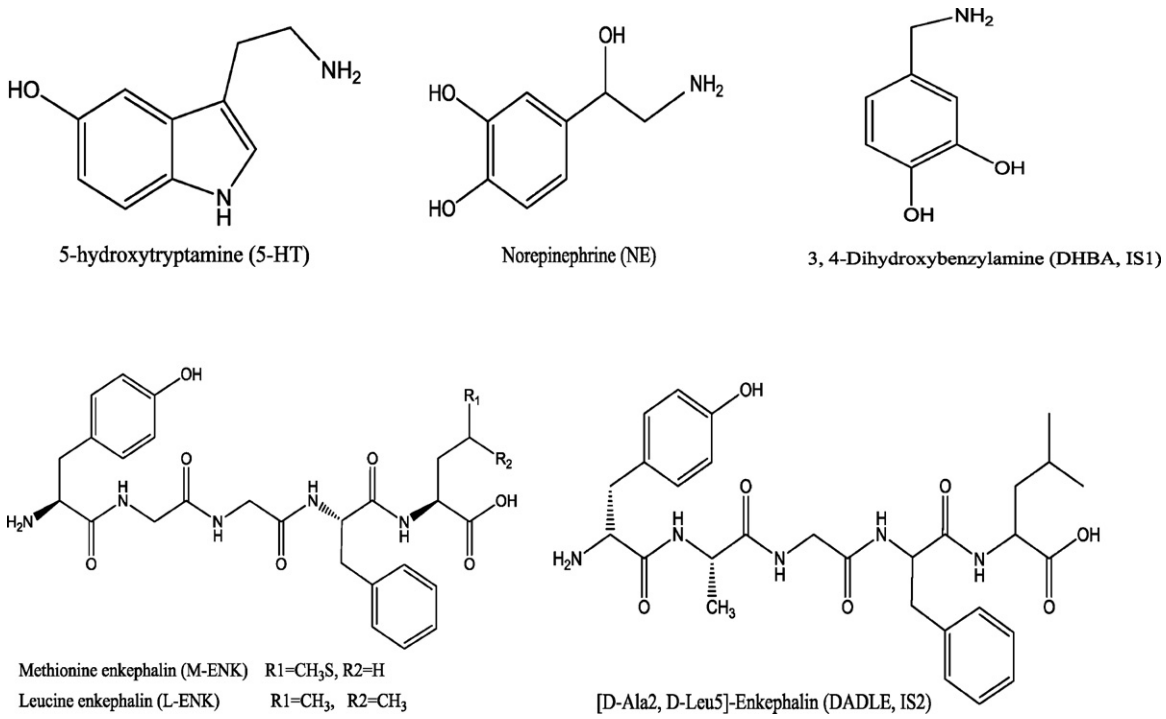

Fig. 1. Structures of 5-HT, NE, M-ENK, L-ENK, DHBA (IS1) and DADLE (IS2).

ing the entire range of concentrations (4, 10, 100 and 500 ng mL^{-1} for NE and 5-HT; 2, 5, 25 and 250 ng mL^{-1} for M-ENK and 2, 5, 25 and 100 ng mL^{-1} L-ENK).

2.6. Validation procedures

2.6.1. Accuracy and precision

The accuracy and precision of the method was determined for intra- and inter-day variations. Intra-day precision (% relative standard derivation, RSD) and accuracy was evaluated five times on the same sample extracted on the same day, while inter-day precision and accuracy was evaluated five times in another independent sample extracted on three separate days.

2.6.2. Extraction recovery

The extraction recoveries of 5-HT, NE, M-ENK and L-ENK from brain were determined by comparing the absolute response of an extract of control brain to which these analytes had been added after extraction, with the absolute response of an extract of brain to which the same amounts had been added before extraction.

