Simultaneous identification of the three active constituents in Lung-Ventilating-Regulating Oral Liquid by RP-HPLC

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Abstract: The study aimed to develop a HPLC method for ephedrine, hesperidin, and baicalin in Lung-Ventilating-Regulating Oral Liquid. The three active constituents were identified in an Agilent TC-C18 (2) chromatographic column (250mm × 4.6mm, 5μm), with 0.2% phosphoric acid solution - methyl cyanides as mobile phase, which was performed at a gradient elution column temperature of 25°C and a flow rate of 0.8 mL•min^{-1}. Then the eluate was detected at detection wavelengths of 207 nm (for ephedrine) and 278 nm (for hesperidin and baicalin). Under the chromatographic conditions, ephedrine, hesperidin, and baicalin were well separated, which showed good linear relationships at 0.158-2.370, 0.164-4.100 and 0.160-4.000μg, respectively. The coefficients of recovery of these three kinds of samples showed 100.2%, 98.7% and 97.8%, respectively. The developed method is convenient, accurate and well repeatable, and consequently can be applied for the quality control of Lung-Ventilating-Regulating Oral Liquid.

Keywords: Baicalin, ephedrine, hesperidin, hplc, chromatographic conditions, phosphoric acid solution.

INTRODUCTION

Lung-Ventilating-Regulating Oral Liquid is an oral fluid, which is extracted and purified mainly from eleven kinds of Chinese medicinal materials. It has effects of relieving exterior syndrome, dissipating cold, facilitating lung, and relieving cough. It is indicated for cough, aversion to cold with fever, nasal obstruction or discharge, headache, anhidrosis and ache (Fan *et al*, 2015). The formula includes cultivated purple perilla leaf (144g), hogfennel root (96g), balloonflower root 175(96g), bitter apricot seed (fried) (72g), Chinese ephedra herb (96g), pinelliae tuber (fried with honey) (72g), hoelen (96g), bitter orange (96g), baikal skullcap root (96g), seasoned orange peel (96g) and licorice root (72g). The quality standard includes identification of ephedrine by HPLC, and inspections of liquid description, relative density, and pH value (Zhang & Zhang 2002). In the formula, ephedrine, the main extract of Chinese ephedra herb, has effects of relaxing bronchial smooth muscle and contracting blood vessel, as well as marked central excitation effect (China 2010); hesperidin, the main extract of seasoned orange peel, has effects of improving deficiency of spirit lifeblood and lymphatic system hypofunction, expelling dampness, and eliminating sputum (Liu *et al*, 2009); and baicalin, the main extract of baikal skullcap root, has effects of inhibiting bacterial growth, promoting urination, eliminating inflammation, anti-anaphylaxis and relieving muscular spasm (Hu & Chen 2012). These effects are accordant with the functions and indications of the preparation. Currently, there are only reports on identification of ephedrine (Yu *et al*, 2013; Wang 2008; Xie & Zhang 2013), hesperidin and baicalin (Xiao *et al*, 2011) in Lung-Ventilating-Regulating Pills or Oral Liquid yet. While in this study, the amount of ephedrine, hesperidin, and baicalin in Lung-Ventilating-Regulating Oral Liquid was simultaneously determined by high performance liquid chromatography (HPLC) gradient elution (Tang 2011), which provided reference foundations for better quality control of the preparation.

MATERIALS AND METHODS

Instruments

Agilent 1200 high performance liquid chromatograph (equipped with online degasser, quaternionic pump, automatic sampler, column incubator, and photo-diode array (PDA) detector; American Agilent company), Advanced-I-24L ultrapure water machine (Chengdu Aike Water Treating Equipment Co., Ltd), FW135 Chinese medicinal herb crusher (Tianjing Taisite Instrument Co., Ltd), and AB204-S electronic analytical balance (Switzerland Mettler Toledo Company).

