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Analysis of Synephrine in Da-cheng-qi Decoction by HPLC Employing Precolumn Derivatization With 9-Fluorenylmethyl Chloroformate

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Abstract

A sensitive and reliable method has been developed to determine synephrine in a famous purgative Chinese medicinal prescription, da-cheng-qi decoction. After purified by solid-phase extraction (SPE), the decoction was derivatized with 9-fluorenylmethyl chloroformate in borate buffer and analyzed by reversed-phase high-performance liquid chromatography equipped with a diode-array detector. The chromatographic separation was carried out on a Shimadzu shim-pack VP-ODS column using acetonitrile–water as mobile phase. This method was validated in terms of linearity, recovery, precision, accuracy, and repeatability. Calibration curves were linear (r^2 > 0.99) over the concentration range of 0.78–100 μg/mL, and the mean recovery was 93.28%. The intra- and inter-batch precisions were less than 2% in terms of relative standard deviations. The accuracy and repeatability were well within the acceptable range. The limit of detection was 0.25 μg/mL. This validated method also can be used for assays of herbal products, fruits, dietary supplements, and biological samples containing synephrine.

Introduction

Da-cheng-qi decoction is a famous purgative Chinese medicinal prescription that fights against ailments such as acute intestinal obstruction without complications, acute cholecystitis, and acute appendicitis (1). Synephrine, an adrenergic amine structurally related to endogenous neurotransmitters (epinephrine and norepinephrine), is the main hydrosoluble compound existed in da-cheng-qi decoction. It has been used for the treatment of obesity by stimulating lipolysis, rising metabolic rate, and oxidating fat (2,3). Nowadays, weight loss dietary supplements containing synephrine have rapidly replaced the products containing ephedrine alkaloids, which have been banned by the United States Food and Drug Administration (FDA) in April 2004 because of an association with serious adverse health effects (4). So the quantitative determination of synephrine is very important.

Up to now, several methods have been reported for the determination of synephrine in herbal products, fruits of various citrus species, and dietary supplements (5–7); however, there are still some problems. What is well-known is that synephrine is so polar that it has no retention in the ODS column. At the same time, it can strongly interact with the free silanols on the surface of stationary phase, which directly causes the band tailing of analytes and affects the resolution and the quantitative analysis. Ion-pair chromatography (8–10) and the capillary electrophoresis with electrochemical detection (11–13) are widely used to determine synephrine. Because the apparatus are generally found in analytical laboratories and easy to use, liquid chromatography with UV (14,15) photodiode array detectors (DAD) are chosen first. Many other methods (16,17) are tried, but all of the mobile phase added many volatile organic solvents, aqueous solutions of room temperature ionic liquids, and volumes of extraction solvents, which are harmful to the environment and operators and consumed a large amount of time and resources. Compared with the previously mentioned methods, gas chromatography with mass spectrometry (GC–MS) (18,19) show very high sensitivity and specificity, but the high temperature derivative condition might cause thermal decomposition. In addition, the sample needs to concentrate and redissolve before injection, which is too complicated. Also the GC–MS apparatus is not available for economic reason.

In the present paper, a simple analytical method using precolumn derivatization high-performance liquid chromatography (HPLC) for quantitative determination of synephrine in da-cheng-qi decoction is reported for the first time.

Experimental

Chemicals and reagents

Raw materials of Dahuang (*Radix et Rhizoma Rhei*), Houpu

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(*Magnolia officinalis Rehd.*), Zhishi (*Fructus Aurantii Immaturus*), and Mirabilite (crystals of sodium sulfate, Na2SO4) were purchased from BaiXin Pharmacy (Nanjing, China) and authenticated by Professor Ping Li (Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China). Synephrine (Batch No: 0727-200105, > 98.5% purity) reference standard was purchased from the Chinese National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, People's Republic China); 97% 9-fluorenylmethyl chloroformate (FMOC-Cl) was supplied by GL Bio Ltd (Shanghai, China). Glycine was purchased from Huixin Biological Company (Shanghai, China). Acetonitrile was of HPLC-grade and purchased from VWR International Company (Darmstadt, Germany). Other chemicals were all of analytical-grade. Water was distilled twice before use.