2.6.3. Matrix effects

Matrix effects were established by assaying six lots of blank control matrix and comparing the response of each blank relative to the LLOQ standards. The specificity was further investigated by preparing six QC pools (one replicate of each) of three concentrations in the range of the calibration curve from individual matrix donors and comparing the measured concentrations to the nominal concentrations.

2.7. Stability

The stability of four neurotransmitters was evaluated under different temperature and storage conditions. As far as the stability of four analytes in rat brain was concerned, there was no significant degradation observed under the storage conditions described in the previous reports [21,24]. In this study the stability of bench-top, −20 °C for 30 days, and three freeze–thaw cycles were investigated. All stability studies were conducted at four concentrations of four neurotransmitters with three determinations each.

2.8. MI model and drug administration

The rats were randomly divided into four groups with eight rats each, which were control group, MI group, propranolol group and SMS group.

The myocardial infarction was induced in experimental rats by intraperitoneal injection of isoproterenol, dissolved in physiological saline, for three consecutive days.

Control group, animals received with 0.9% normal saline once daily for 7 days and in addition received saline (5 mg/kg/d) on the 8th, 9th and 10th day at an interval of 24 h. MI group, rats were orally fed with 0.9% normal saline (180 mg/kg/d) once daily for 7 days and in addition received isoproterenol (5 mg/kg/d) on the 8th, 9th and 10th day at an interval of 24 h. Propranolol group, rats were pretreated with propranolol (10 mg/kg/d) for a period of 7 days and in addition received isoproterenol (5 mg/kg/d) on the 8th, 9th and 10th day at an interval of 24 h. SMS group, rats were pretreated with SMS extract (11.6 g/kg/d) for a period of 7 days and in addition received isoproterenol (5 mg/kg/d) on the 8th, 9th and 10th day at an interval of 24 h.

3. Results and discussion

3.1. LC–MS/MS optimization

MS optimization was performed in both negative and positive ionization (PI) modes, and different mobile phase compositions were investigated to find compatible ESI and fragmentation conditions with the chromatographic separation. PI mode could provide higher sensitivity and was selected for the following study. Fig. 3 shows the representative SRM chromatograms from MI model rat brains.