Drugs

Ephedrine (lot No. 110749-200410), hesperidin (lot No. 110736-200933), and baicalin (lot No. 110715-201016) reference substances were all bought from National Institute for the Control of Pharmaceutical and Biological Products of China. Lung-Ventilating-Regulating Oral Liquid (specification: 10ml/vial; Tongrentang Drug Plant of Beijing Tongrentang Corporation, Lot No.4260064,

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4260087, and 4260094, respectively). Methyl cyanides and methanol were both chromatographic pure (Chemical Industry Branch Office, Shangdong Yuwang Industry Co., Ltd). Water was bidistilled water. And phosphoric acid was analytical pure (Yantai Shuangshuang Chemical Industry Co., Ltd).

Chromatographic conditions

The three active constituents were identified in an Agilent TC-C18 (2) chromatographic column (250 mm × 4.6 mm, 5μm), with 0.2% phosphoric acid water solution (A) - methyl cyanides (B) as mobile phase, which was performed at a gradient elution column temperature of 25^{o}C (0-5min, 100% A; 5-10min, 97% A; 10-11min, 95% A; 11-20min, 98% A; 20 - 25min, 92% A; 25-30min, 80% A; 30 - 45min, 80% A and 45 - 50min, 75% A), and a flow rate of 0.8 mL•min^{-1}. Then the eluate was detected at detection wavelengths of 207nm (for ephedrine) and 278nm for hesperidin and baicalin respectively.

Reference solution

An appropriate amount of ephedrine, hesperidin, and baicalin reference substances were precisely taken, respectively; and then diluted in analytical pure methanol to make reference stock solutions with concentrations of 0.7900, 0.8200 and 0.8000 g•L^{-1}, respectively. And the stock solutions were stored at 4^{o}C. An appropriate amount of the above reference stock solutions were taken, respectively; then diluted in methanol to make a mixed reference working solution with concentrations of 0.1580, 0.1640, and 0.1600 g•L^{-1}, respectively. And the working solution was stored at 4^{o}C for further use.

Test solution

0.2% phosphoric acid water solution and methanol were made into a mixed solvent with a certain volume according to a proportion of 4:1.4mL of Lung-Ventilating-Regulating Oral Liquid with 3 lot numbers (4260064, 4260087 and 4260094) were precisely taken, respectively and then infused into three 25mL volumetric flasks, respectively. 20mL of the mixed solvent was taken and added into the volumetric flasks. The volumetric flasks were treated with ultrasound in an ultrasonic cleaner for 30 min. The flasks were then taken out and cooled in open air to room temperature. The liquids were metered to volume with the mixed solvent. 2 h later, the flasks were treated with ultrasound in the ultrasonic cleaner for another 30 min. The flasks were then taken out and cooled in open air. The solutions were filtrated. The subsequent filtrates were taken and placed at 4^{o}C for further use.

Negative sample solution

In the study, the 3 constituents in Lung-Ventilating-Regulating Oral Liquid were identified, which were ephedrine, hesperidin, and baicalin, respectively and were from Chinese crude drug Chinese ephedra herb, seasoned orange peel, and baikal skullcap root, respectively. Negative reference substances short of Chinese ephedra herb, seasoned orange peel and baikal skullcap root were made according to the formula and preparation method of the product, respectively. Then negative sample solutions were made according to the preparation method of the test solution. The negative sample solutions were mixed and filtrated, and the subsequent filtrate was taken and placed at 4^{o}C for further use.

RESULTS

System suitability test and specificity test

6μL of the mixed reference solution, 12μL of the test solution, and 12μL of the negative sample solution were precisely taken, respectively. The three solutions were tested under the chromatographic conditions in Chromatographic conditions. The separating degrees between the 3 targeted constituents and neighboring chromatographic peaks were all >1.5; symmetry factors were all 0.95-1.05; theoretical plate numbers were all >20000 calculated based on chromatographic peaks. The identification of the 3 constituents was not interfered by the other constituents in Lung-Ventilating-Regulating Oral Liquid, suggesting the method performed good specificity. The details were shown in fig. 1.

Linear relationship test

1, 3, 6, 9, 12, 15, 20 and 25μL of the mixed reference solutions were precisely taken, respectively, which were then infused into a high performance liquid chromatograph, respectively. The solutions were tested under the chromatographic conditions. With peak area as Y-axis and sample size as X-axis, the regression equations of ephedrine, hesperidin, and baicalin were Y = 3001.6X - 80.029 (r = 0.9998), Y = 1369.3X - 21.673 (r = 0.9999), and Y = 3367.7X-21.08 (r=1.0000), respectively. And the linear ranges were 0.158-2.370, 0.164 -4.100 and 0.160 - 4.000 μg, respectively.

