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Simultaneous analysis of 4 specific antihypertensive adulterants in herbal products using LC-MS/MS

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Abstract

A sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the simultaneous analysis of 4 compounds used for treating hypertension as adulterants in herbal products. The method was validated in terms of selectivity, linearity, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and precision in accordance with AOAC and ICH guidelines. The LOD and LOQ of all compounds were about 0.3 ng/mL and 1 ng/mL, respectively. The linearity was good (R^2 > 0.999), with intra-day and inter-day precision levels not more than 9.85% and 8.51%, respectively, and 81.9 – 109.8% accuracies. Thirty commercial herbal products consisting of traditional medicines and dietary supplements available in Vietnamese markets were tested. While none contained detectable amounts of the 4 antihypertensive compounds, the developed LC-MS/MS procedure can be used for routine analysis to monitor illegal adulteration in various forms of herbal products.

Keywords: *LC-MS/MS, analysis, anti-hypertensive, adulteration, herbal products.*

1. INTRODUCTION

Hypertension is a common chronic medical condition today and is the leading cause of many cardiovascular complications, such as cerebrovascular accidents, myocardial infarction, arrhythmia, and heart failure [1]. In addition to the use of pharmaceuticals in treatment, many traditional herbal products have been used by hypertensive patients because

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they believe that their therapeutic effects are more sustainable and safer than synthetic compounds. However, in order to increase the effectiveness of treatment to improve profits, some manufacturers have illegally mixed chemical drugs into herbal products. There have been a number of researches to detect chemical drugs in antihypertensive preparations with natural source such as atenolol, furosemide, clonidine, etc [2-4]. In Vietnam, in the list of pharmaceutical compounds and derivatives banned from use in the production and trading of dietary supplements of the Ministry of Health, there are 4 drugs commonly used in the treatment of high blood pressure, namely amlodipine, felodipine, nifedipine, furosemide [5].

In order to detect illegal mixing of antihypertensive drugs in herbal preparations, high performance liquid chromatography with UV detector [2, 6] or liquid chromatography-mass spectrometry (LC-MS/MS) can be used [3-4, 7]. In particular, LC-MS/MS has higher reliability because the method has high specificity, sensitivity, precision, accuracy, and suitable for analyzing many substances in complex matrices such as herbal preparations.

So that, this research was conducted with the goal of establishing a method of LC-MS/MS analyzes simultaneously amlodipine, felodipine, nifedipine, furosemide in traditional preparations, and application to detect these substances illegally mixed in herbal preparations circulating on the market.

2. MATERIALS AND METHODS

2.1. Subjects

Subjects of this research were 4 antihypertensive compounds including amlodipine, felodipine, nifedipine, furosemide.

Placebo: The placebos using for method validation were prepared from 14 medicinal herbs that have the effect on anttihypertension, sedation, diuretic, vascular stability. They are: Dioscorea persimilis Dioscoreaceae, Paeonia suffruticosa Paeoniaceae, Rehmannia glutinosa Scrophulariaceae, Cornus officinalis Comaceae, Alisma orientalis Alismataceae, Porta cocos Polyporaceae, Zea mays Poaceae, Prunella vulgaris Lamiaceae, Morinda citrifolia Rubiaceae, Rehmannia glutinosa Scrophulariaceae, Plantago major Plantaginaceae, Styphnolobium japonicum Fabaceae, Achyranthes bidentata Amaranthaceae, Ziziphus mauritiana Rhamnaceae. The composition of the remedy and ratio of ingredients were chosen based on the principles of traditional medicine. These herbs were prepared in liquid extract (liquid form: L matrix) and solid extract (solid form: R matrix) according to the instructions in the Vietnamese Pharmacopoeia [8].

Spiked samples: Standards were spiked with different concentrations into the placebo.

Analytical samples: 30 samples traditional medicines and dietary supplements products collected from pharmacies, online shops or traditional medicine clinics.

2.2. Instruments, chemical and standard material

2.2.1. Instruments

The main instrument used in this research was LC-MS/MS Agilent 6460 Triple Quad (Agilent, USA).

2.2.2. Chemical and standard

Standard materials: Amlodipine besylate (AML) 100.43%, lot. QT145090516; felodipine (FEL) 99.30%. water content 0.06%. lot. WS.0107222; nifedipine (NIF) 99.68%, lot. C0319200.03; furosemide (FUR) 99.51%, water content 0.07%, lot. 0103128 purchased from Vietnam National Institute of Drug Quality Control.