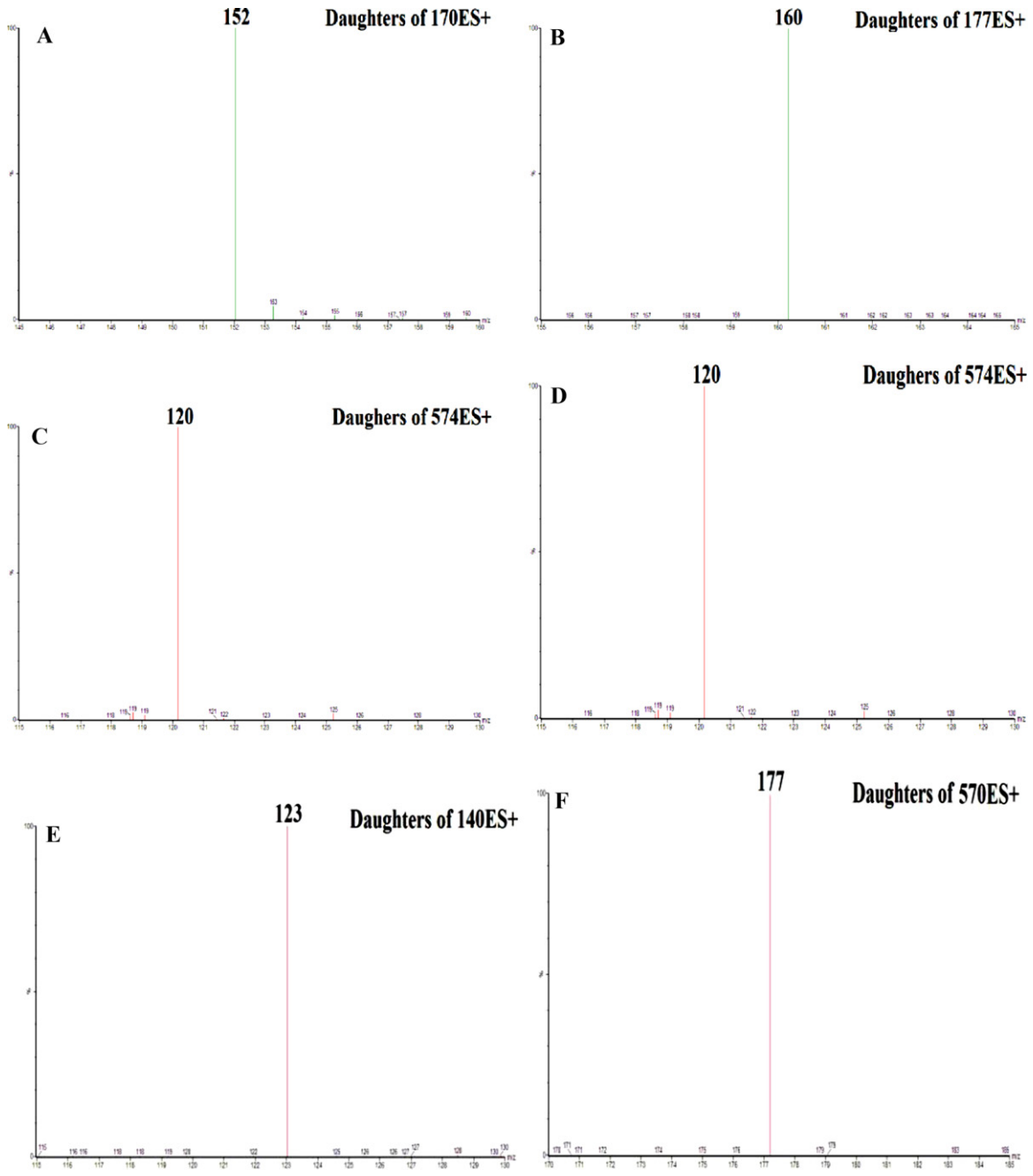

Fig. 2. Full daughter scan ESI+ mass spectra of NE, 5-HT, M-ENK, L-ENK, DHBA (IS1) and DADLE (IS2). A: daughters of 170.0: NE, B: daughters of 177.0: 5-HT, C: daughters of 574.0: M-ENK, D: daughters of 556.0: L-ENK, E: daughters of 140.0: DHBA (IS1), F: daughters of 570.0: DADLE (IS2).

5-HT and NE are high polar analytes, which were difficult to be retained in the column. The progress of acidification is required to separate the analytes from the specimen matrix and improve protonation in positive ESI, but also results in signal suppression in negative ESI. Formic acid was chosen as modifier to adjust the pH of the mobile phase because it could improve the ionization of analytes in positive ESI. In addition, mobile phases used in RPLC for the analysis of peptides usually contain an additive which is working as an ion-pairing agent. The additive increases the hydrophobicity of molecules by forming ionic pairs with their charged groups. Moreover, it usually yields high or low pHs and promote protein unfolding and denaturation. Formic acid is one of important ion-pairing agents most widely used for the separation of proteins and peptides. So formic acid used as not only a modifier to analyze 5-HT and NE but also an ion-pairing agent to analyze M-ENK and L-ENK in our study.

3.2. Method validation

The lower limit of quantification (LLOQ) for both 5-HT and NE were found to be 4.0 ng mL^{-1}; and 2.0 ng mL^{-1} for M-ENK and L-ENK. Good linear relationship with coefficients $\geq$0.99 for all 4 neurotransmitters was observed over the concentration range of 10–1000 ng mL^{-1} for 5-HT and NE, and 2–250 ng mL^{-1} for M-ENK and L-ENK.