Precision test

6μL of the mixed reference solution was taken precisely, and then infused into the high performance liquid chromatograph. The solution was tested complying with the chromatographic conditions for 6 continuous times. Relative standard deviation (RSD) of ephedrine, hesperidin and baicalin calculated based on the peak area of each chromatographic peak were 1.48%, 1.25% and 1.20%, respectively, suggesting the instruments had good precisions and could meet with the requirements of quantitative determination.

Repeatability test

6 samples of Lung-Ventilating-Regulating Oral Liquid with the same Lot No.4260064 were precisely taken, and made into test solutions complying with the method.

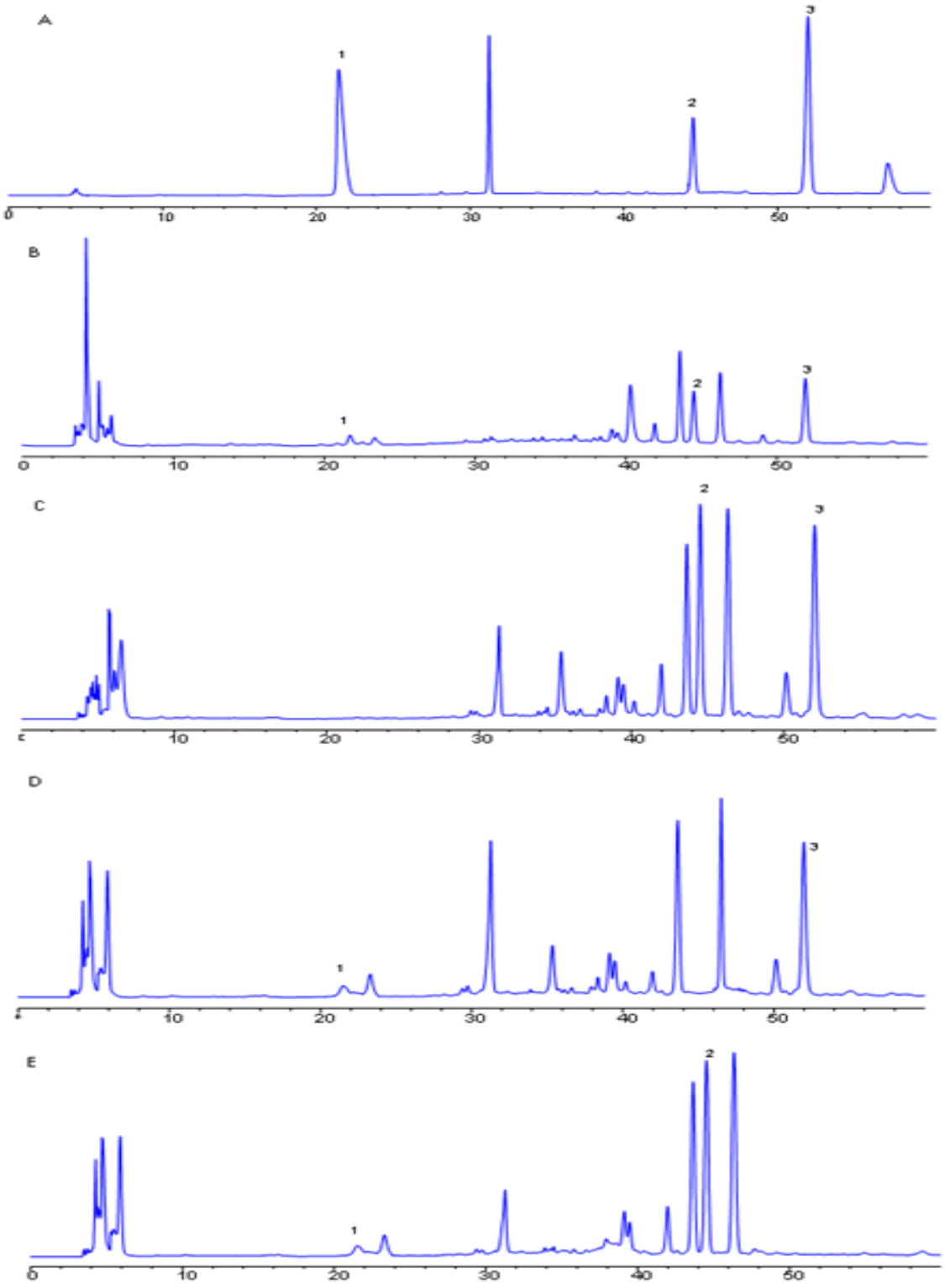

Fig. 1: Lung-Ventilating-Regulating Oral Liquid tested by HPLC. Reference substance; B. Test substance; C. Negative reference substance short of Chinese ephedra herb; D. Negative reference substance short of seasoned orange peel; E. Negative reference substance short of baikal skullcap root; 1. Ephedrine; 2. Hesperidin; 3. Baicalin

Table 1: Test of coefficients of recovery of the 3 constituents in Lung-Ventilating-Regulating Oral Liquid

Constituent	Amount in sample (mg)	Added amount (mg)	Determined Amount (mg)	Coefficient of recovery (%)	Average (%)	RSD (%)
Ephedrine	0.2094	0.3160	0.5200	98.29	100.2	2.6
	0.2094	0.3160	0.5214	98.73		
	0.2094	0.3160	0.5239	99.53		
	0.2094	0.3160	0.5320	102.09		
	0.2094	0.3160	0.5189	97.94		
	0.2094	0.3160	0.5400	104.62		
Hesperidin	2.7178	2.4600	5.1709	99.72	98.8	1.9
	2.7178	2.4600	5.0602	95.22		
	2.7178	2.4600	5.1468	98.74		
	2.7178	2.4600	5.2019	100.98		
	2.7178	2.4600	5.1547	99.06		
	2.7178	2.4600	5.1530	98.99		