Preparation of stock and working solutions

The stock solution of synephrine (0.5 mg/mL) was prepared by dissolving appropriate amounts in 10 mL of 30% methanol, and the stock solution of glycine reagent was prepared with distilled water at a concentration of 2.0 mg/mL. Both of the stock solutions were stored at 4°C and further diluted to make working solutions at room temperature.

A 0.4 M borate buffer was prepared from boric acid adjusted with hydrochloric acid to pH 6.0. The FMOC-Cl reagent was prepared freshly each time by dissolving 20.0 mg of FMOC-Cl in 10 mL acetonitrile to give a concentration of 2.0 mg/mL.

Sample preparation and purification

The decoction was prepared according to the procedure described in *Shang-Han-Za-Bing-Lun*: 15 g Zhishi (*Fructus Aurantii Immaturus*) and 24 g Houpu (*Magnolia officinalis Rehd.*) were immersed in 500 mL distilled water and boiled until half of the original amount was left. This procedure was repeated. Then, the extracts were combined. 12 g Dahuang (*Radix et Rhizoma Rhei*) was immersed in the extract water mentioned earlier and boiled until half of the amount was left. At last, 6 g mirabilite (crystal of sodium sulfate, Na_2SO_4) was dissolved in the extract water. The decoction was then filtered and diluted to 1000 mL with distilled water and stored at 4°C until use.

The solid-phase extraction (SPE) column used for sample purification contained 250 mg of C_{18} packing material (PT-C_{18}, Hanbang, Jiangsu, China). The column was preconditioned by rinsing with 2.0 mL of methanol followed by 2.0 mL of water. 1 mL of the da-cheng-qi decoction was allowed to percolate through the preconditioned SPE column, then the column was washed with 2.0 mL of methanol–water (30:70, v/v), and the effluent was collected and diluted to the mark with 30% methanol in a 5-mL flask for subsequent derivation. The rest of the liposolubile was washed with 50% methanol and pure methanol for the research of other compounds.

Sample derivatization

0.5 mL synephrine standard solution or da-cheng-qi effluent was mixed with 0.5 mL acetoritrile and 200 µL of 0.4 M borate buffer (pH 6.0) in a 10-mL centrifuge tube. Then, 50 µL FMOC-Cl reagent (2.0 mg/mL) in acetonitrile was added, vortexed immediately for 2 min, and allowed to react at room temperature for 15 min. Afterwards, 150 µL glycine (2.0 mg/mL) was added and vortexed for 2 min to terminate the reaction by quenching the remaining FMOC-Cl. The mixture was centrifuged at 14,770 g for 8 min; an aliquot of 20 µL was injected into the HPLC system. The conditions used to optimize the derivatization by FMOC-Cl were the same as stated earlier unless stated otherwise.

Chromatographic equipment and conditions

HPLC analyses were carried out on an Agilent 1100 series instrument (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump, a four-channel-online degasser, an autosampler, a column oven, and a DAD. Data collection was performed using HP ChemStation software. Chromatography was performed on a Shimadzu shim-pack VP-ODS (150 L × 4.6 mm i.d., 5 µm, Kyoto, Japan) column with a flow rate of 1.0 mL/min. Some preliminary work was carried out on a Guard reversed phase C_{18} column (Agilent).

Acetonitrile–water was used as the mobile phase, and the following gradient elution was used: 0–11 min 44% acetonitrile, 11–15 min from 44% to 100% acetonitrile. At the end of the run, 100% of acetonitrile was allowed to flush the column for 5 min, and an additional 15 min of post-run time were set to allow for equilibration of the column with the starting eluant to obtain reproducible chromatograms. The column temperature was constant at 45°C. The sample volume injected was 20 µL, and the UV spectra were collected at 265 nm.