The QC intra- and inter-day precision and the accuracy results of the four neurotransmitters are listed in Table 1. The intra-day precision is in the range of 2.8–8.4%, while the inter-day precision is in

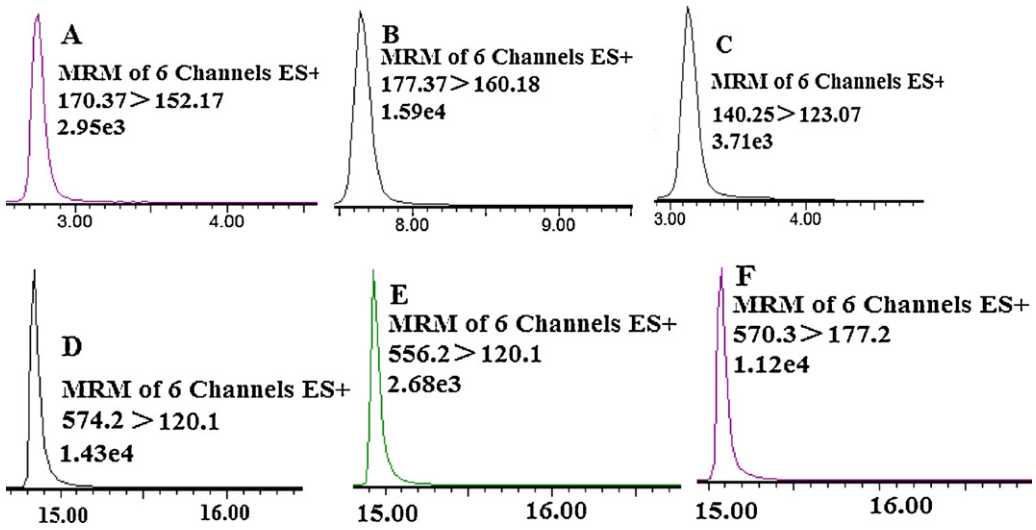

Fig. 3. SRM chromatograms of four neurotransmitters, IS1 and IS2 in a MI model rats brain sample: (a) M-ENK, (b) IS2, (c) L-ENK, (d) 5-HT, (e) NE and (f) IS1.

the range of 3.1–8.9%. The intra-day accuracy is within 86.5–95.3%, and the inter-day accuracy is within 88.5–94.9%.

The extraction recoveries of the four neurotransmitters were found to be consistent, precise and reproducible. The mean recoveries of the LLOQ, low, middle and high QC levels and their precisions are shown in Table 2.

Matrix effects were further investigated by spiking NE, 5-HT, M-ENK and L-ENK at the LLOQ, low, middle and high QC concentration into six different brain lots and comparing the measured concentrations with the nominal concentrations. The results are summarized in Table 2. The %CV between the concentrations measured for the six different spiked QC specificity samples was not more than 2.9%, and a bias to the theoretical concentration value was not more than −5.9%, indicating there is no significant matrix effect.

Based on the data of the stability study obtained (Table 3), the working solutions of four analytes are intact within 4 h and may entail freeze–thaw cycles, can be undertaken if the number of cycles is three or less.

3.3. Determination of neurotransmitters in MI model

Effect of MI model group and SMS group on NE content is shown in Fig. 4A. The concentration of NE in the MI model group ($79.69 \pm 9.47\ ng\ mL^{-1}$) was significantly increased in rat brain at 30 min ($p < 0.01$) compared with the control group ($45.07 \pm 4.36\ ng\ mL^{-1}$). Both SMS group ($59.68 \pm 3.71\ ng\ mL^{-1}$) and propranolol group ($60.52 \pm 11.87\ ng\ mL^{-1}$) reduced the content of NE in rat brain, but SMS pretreatment with isoproterenol could significantly decrease the content of NE which was found to approach basal values closely.