Baicalin	1.6132	1.6000	3.1906	98.59	100.1	2.5
	1.6132	1.6000	3.1801	97.93		
	1.6132	1.6000	3.2456	102.03		
	1.6132	1.6000	3.2698	103.54		
	1.6132	1.6000	3.2332	101.25		
	1.6132	1.6000	3.1723	97.44		

Table 2: Identification of the 3 constituents in Lung-Ventilating-Regulating Oral Liquid

Lot No.	Ephedrine			Hesperidin			Baicalin		
	Amount ($g•L^{-1}$)	Mean Amount ($g•L^{-1}$)	RSD ($g•L^{-1}$)	Amount ($g•L^{-1}$)	Mean Amount ($g•L^{-1}$)	RSD ($g•L^{-1}$)	Amount ($g•L^{-1}$)	Mean Amount ($g•L^{-1}$)	RSD ($g•L^{-1}$)
4260064	0.1065	0.1046	1.66	1.3359	1.3605	1.72	0.7974	0.8041	0.72
	0.1044			1.3823			0.8080		
	0.1031			1.3634			0.8069		
4260087	0.0976	0.0982	0.7	1.4316	1.4391	0.59	0.8250	0.8265	0.21
	0.0982			1.4373			0.8262		
	0.0989			1.4484			0.8283		
4260094	0.1088	0.1098	0.89	1.4661	1.472	0.48	0.8759	0.8819	0.58
	0.1108			1.4700			0.8849		
	0.1098			1.4799			0.8848		

The solutions were tested under the above-mentioned chromatographic conditions, with sample size of 12μL. The peak area of each solution was recorded, and RSD calculated. The amount of ephedrine, hesperidin, and baicalin was 0.1047, 1.3589 and 0.8066 $g•L^{-1}$, respectively and the RSD were 1.41%, 1.40% and 0.84 %, respectively. These suggested that the method had good repeatability.

Stability test

One sample of Lung-Ventilating-Regulating Oral Liquid with the same Lot No.4260087 was precisely taken, and then made into a test solution complying with the method. The solution was tested at 0, 2, 4, 6, 8, and 10h after preparation under the above-mentioned chromatographic conditions, respectively. The RSD of the areas of each chromatographic peak were 1.12 %, 0.48% and 0.23 %, respectively, suggesting the test solution was stable within 10 h after preparation.