Solvents, reagents: Solvents, reagents were LC-MS or HPLC grade; deionized water had a conductivity ≥ 18 MΩ.

2.3. Methods

2.3.1. Sample treatment

Based on reference [4, 6-7] and experimental results, the sample treatment was carried out in following steps: i) Sample homogenization: Solid samples was ground and liquid samples was thoroughly shaken; ii) Accurately weigh about 0.25 g sample in to a falcon tube; iii) Add about 20 mL of methanol, vortex 5 min, sonication 15 min at room temperature. Transfer the entire mixture to a 25 mL volumetric flask, rinse the tube twice, with approximately 2 mL of methanol each time, concentrate the washings in the volumetric flask, and make up to the mark with methanol. Shaken. iv) Transfer about 10 mL of the mixture to a centrifuge tube and centrifuge at 6,000 rpm for 10 min, then dilute the clear supernatant with methanol to the proper concentration and filter through a membrane with pore size of 0.22 µm.

2.3.2. LC-MS conditions

Mass spectrometer parameters, m/z of precursor ion and daughter ion, ions intensity ratio are optimized by directly injecting single standard solutions into the MS system.

Based on references [4, 7] and available conditions, chromatographic conditions were: Agilent Zorbax Eclipse Plus C18 (150 mm × 3.0 mm; 3.5 µm) column; mobile phase consisting of formic acid 0.1% in water (A) and acetonitrile (B) in gradient (0 → 7.5 min: 15 → 75 % B; 7.5 → 8.5 min: 75 → 15% B; 8.5 → 12 min: 15 % B); injection volume of 5 µL; flow rate of 0.5 mL/min.

2.3.3. Method validation

Method was validated according to AOAC [9] and ICH [10] guideline in the following criteria: System suitablility, specificity/selectivity, linearity range, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision.

2.3.4. Application

Samples were treated and analysed according to the developed method. Identification of targeted compound based on MS spectrum, parent ion, daughter ion and ions intensity ratio. Concentration of the analytes in the samples are calculated based on the calibration curve established in the same day with $R^2 \geq 0.995$.

III. RESULTS AND DISCUSSIONS

3.1. Optimization of LC-MS/MS conditions

Single standard solutions of AML, FEL, FUR and NIF at concentration of 5 µg/mL in methanol were injected without column into the MS system to identify mother and daughter ions. The fragmentation collision energy (CE), nozzle voltage, etc were automatically optimized to obtain a high and stable signal. The selected mass spectrometer (MS) parameters were ionization in ESI (+) mode, nozzle voltage +4500 V; cone temperature 350^{o}C, CID gas: Ar 1,5 mTorr, multiple reaction monitoring (MRM) transition mode. Other conditions are shown in Table 1.

Table 1. *Mass spectrometry conditions*

Substance	***Precursor ion (m/z)***	***Product ions (m/z)***	***CE (eV)***	***Ion ratio (%)***	***Fragmentor (V)***
Amlodipine	409.2	237.8*	12	100	97
		293.9	10	37.4	
Felodipine	384.1	337.8*	14	100	83
		197.9	32	0.3	
Furosemide	329.0	252.9*	20	100	132
		151.9	44	35.5	
Nifedipine	347.0	315.3*	60	100	132
		253.5	80	38.5	

** : Quantitative ion*

The MS parameters of the method meet the requirements of European regulations (2002/657/EC) when there are at least 4 IP points (1 parent ion, 2 daughter ions for qualitative and quantitative purposes) [11].

3.2. Method validation

3.2.1. System suitability

A standard solution containing 4 analytes at concentration of 50 ng/mL in methanol was repeatedly injected for 6 times into the chromatographic system. Retention time (minutes) of AML, FEL, FUR and NIF was 7.398; 8.917; 8.159 and 8.145, respectively, and RSD (%) of peak area was 2.20; 1.99; 2.03 and 1.85, respectively. Thus, the analytical system meets the requirements according to AOAC [9] (RSD < 15% with concentration level ≤ 100 ppb).