In comparison with the control group ($24.18 \pm 4.48\ ng\ mL^{-1}$), the contents of 5-HT in MI group ($46.58 \pm 9.63\ ng\ mL^{-1}$) was significantly increased, and the number of errors was significantly ($p < 0.01$) reduced. Propranolol group ($31.83 \pm 7.99\ ng\ mL^{-1}$) and SMS group ($28.86 \pm 9.81\ ng\ mL^{-1}$) reduced significantly ($p < 0.01$) the number of errors and protected the rat against myocardial ischemia induced by isoproterenol (Fig. 4B).

Table 1
Intra- and inter-day precision and accuracy of the assay ($n = 5$).

Sample	Amount of homogenate ($ng\ mL^{-1}$)	Amount added ($ng\ mL^{-1}$)	Intra-day			Inter-day		
			Amount detected (mean ± S.D.%, $ng\ mL^{-1}$)	Accuracy (%)	RSD (%)	Amount detected (mean ± S.D.%, $ng\ mL^{-1}$)	Accuracy (%)	RSD (%)
5-HT	42.57	4	3.47 ± 8.6	86.8	7.6	3.55 ± 5.6	88.8	6.3
		10	8.65 ± 5.7	86.5	4.7	8.97 ± 5.6	89.7	4.9
		200	183.6 ± 5.3	91.8	3.7	184.8 ± 4.9	92.4	4.1
		1000	953.2 ± 4.8	95.3	3.1	949.3 ± 4.3	94.9	3.6
NE	24.12	4	3.51 ± 5.6	87.8	8.2	3.49 ± 5.6	87.3	7.5
		10	8.96 ± 5.6	89.6	3.6	8.99 ± 6.7	89.9	3.8
		200	180.6 ± 4.7	90.3	3.1	182.4 ± 5.2	91.2	3.4
		1000	921.5 ± 4.5	92.2	2.8	934.2 ± 4.9	93.4	3.1
M-ENK	40.03	2	1.74 ± 5.6	87.0	7.9	1.77 ± 5.6	88.5	7.3
		5	4.42 ± 4.9	88.3	6.4	4.44 ± 7.4	88.9	7.2
		25	22.7 ± 4.5	90.6	4.5	22.3 ± 5.8	89.3	4.7
		250	226.2 ± 4.4	90.5	3.7	223.2 ± 5.3	89.3	3.8
L-ENK	5.03	2	1.78 ± 5.6	89.0	8.4	1.77 ± 5.6	88.5	8.9
		5	4.48 ± 5.9	89.5	7.1	4.43 ± 7.2	88.6	7.8
		25	22.53 ± 5.2	90.1	3.9	22.38 ± 5.9	89.5	4.3
		100	90.3 ± 4.8	90.3	3.6	90.1 ± 5.4	90.1	3.8

Table 2
Recoveries and specificity of four analytes from rat brains ($n = 5$).

Analyte	Amount added ($ng\,mL^{-1}$)	Recovery (mean ± S.D.%)	Specificity (Con ± S.D.%)	CV (%)	Bias (%)
5-HT	4	71.3 ± 7.6	3.61 ± 6.3	2.7	−5.9
	10	70.2 ± 5.2	9.30 ± 3.1	1.9	−4.8
	200	74.1 ± 5.9	192 ± 3.7	2.6	−3.5
	1000	74.0 ± 4.8	983 ± 3.8	2.9	−5.8
NE	4	73.5 ± 6.9	3.72 ± 5.7	2.3	−5.4
	10	73.3 ± 3.7	9.20 ± 5.6	1.8	−4.2
	200	78.7 ± 6.6	187 ± 4.9	1.6	−3.9
	1000	77.7 ± 4.2	992 ± 6.2	1.8	−5.7
M-ENK	2	72.6 ± 8.1	1.71 ± 7.1	2.4	−5.9
	5	74.4 ± 4.2	4.30 ± 3.6	2.1	−4.8
	25	74.1 ± 3.6	23.4 ± 4.8	2.5	−3.7
	250	75.7 ± 5.9	239 ± 5.7	2.4	−5.1
L-ENK	2	73.8 ± 7.4	1.81 ± 5.1	2.6	−5.6
	5	75.3 ± 5.1	4.10 ± 5.3	1.9	−4.6
	25	74.6 ± 4.6	24.2 ± 4.9	2.5	−3.8
	100	73.9 ± 3.9	242 ± 3.7	2.8	−3.6