Test of coefficients of recovery of the three kinds of samples

2 mL of Lung-Ventilating-Regulating Oral Liquid (Lot No.4260064) with known amount (the amount of ephedrine, hesperidin and baicalin was 0.1047 $g•L^{-1}$, 1.3589 $g•L^{-1}$ and 0.8066 $g•L^{-1}$, respectively) was precisely

taken and then placed in a 25 mL volumetric flask. 2ml of 0.158 g•L^{-1} ephedrine reference solution (by diluting 0.790 g•L^{-1} ephedrine reference stock solution in methanol), 3 mL of hesperidin reference stock solution, and 2 mL of baicalin reference stock solution were precisely added, respectively. Then the solution was metered to volume with the mixed solvent of 0.2% phosphoric acid water solution - methanol (4:1). And an applied test solution was made complying with the method. 12μL of the applied test solution was precisely taken, and then infused into the liquid chromatograph. The peak area was determined under the chromatographic conditions, and then the coefficient of recovery was calculated. The results suggested the method had good recovery, and the details were shown in table 1.

Sample test

Appropriate amounts of 3 samples of Lung-Ventilating-Regulating Oral Liquid with 3 different lot numbers (3 samples each lot number) were precisely taken and made into test solutions. A total of 12μL of the test solutions were applied, separately, and then tested under the chromatographic conditions. The amount was calculated. The results were shown in table 2.

DISCUSSION

In this study, dual wavelength was selected to simultaneously determine the amount of ephedrine, hesperidin, and baicalin (207 nm for ephedrine and 278 nm for hesperidin and baicalin). The extraction condition is 30 min for the second time after the first extraction and placement at a room temperature for 2 h. There was a research also showed a determination method for traditional Chinese medicine formula that determined three chemical markers, including *Ephedra intermedia*, *Rheum palmatum*, and *Lithospermum erythrorhizon* (Jeong *et al*, 2018). A method for the separation of diosmin, hesperidin, diosmetin and hesperitin in different pharmaceutical preparations by high performance liquid chromatography with UV-DAD was successfully applied to determine the target flavonoids in pharmaceutical preparations with satisfactory recoveries (Sammani 2017). These results were similar for our research. In their research, the intraday variability for all the chemical markers was less than a RSD of 3%.

In this study, we established an HPLC method to evaluate the quality of Lung-Ventilating-Regulating Oral Liquid. Due to the variety of components of traditional Chinese medicine preparations, any one of the active ingredients cannot reflect the overall curative effect of traditional Chinese medicine. Therefore, a comprehensive macroscopic analysis will become an inevitable trend. Many researches reported that different HPLC methods for assessing the quality of traditional Chinese medicine. A valid and encyclopaedic evaluation method for assessing the quality of Sanhuang Gypsum Soup (SGS) has been set up based on analysis of HPLC fingerprint combined with the quantitative analysis of multicomponents by single marker (QAMS) method, hierarchical cluster analysis (HCA), and similarity analysis (Peng *et al*, 2018). A rapid and reliable HPLC-quadrupole-time-of-flight(Q-TOF)-MS method was used for the simultaneous determination of alpinetin, apigenin-7-O-β-D-glucopyranoside, scutellarein, apigenin, quercetin-3-O-β-D-glucopyranoside, wogonoside, quercetin, amentoflavone, wogonin, chrysin, luteolin, rutin, naringenin, baicalein, and baicalin in the *S. barbata-H. diffusa* herb pair (Yang *et al*, 2018). A powerful method based on HPLC with photodiode array (PDA) detection was established and validated for the quantitative analysis of eight components: chlorogenic acid, gentiopicroside, liquiritin apioside, liquiritin, nodakenin, baicalin, wogonoside and glycyrrhizin in Yongdamsagan-tang extract (Seo & Shin 2017). Considering the variability of chromatographic conditions involved in the analysis of the target flavonoids and the necessity of cost reduction in routine pharmaceutical analysis, the aim of the present work was to develop and validate a simple and reliable HPLC method for analysing these flavonoids in Lung-Ventilating-Regulating Oral Liquid.