3.2.2. Specificity

The specificity of the method was evaluated based on the analysis of the placebo (L and R matrix), the mixture standard (containing 4 hypertention standards at concentration of 1 ng/mL) and the spiked placebo (placebo spiked with 4 hypertention standards at concentration of 1 ng/mL). The results are illustrated in Figure 1. On the placebo chromatogram, no peaks appear at the retention time of the analytes. On the mixture standard chromatogram, the peaks appear with the same retention time as the spiked placebo with good shape. Thus, the method meets the requirements of specificity.

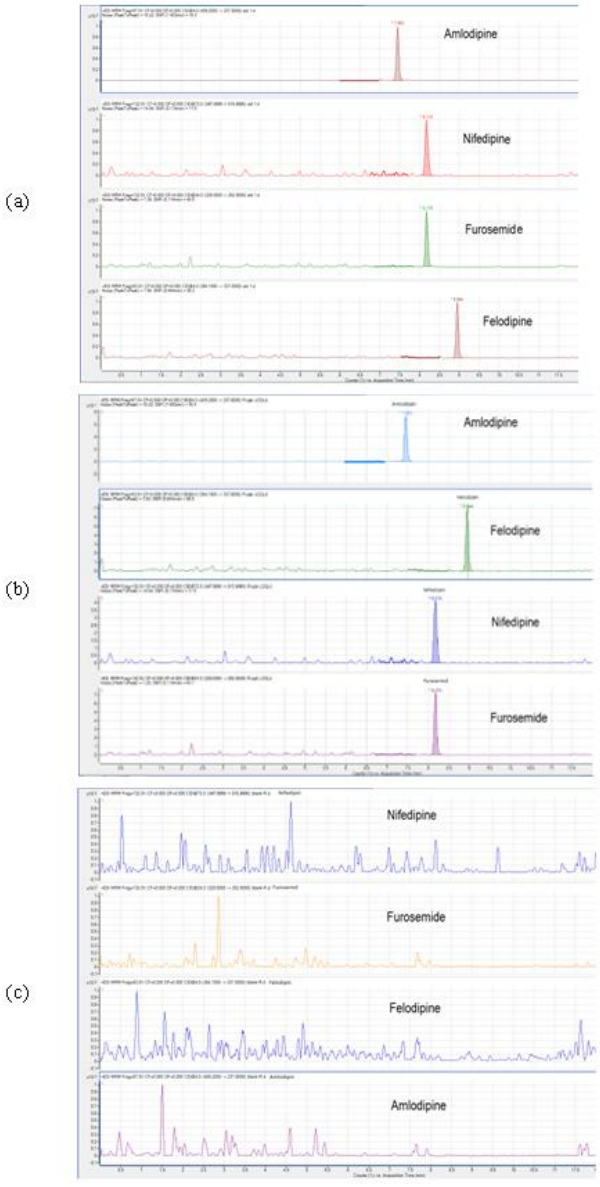

Figure 1. *Chromatogram of specificity validation*

a) Mixture standard; b) Spiked placebo (R matrix); c) Placebo (R matrix)

3.2.3. Linearity

Series of mixture standard solutions in methanol at concentration of 1, 2, 5, 10, 20, and 50 ng/mL were prepared and analysed. Then, calibration curves showing the dependence between the peak areas and the concentrations of each substance were established. The regression correlation coefficient for each substance was calculated. The results shows that there was a good linear correlation between the peak area and the concentration in the investigated range and the accuracy calculated from the calibration curve of the analytes are less than 15%, meeting the requirements according to AOAC [9] (Table 2).

Table 2. *Linearity evaluation*

Analyte	***Concentration range (ng/mL)***	***Equation***		***R^2***	***Accuracy***	
		Slope	***Intercept***		***Min (%)***	***Max (%)***
AML	1 - 50	1067.5	446.61	0.9996	92.3	107.3
FEL	1 - 50	353.12	20.50	0.9999	94.5	110.3
FUR	1 - 50	3027.2	-308.64	0.9995	90.4	112.6
NIF	1 - 50	1342.96	-86.22	0.9998	95.4	108.8

3.2.4. Accuracy and precision

Accuracy and intraday precision were determined by accurately adding 4 analyte standards into the matrix at 3 concentrations (LOQ, low, medium, and high). The standard concentration at 3 levels was 5, 20, and 50 ng/mL for all 4 substances, respectively. Each concentration level was repeated 6 times. Accuracy and intraday precision were performed similarly but on different days. The results show that the method meets the requirement of AOAC [9] on the accuracy and precision on both L and R matrix (Table 3).