Fig. 4C and D shows the levels of M-ENK ($48.69 \pm 6.45\,ng\,mL^{-1}$) as well as the levels of L-ENK ($5.30 \pm 0.39\,ng\,mL^{-1}$), which were increased significantly in control group compared to MI group ($52.30 \pm 7.95\,ng\,mL^{-1}$ for M-ENK and $10.15 \pm 4.52\,ng\,mL^{-1}$ for L-ENK), while this increase was almost abolished in the group treated with SMS ($51.03 \pm 7.70\,ng\,mL^{-1}$ for M-ENK and $6.03 \pm 2.33\,ng\,mL^{-1}$ for L-ENK). The observations from Fig. 4C and D indicated that SMS might be beneficial in experimental myocardial infarction.

3.4. SMS protection

In order to evaluate protective effects of SMS on MI associated with neurotransmitters, a MI model was built to analyze the four neurotransmitters in rat brains. According to the results in Section 3.3, the contents of four neurotransmitters were changed in MI model, and SMS could protect experimental myocardial infarction associated with decreased concentrations of neurotransmitters in brain.

The increase in NE and 5-HT concentration in rat brains caused by MI could contribute to the effects of, on the one hand, the stimulated sympathetic nervous system during ischemia on the transmitter levels by affecting the activities of aminergic brain neurons in brain; on the other hand, local ischemia could sufficiently damage monoaminergic axons or terminal within the ischemic area to render them incapable of storing the monoamines against the physiological concentration gradient, as a result the monoamines would be released into synapses and into the surrounding extracellular region.

In the case of M-ENK and L-ENK, quantitative analyses of rat brains demonstrated a deficit in neuropeptides in the SMS group relative to the MI group. Studies [5,6] have shown acute ischemic myocardial could influence the central nervous system through activating the inflammatory response, the increasing of contents of M-ENK and L-ENK can be act as a result of activated the inflammatory response. Moreover, the increase in M-ENK and L-ENK concentration in rat brains due to control sympathetic nervous system reduce the force of contraction during ischemia.

Our results suggested that SMS decrease the secretion of neurotransmitters in MI rat brains via three different pathways. Firstly, SMS could significantly regulate the sympathetic nervous system activity and the decrease of the myocardial contraction and heart rate [6], which may lead to the decreased concentration of monoamine neurotransmitters and neuropeptides. Secondly, SMS can meliorate the level of local ischemia through improving the tolerance of myocardial hypoxia and decrease the cost of oxygen and chemical energy, which could control secretion of monoamine

Table 3
Stability of four analytes in rat brains ($n = 3$).

Analyte	Added concentration ($ng\,mL^{-1}$)	Accuracy (mean ± S.D.%)		
		Bench-top stability (8 h, room temperature)	Freeze–thaw stability (3 cycles, −20 °C, room temperature)	Long-term stability (30 days, −20 °C)
5-HT	4	87.9 ± 10.4	86.8 ± 10.2	85.9 ± 10.7
	10	93.6 ± 9.2	98.6 ± 9.6	96.3 ± 9.8
	200	91.5 ± 7.6	97.6 ± 7.9	102.2 ± 7.4
	1000	106.9 ± 3.9	91.1 ± 4.8	93.1 ± 3.1
NE	4	86.9 ± 10.2	87.2 ± 10.5	88.1 ± 9.9
	10	104.2 ± 9.1	91.0 ± 9.9	91.0 ± 10.8
	200	105.8 ± 7.6	95.1 ± 6.9	97.9 ± 7.3
	1000	93.5 ± 3.9	95.7 ± 4.2	92.4 ± 5.2
M-ENK	2	90.3 ± 9.8	89.1 ± 11.2	90.4 ± 12.4
	5	103.6 ± 9.2	95.6 ± 9.2	96.3 ± 9.8
	25	91.5 ± 5.6	97.6 ± 5.9	102.2 ± 7.4
	250	106.9 ± 4.9	91.1 ± 4.8	93.1 ± 3.1
L-ENK	2	88.9 ± 12.5	86.5 ± 13.2	89.5 ± 11.7
	5	104.2 ± 10.1	91.0 ± 10.3	91.0 ± 10.8
	25	105.8 ± 7.6	95.1 ± 6.9	97.9 ± 7.1
	100	93.5 ± 3.9	95.7 ± 4.2	92.4 ± 5.2