CONCLUSION

In this study, the determination method of three chemical markers in Lung-Ventilating-Regulating Oral Liquid was successfully established. The analytical conditions were optimized according to the physiochemical properties of the chemical markers and different proportions of three herbs. These results provide scientific information for further exploration of herbal medicines that comprised several herbs or have unstable gradient elution derivatives.

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REFERENCES

Fan C, Li N and Cao X (2015). Determination of chlorophenols in honey samples using in-situ ionic liquid-dispersive liquid–liquid microextraction as a pretreatment method followed by high-performance liquid chromatography. *Food Chem.*, **174**(1): 446-451.

Hu ZJ and Chen JQ (2012). Identification of hesperidin in different seasoned orange peel drugs by HPLC. *Chinese J. Exp. Traditional Med. Formula*, **18**(10): 95-98.

Jeong B, Roh JS, Yoon M, Yoon Y, Shin SS, Cho HJ, Kwon YS and Yang H (2018). Determination of five chemical markers in DF formula, the herbal composition of *Ephedra intermedia, Rheum palmatum*, and *Lithospermum erythrorhizon*, using high-performance liquid chromatography-ultraviolet detection. *Pharmacogn Mag*, **14**(54): 162-166.

Liu Z, Shi Q, Yang Y, Li YJ, Zhu YY and Bai G (2009). Comparison of antiasthma effects and Mechanisms between ephedrine and pseudoephedrine. *Chinese Traditional Herbal Drugs*, **40**(5): 771-774.

Tian YQ, Ding P, Yan XH and Hu WJ (2010). Pharmacopoeia of the People's Republic of China-2010, Ed 1.China Medical Science Press, Beijing, **282**: 1056.

Peng Y, Dong M, Zou J and Liu Z (2018). Analysis of the HPLC fingerprint and QAMS for Sanhuang Gypsum Soup. *J. Anal. Methods Chem*, **2:** 5890973.

Sammani MS, Clavijo S, Portugal L, Suarez R, Seddik H and Cerda V (2017). Use of multiresponse statistical techniques to optimize the separation of diosmin, hesperidin, diosmetin and hesperitin in different pharmaceutical preparations by high performance liquid chromatography with UV-DAD. *Talanta*, **167**(15): 695-702.

Seo CS and Shin HK (2017). Simultaneous quantification of eight marker compounds in Yongdamsagan-Tang using a high-performance liquid chromatography equipped with photodiode array detector. *J. Chromatogr. Sci*., **55(**9): 926-933.

Tang DZ (2011). Identification of naringin, hesperidin, and baicalin in Lung-Ventilating-Regulating Capsules. *Chinese J. Exp. Traditional Med. Formula*, **17**(15): 93-95.

Wang Z (2008). Identification of ephedrine hydrochloride in Lung-Ventilating-Regulating Pills. *Shandong J. Traditional Chinese Med.*, **27**(8): 490-491.

Xie YY and Zhang J (2013). Identification of ephedrine hydrochloride and pseudoephedrine hydrochloride in Lung-Ventilating-Regulating Granules. *J. Pediatric Pharmacy*, **19**(10):44-46.

Xiao ST, Fu XT and Chang ZR (2011). Identification method for ephedrine in Lung-Ventilating-Regulating Granules. *Chinese J Informat Traditional Chinese Med*., **18 (12)**:53-55.

Yu Y, Wang YJ, Pi JX, Wang DH, Xuan XY and Zheng Y (2013). Bacteriostasis of coadministration of baicalin-borneol on Staphylococcus epidermidis. *Chinese J. Exp. Traditional Med. Formula*, **19**(10): 191-194.

Yang W, He S, Xiao N, Qiao Y, Sui H, Liang L, Chen J, Li W and Zhang L(2018). Simultaneous determination of 15 flavonoids in Scutellaria barbata-Hedyotis diffusa herb pair by HPLC Q-TOF MS. *J AOAC Int*, 20.

Zhang XZ and Zhang PJ (2002). Elevating the quality of Lung-Ventilating-Regulating Oral Liquid by ultrafiltration. *Chinese J. Med. Pharmaceutical Industry*, **33**(6): 283-285.