Liquid chromatography tandem mass spectrometry (LC/MS/MS) experiments were conducted using a Finnigan Surveyor HPLC system (Thermo Electron, san Jose, CA) equipped with a Finnigan autosampler. The HPLC eluant from the column was introduced into the mass spectrometer via a 1:4 split. The mass detection was conducted using a Finnigan TSQ Quantum Discovery max system (Thermo Electron) equipped with an electrospray ionization source. The spray voltage was 4 kv, and the capillary temperature was 350°C. Nitrogen was used as nebulizing and auxiliary gas. The nebulizing gas back-pressure was set at 40 psi and auxiliary gas at 15 (arbitrary units). For MS/MS, argon was used as the collision gas. Data acquisition was performed with Xcalibur 1.2 software (Thermo Finnigan).

Assay validation

This method was validated in terms of linearity, recovery, accuracy, precision, and repeatability. Five calibration curves were constructed by analyzing eight different concentrations of standard solutions. Standard samples were prepared by dissolving synephrine standard solution in appropriate volumes of distilled water to produce the standard curve point equivalent to 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL. The following assay procedures were the same as that described earlier. In each run, a blank sample without synephrine was analyzed to confirm absence of interferences but not used to construct the calibration function. The calibration curves were carried out using the external standard method and were investigated between the peak area (y) and the quantity of compound (x, µg/mL). The limit of detection (LOD) and limit of quantitation (LOQ) under the present chromatographic con-

ditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

The precision and accuracy were measured by performing five sets of three different standard concentrations (1.56, 12.5, and 50 µg/mL) and da-cheng-qi decoction samples on the five different validation batches. The repeatability of the chromatographic determination for the proposed method was calculated by five parallel samples injection continuously. The precision and repeatability were expressed in terms of relative standard deviation [RSD (%) = (SD/mean) × 100%]. The accuracy was determined by calculating the percentage accuracy observed in the analytes [(mean observed concentration/theoretical concentration) × 100%].

0.5 mL of 12.5 mg/mL synephrine was added into 0.5 mL da-cheng-qi decoction, then extracted and quantified as described previously. The absolute recovery was calculated by comparing the determined amount of the standard with the amount originally added. This procedure was repeated five times, and the recovery was calculated by the formula: recovery (%) = (amount found – original amount)/amount spiked × 100%.

Results and Discussion

Optimization of the chromatographic separation and sample preparation

The formation of FMOC-synephrine is shown in Figure 1. HPLC analysis of the reaction mixture revealed the peaks of FMOC-Cl, FMOC-synephrine, and hydrolyzed FMOC-Cl (FMOC-OH) (Figure 2). The chromatographic conditions were optimized to obtain good separation of the target compound from other co-existing components in da-cheng-qi decoction, giving symmetrical and sharp peaks. Figure 3 shows the representative chromatograms of standard FMOC-synephrine (A) and FMOC-synephrine in da-cheng-qi decoction (B). The retention time of FMOC-synephrine was 11 min. The UV spectrum of FMOC-synephrine was monitored by the DAD detector (Figure 4), the maximum wavelength of 265 nm was adopted. Then a triple stage quadrupole (TSQ) tandem mass spectrometry was used to give sufficient structure information of FMOC-synephrine. The atmospheric pressure chemical ionization (APCI) source and the electrospray ionization (ESI) source were both utilized in positive ion mode. Figure 5 shows the MS–MS spectra of FMOC-synephrine. Combined with the differences of the retention time, UV spectrum and assisted MS–MS chromatography, the FMOC-synephrine can be distinguished from FMOC-Cl and FMOC-OH distinctly.

There are many components in da-cheng-qi decoction, which would severely interfere with the analyte. To obtain a clean chromatogram and achieve a sufficient recovery, the liquid–liquid extraction and the SPE were investigated. Eventually, the SPE was proved to be better than the liquid–liquid extraction method in terms of the higher recovery and absences of interference at the retention time of FMOC-synephrine in the chromatogram.