Table 3. *Accuracy and precision results*

Analyte	***Concentration in solution*** *(ng/ml)*	***Concentration in sample (ng/g)***	***Accuracy (% recovery)***	***Precision***	
				Intraday (Max value of RSD (%)	***Interday (Max value of RSD (%)***
AML	5	500	85.1 - 109.8	9.85	7.97
	20	2.000	81.9 - 108.0	6.93	8.51
	50	5.000	83.5 - 101.9	5.28	4.70
FEL	5	500	84.2 - 103.8	8.66	6.95
	20	2.000	91.6 - 109.1	3.40	5.11
	50	5.000	93.1 - 108.5	3.95	3.76
FUR	5	500	84.6 - 102.7	6.43	7.02
	20	2.000	89.1 - 106.3	7.20	7.20
	50	5.000	90.7 - 106.7	5.26	5.06

Analyte	*Concentration in solution (ng/ml)*	*Concentration in sample (ng/g)*	*Accuracy (% recovery)*	*Precision*	
				Intraday (Max value of RSD (%)	*Interday (Max value of RSD (%)*
NIF	5	500	83.0 - 104.2	5.47	6.16
	20	2.000	93.7 - 108.3	6.72	5.73
	50	5.000	86.0 - 108.4	5.26	6.91

Notes:

- Requirement for recovery: 80 - 110%

- Requirement for intraday and interday precision:

+ RSD is not more than 11 and 16%, respectively, at concentration of 5 (500 ppb calculated on matrix);

+ RSD is not more than 7.3 and 11%, respectively, at concentration of 20 and 50 ppb (1.25 ppm and 5 ppm calculated on matrix).

3.2.5. LOD và LOQ

The LOD and LOQ of the method were determined by analyzing spiked placebo at different concentrations. Samples were then treated and analysed according to the method. The concentration at which a signal-to-noise ratio (S/N) of about 3 is the LOD, and the concentration that gives an S/N about 10 is the LOQ [10]. The results show that all 4 analytes have a LOD of about 0.3 ng/mL equivalent to 30 ng/g in the analytical sample. LOQ calculated in the chromatographic injection solution is of about 1 ng/mL, equivalent to 100 ng/g in the analytical sample. Figure 2 is the chromatograms of nifedipine at the LOQ concentration determined on the L and R matrix.

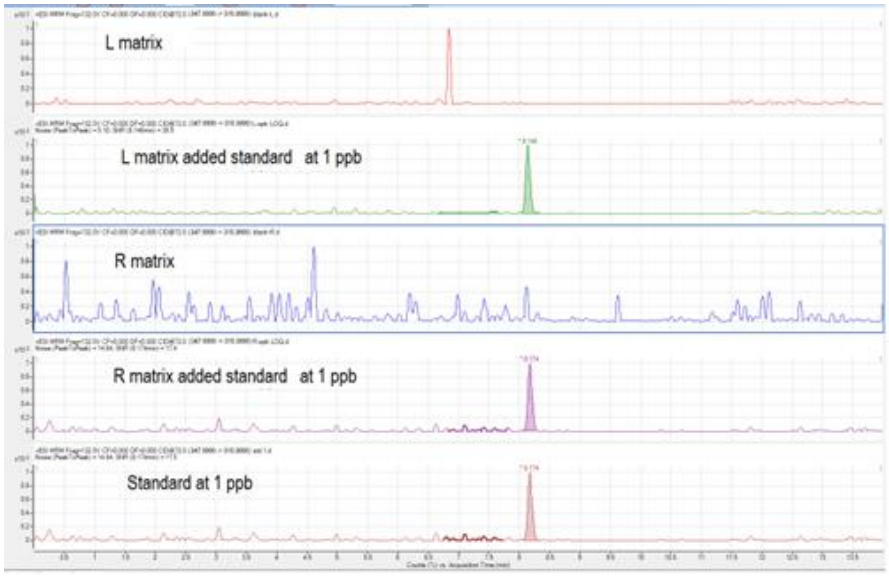

Figure 2. *Chromatograms of nifedipine at the LOQ concentration*

The LOQ values obtained were higher than that obtained by HPLC-PDA analysis for all 4 substances (0.1 - 0.24 ppm) [6] or 0.9 - 3.0 ppm for 3 substances AML, NIF and FEL [2]. When analyzed by LC-MS/MS as used in the research, the LOQ values were quite similar to study [4] with a LOQ of FUR of 1.57 µg/L and research [7] with a LOQ of AML of 1.5 ng/mL; but lower for FEL and NIF with LOQ of 0.25 ng/mL and 0.15 ng/mL, respectively [7]. With the LOQ values were determined for 4 drugs of AML, FEL, FUR, NIF in the traditional medicine matrix, the method is possible to detect the mixing level of these pharmaceutical drugs at a very small level compared to the therapeutic dose, for example, common dose of AML is 5 mg/time, the method can quantify this substance if mixed at 0.02% of the dose when taking 1 g of the preparation.