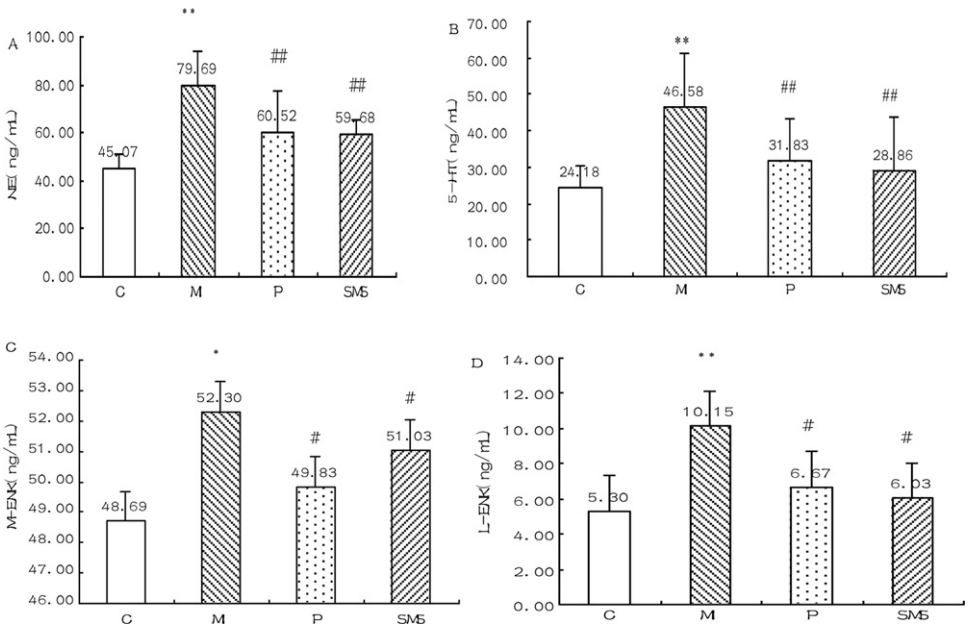

Fig. 4. Effect of SMS and propranolol on content of NE (A), 5-HT (B), M-ENK (C) and L-ENK (D) in rats brain in isoproterenol induced myocardial infarction. Values are mean ± S.E. of eight experiments. Columns indicate means and vertical bars indicate S.E. C – control; MI – ISO; P – propranolol. $^{*}p < 0.05$; $^{**}p < 0.01$ as compared to control, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ as compared to MI ($n = 8$).

neurotransmitters and neuropeptides. Last but not least, SMS may enhance human immunity through decreasing the level of IL-6 and TNF-α in treated heart failure patients [25], which in turn control the activities of HPA axis and the secretion of neuropeptides.

4. Conclusions

In this paper, HPLC/MS/MS method was developed for the simultaneous determination of two types of neurotransmitters, which closely relate to myocardial ischemia. The results indicated that the method could successfully measure 4 major neurotransmitters in brain of mature rats after the induction of acute myocardial injury. The protective effects of SMS might be mediated by modulating neurotransmitters release in myocardial injury rat brains.

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