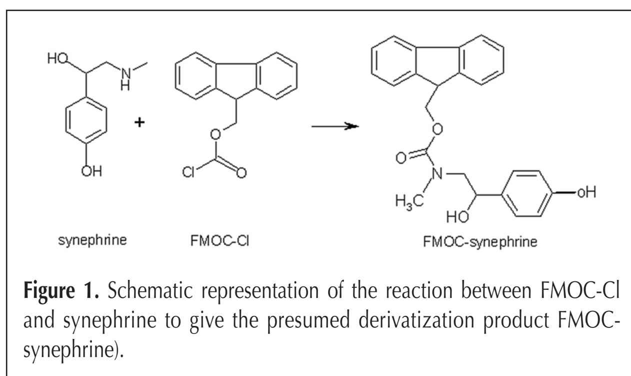

Figure 1. Schematic representation of the reaction between FMOC-Cl and synephrine to give the presumed derivatization product FMOC-synephrine).

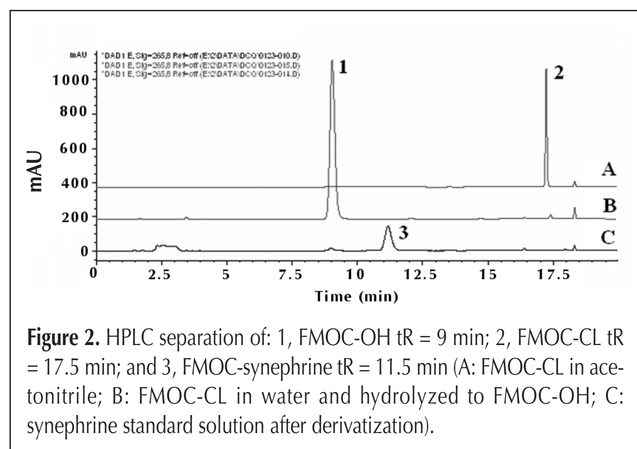

Figure 2. HPLC separation of: 1, FMOC-OH tR = 9 min; 2, FMOC-CL tR = 17.5 min; and 3, FMOC-synephrine tR = 11.5 min (A: FMOC-CL in acetonitrile; B: FMOC-CL in water and hydrolyzed to FMOC-OH; C: synephrine standard solution after derivatization).

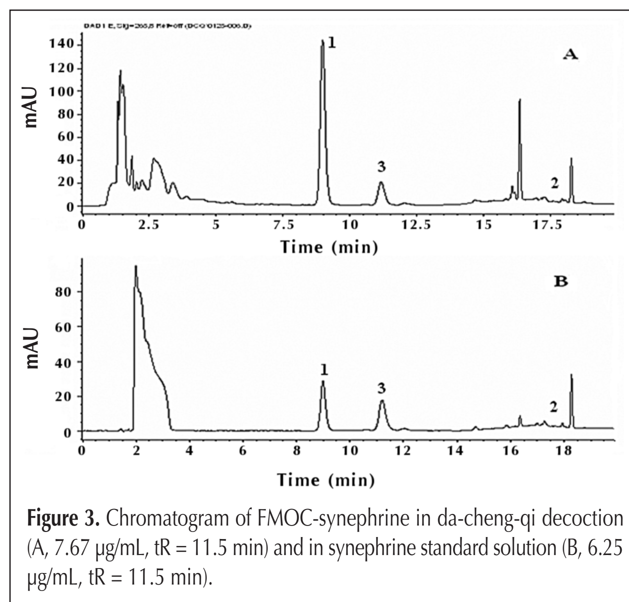

Figure 3. Chromatogram of FMOC-synephrine in da-cheng-qi decoction (A, 7.67 µg/mL, tR = 11.5 min) and in synephrine standard solution (B, 6.25 µg/mL, tR = 11.5 min).