3.3. Application

The described procedure was applied to the routine examination of various herbal products. Thirty samples, including those from tablets (4), hard capsules (14), hard pills (12), were analyzed with the validated simultaneous LC-MS/MS analysis method used for the 4 anti-hypertensive compounds. In which, 16 samples have indications for treatment or support for the treatment of high blood pressure or used to activate blood and nourish the heart; 14 samples indicated for treatment or support for the treatment of kidney stones or for diuretic use. The results showed that no samples were detected positive for the targeted substances (ie, they were all smaller than the LOD of the analytical method).

Acitive Pharmaceutical Ingredients with antihypertensive and diuretic effects have not been detected in the real samples. The cause may be that these are prescription drugs with strong pharmacological effects, serious side effects, even life-threatening immediately after use, so manufacturers are afraid to add them into their products. Another cause may be that due to the small number of samples, the representativeness is still limited. This result is similar to the results published by a research group in Korea that did not detect positive samples when analyzing 97 samples of traditional medicinal products [7]. But in other studies, it has been found that in herbal preparations illegally mixed atenolol [2]; hydrochlorothiazide [3-4]; clonidine and triamterene [3] and furosemide [4] with different levels such as 0.01% for clonidine, 10.49% for hydrochlorothiazide. Thus, it is necessary to expand the method to other compounds with antihypertensive effects in order to be able to more stricly control the quality of products circulating on the market serving the community.

4. CONCLUSION

The study has established an LC-MS/MS method to simultaneously determine amlodipine, felodipine, furosemide and nifedipine mixed in herbal preparations. Sample treatment is quick and simple. The analytical method has high specificity, low detection

limit, accuracy and precision met the requirements of AOAC 2016 and ICH. The method has high feasibility in analyzing real samples circulating in the market. While none contained detectable amounts of the 4 antihypertensive compounds, the developed LC-MS/MS procedure can be used for routine analysis to monitor illegal adulteration in various forms of herbal products.

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Phân tích đồng thời 4 thuốc hạ huyết áp trộn trong chế phẩm đông dược bằng LC-MS/MS

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Tóm tắt

Phương pháp sắc ký lỏng ghép nối khối phổ (LC-MS/MS) nhạy và đặc hiệu đã được phát triển để phân tích đồng thời 4 thuốc hóa dược có tác dụng hạ huyết áp trộn trong các chế phẩm đông dược. Phương pháp được thẩm định về độ chọn lọc, độ tuyến tính, độ chính xác, giới hạn phát hiện (LOD), giới hạn định lượng (LOQ) và độ đúng theo hướng dẫn của AOAC và ICH. LOD và LOQ của tất cả các chất phân tích khoảng 0,3 ng/mL và 1 ng/mL. Phương pháp có độ tuyến tính tốt ($R^2 > 0,999$) với độ chính xác trong ngày và độ chính xác khác ngày lần lượt là 1,20 - 9,85% và 3,76 - 8,51%, độ đúng nằm trong khoảng 81,9 - 109,8%. Phương pháp đã ứng dụng kiểm tra ba mươi mẫu chế phẩm đông dược bao gồm thuốc cổ truyền và thực phẩm bảo vệ sức khỏe đang lưu hành trên thị trường Việt Nam. Mặc dù không có chất nào trong 4 thuốc có tác dụng hạ huyết áp được phát hiện trong mẫu thử, nhưng quy trình LC-MS/MS đã xây dựng có thể sử dụng cho phân tích thường qui nhằm theo dõi sự trộn trái phép các chất này trong chế phẩm đông dược ở các dạng bào chế khác nhau.

Từ khóa: *LC-MS/MS, phân tích, thuốc hạ huyết áp, trộn trái phép, chế phẩm đông dược.*