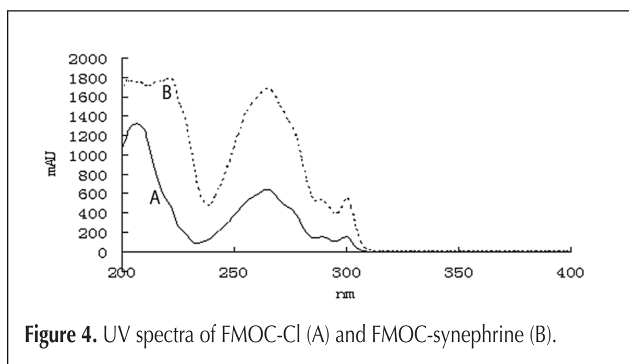

Figure 4. UV spectra of FMOC-Cl (A) and FMOC-synephrine (B).

Optimization of the derivatization conditions

Analytical parameters have been investigated to obtain an optimal condition for FMOC-synephrine derivative and to achieve effective HPLC separation. The derivatization reagent, the pH value and concentration of borate buffer, the concentration of FMOC-Cl and glycine, the proportion of acetonitrile–water, and the reaction time were validated around the expected optimal values.

The ideal derivatization reagent should react rapidly and quantitatively, under mild conditions, without being disturbed by sample matrix components. Furthermore, the derivatives should be detectable with high sensitivity, and the reagent itself or its degradation products should not interfere with the chromatographic separation. Numerous precolumn derivatization reagents have been introduced in which *O*-phthaldialdehyde (OPA), dansyl chloride (Dansyl-Cl), phenyl isothiocyanate (PITC), and FMOC-Cl are the most widely used (20), but all of them show various disadvantages. The OPA and its derivate products are very unstable and degrade within 30 min. The Dansyl-Cl derivate products are more stable than OPA; however, the low sensitivity is incompatible with the UV detector, and the derivatization procedure requires time (usually > 35 min) because of the low reaction activity. FMOC-Cl was finally chosen because it meets most of ideal reagent criteria, but it also has some drawbacks. A disadvantage is that FMOC-Cl is very unstable and can hydrolyze spontaneously to FMOC-OH, which would interfere with the target derivates. So the excess reagent should be removed before chromatography by reacting with glycine.

The borate buffer at different pH values and concentrations were tried to obtain best result, respectively. In the early stage of the method development, 0.1–0.4 M borate buffer in pH 7.0 were examined, but the borate buffer concentrations did not influence any appreciable degree of the derivatization yield. To achieve maximum buffering capacity of the borate buffer, 0.4 M was selected. Then, 0.4 M borate buffer from pH 4.0 to 8.5 were tried. The production of FMOC-synephrine increased from pH 4.0 to 5.5 and decreased from pH 6.0 to 8.5 (Figure 6A). This suggests that the pH value is an important factor in controlling the derivatization and the hydrolysis. The formation of FMOC-OH and FMOC-synephrine proceed simultaneously, and the peak area of FMOC-synephrine starts to decrease when the rate of FMOC-OH formation becomes quicker than that of FMOC-synephrine. So 0.4 M borate buffer in pH 6.0 was used at last.

The effect of FMOC-Cl and glycine concentration on derivatization was examined. Figure 6B shows that the derivatization yield of synephrine increased from 0.25 to 2.0 mg/mL of FMOC-Cl concentrations and did not significantly change from 2.0 up to 10.0 mg/mL as the reactions were complete. 2.0 mg/mL was chosen as the experimental concentration of FMOC-Cl to supply sufficient FMOC-Cl to synephrine during the reaction. After the FMOC-Cl concentration has been optimized, excess glycine was used to terminate the reaction. If the glycine was not added in the reaction, it would require some time to get a stable value of FMOC-synephrine. Then 1.0 mg/mL–10.0 mg/mL glycine was added to the reaction system to optimize the best concentration of glycine, and the production of FMOC-Gly formed a plateau. So, 2.0 mg/mL glycine was adopted to quench the remaining FMOC-Cl.

There was some dependency of the reaction yield on the composition of the reaction mixture. 10–60% (v/v) acetonitrile

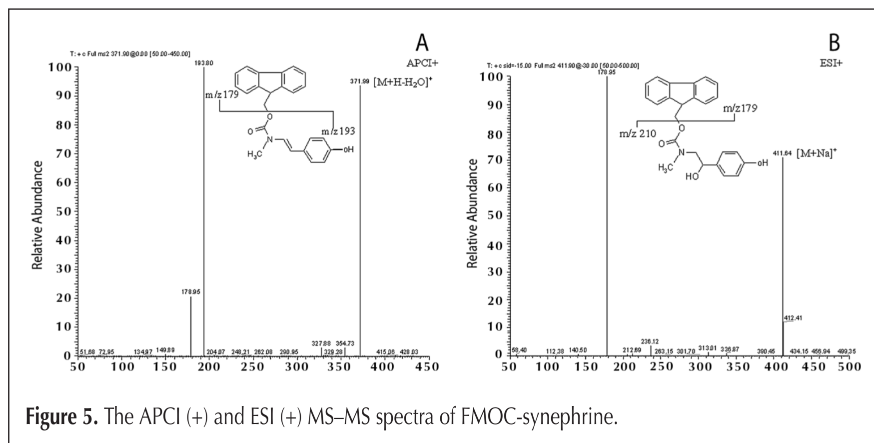

Figure 5. The APCI (+) and ESI (+) MS–MS spectra of FMOC-synephrine.

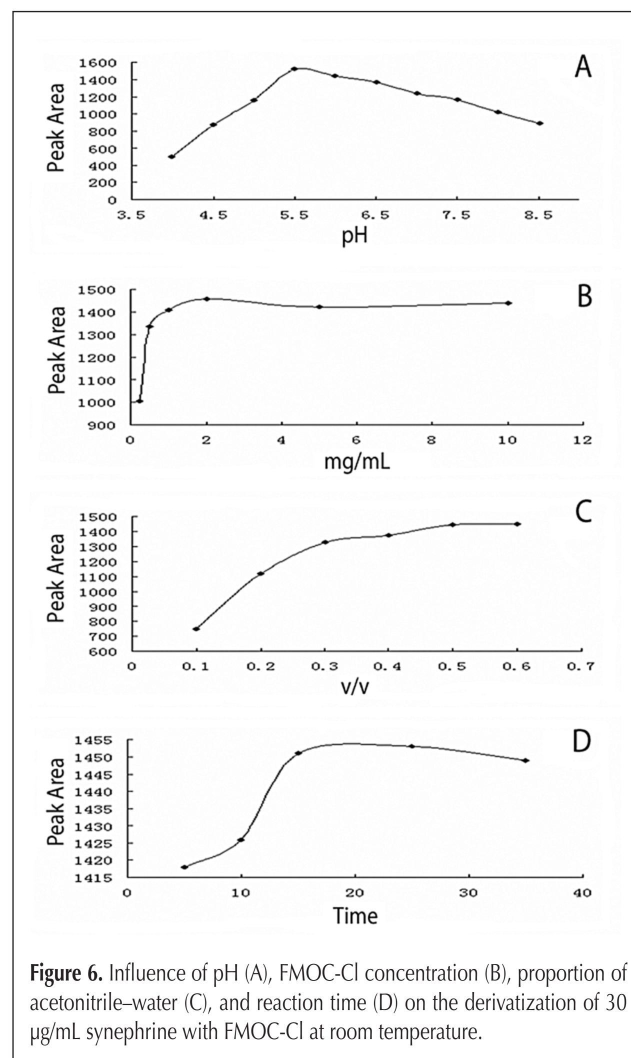

Figure 6. Influence of pH (A), FMOC-Cl concentration (B), proportion of acetonitrile–water (C), and reaction time (D) on the derivatization of 30 µg/mL synephrine with FMOC-Cl at room temperature.

concentration was considered (Figure 6C), and the result showed that at least 50% acetonitrile must be present to assure highest reactivity because FMOC-Cl and FMOC-synephrine are low polar. If they were not dissolved in acetonitrile > 30%, they would precipitate. At the same time, synephrine is water solubile and unavailable in the high acetonitrile concentration. Thus, 50% acetonitrile–water was selected for convenience.

A time course study of the derivatization of FMOC-Cl was performed at 5.0, 10.0, 15.0, 25.0, and 35.0 min at room temperature under the optimal conditions reported previously (Figure 6D). The FMOC-synephrine response was not changed when the reaction time was more than 15.0 min. Finally, 15.0 min reaction time at room temperature was chosen.

Method comparation

In this paper a SPE-HPLC method employing pre-column FMOC-Cl derivatization was developed to determine synephrine in da-cheng-qi decoction for the first time. There were several significant features in this study. One feature is the using of SPE procedure to separate synephrine from other co-existing compounds existing in da-cheng-qi decoction, a very complicated TCM formula, which made the chromatogram much cleaner with less interference. Compared with previously published synephrine analysis methods, the second advantage has the highest sensitivity of derivatization with FMOC-Cl. The LOD of this method was 0.25 µg/mL, much lower than other UV method (14,15). Another advantage of this method was the simplicity of the mobile phase compared with the ion-pair chromatography reported (8–10). The last advantage of this method was the use of the more routine HPLC–UV system than the use of GC–MS.

The whole method was harmless to the operators, “green” to the environment, and can be generalized to determine synephrine in herbal products, fruits, dietary supplements, and biological samples.

Table I. Paraments of the Linearity

Time (h)	Linearity range (µg/mL)	r^2 (correlation coefficient)
0	$y = 49.093x + 5.9981$	0.9997
24	$y = 48.545x + 10.063$	0.9998
48	$y = 49.355x + 7.8992$	0.9996
mean	$y = 48.998x + 7.9867$	0.9997

Table II. The Precision and Accuracy of the Method for Determining Synephrine in Standard Solution and Da-Cheng-Qi Decoction

	Theoretical concentration (µg/mL)	Accuracy (%)	RSD (%)
Intra-batch (n = 5)	1.56	94.28	1.01
	12.5	99.42	0.21
	50	99.20	0.16
Inter-batch (n = 5)	1.56	89.24	1.36
	12.5	102.6	0.53
	50	98.76	0.55
	Concentration (µg/mL)	**RSD (%)**	
Decoction sample	22.89	0.62	
	22.93		
	22.82		
	22.90		
	22.82		

Method validation

A good linearity was achieved over the range of 0.78–100 µg/mL. The average equation of calibration curves (n = 5) was $y = 47.944x + 10.862$, and the correlation coefficients (r^2) of the mean standard curves was 0.9994. The LOD is 0.25 µg/mL, and the LOQ is 0.78 mg/mL. Repeated measurement of one calibration curves are not expressed variations, indicating that the standard derivative solution was stable for at least two days at room temperature. The calibration equations and correlation coefficients are shown in Table I.

As demonstrated in Table II, the results showed good precision, accuracy of the method. In this assay, the intra- and inter-precision of synephrine were both below 1.5%. The intra- and inter-accuracies data were between 85–105%, and the RSDs of repeatability were lower than 2%. The mean recoveries (n = 5) was 93.28%.

Method application

The HPLC–DAD analytical method was subsequently applied to determine synephrine in 10 batches of da-cheng-qi decoction samples. The average content was 22.95 µg/mL.

Conclusions

The results show that the HPLC method described in this report is suitable for separation and quantification of synephrine in the da-cheng-qi decoction. The derivatization, SPE extraction clean-up procedure, and DAD detection gives a very clean chromatogram. The LOD of this method is 0.25 µg/mL, which is enough to detect the concentrations of synephrine in the da-cheng-qi decoction. This precise, accurate, and reliable method could be very useful for quality control of herbal formulations and assay of fruits, dietary supplements, and even biological samples containing synephrine.

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