Original Research

VALIDATION OF LC-MS/MS METHOD FOR DETERMINATION OF CYPROHEPTADINE IN DIETARY SUPPLEMENTS

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ABSTRACT

Aims: A rapid and sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was validated for determination of prohibited cyproheptadine hydrochloride (CP) in dietary supplements.

Methods: CP was extracted by sonication in methanol for 30 min. The chromatography separation of CP took place on C18 column (100 mm x 2.1 mm, 1.8 µm) with gradient mobile phase of both of acetonitrile and water containing 0.1% formic acid, CP was detected and quantified by mass spectrometric detector. Multiple reaction monitoring (MRM) in the positive mode was used to quantify and confirm CP at m/z 288.2/191.1 and 288.2/96.0, respectively.

Results: The method was validated according to the AOAC requirements. The linearity ranges were found from 0.1 to 50 ng. mL^{-1} of CP (R^2 = 1). The limit of detection and limit of quantification were 1.5 ng/g or ng/mL and 5 ng/g or ng/mL, respectively. The accuracy was within the range from 92 to 99%, with the relative standard deviation (RSD%) of 2.0-5.9%.

Conclusions: The validated parameters have met the requirement of Association of Official Analytical Collaboration (AOAC). This reliable method would be useful for the monitoring of cyproheptadine in dietary supplements.

Keywords: *cyproheptadine, dietary supplements, liquid chromatography-mass spectrometry*

I. INTRODUCTION

Cyproheptadine hydrochloride (CP, Figure 1) is a white or slightly yellow, crystalline powder, which is slightly soluble in water, sparingly soluble in ethanol, freely soluble in methanol, soluble in chloroform and practically insoluble in ether. It is an antihistaminic drug, antagonist of histamine and serotonin with appetite stimulating effect and historically used as prophylactic treatment for migraine. The major reported side effects of CP are increased appetite and weight gain, sedation and sleepiness [1]. The literature survey revealed some methods for CP determination in pure form and in pharmaceutical formulations such as gas chromatography [2] spectrophotometric [3], HPLC [4] [5] and LC-MS/MS [6].

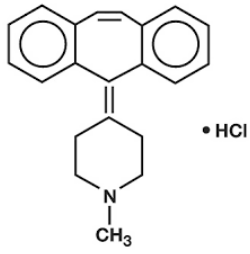

Figure 1. *Chemical structure of cyproheptadine hydrochloride (CP)*

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In Vietnam, The Ministry of Health has issued Circular 10/2011/TT-BYT dated June 30, 2021 [7] regulating the list of substances banned from use in dietary supplement including cyproheptadine. Accordingly, the objective of this study is to develop a selective, sensitive, and accurate method to determine cyproheptadine in dietary supplement. The validated LC-MS/MS method will be applied to monitor the CP illegally mixed in dietary supplements.

II. MATERIALS AND METHODS

2.1. Materials and chemicals

Dietary supplement including solid, hydrophilic, lipophilic samples were collected from pharmacies in Hanoi, Vietnam.

Cyproheptadine hydrochloride (pure 100.64%) was supplied by National Institute of Drug Quality Control, Vietnam. Acetonitrile, methanol, and formic acid were purchased from Merck, Germany. Deionized-water was used for preparation of aqueous solutions and mobile phases.

2.2. Preparation of standard solutions

Stock standard solution 1mg/ml: Ten milligrams of cyproheptadine hydrochloride standard was weighed in a beaker and then transferred to a 10 mL glass volumetric flask and dissolved by sonication in methanol. This solution was stored at -20 $^{\circ}$C for no longer than 1 month. From the stock standard solution, CP standard working solutions were freshly prepared by an appropriate dilution in methanol.

2.3. Preparation of samples

Cyproheptadine in dietary supplement samples were extracted using the method described by Feas X et al (2009) [6], with some modifications. A 1-g or 1-ml portions of dietary supplement samples was weighed in a 50 mL volumetric flask and sonicated for 30 min with 30 mL of methanol. After that, 1 mL of formic acid 0.1% was added. Finally, the volume was made up to 50 mL with methanol. The extract was filtered through a hydrophilic polytetrafluorethylene (PTFE) membrane (0.22 µm. 13 mm) before the LC-MS/MS injection.

2.4. LC-MS/MS equipment and chromatography conditions

The Ekspert ultraLC 110 system (Eksigent, AB Sciex) equipped with autosampler and column oven were used for cyproheptadine separation. The UPLC was coupled with QTRAP 5500 LCMSMS (AB Sciex, USA) for detection and quantification of cyproheptadine, using Electrospray Ionization probe (ESI). Analyst software and Multiquant software (AB Sciex, USA) were used for data acquisition and processing. The UPLC separation of cyproheptadine was implemented on Agilent Eclipse Plus C18 column (2.1 mm x 100mm, 1.8 µm) by using mobile phase A (0,1% formic acid in water) and B (0,1% formic acid in acetonitrile), flow rate of 0.3 mL/min with following

gradient: 10–70% B (3 min), 70% B (2 min), 70–10% B (3 min), 10% B (2 min). Sample injection volume was 10 µL.

The mass spectrometry worked with electrospray ionization in positive mode for cyproheptadine. The following MS/MS parameters were kept constant during the whole acquisition: source temperature: 550ºC; curtain gas: 25 psi; Gas 1: 60 psi; Gas 2: 70 psi; CAD: medium; IS (positive polarity): +5000V.

2.5. Method validation

Validation was established according to the guidelines of AOAC International. Method validation was carried out by performing specificity, linearity, limit of detection (LOD), limit of quatification (LOQ), precision (repeatability) and recovery test

2.5.1. Specificity

The specificity of the method was evaluated by comparing the chromatography of the analytes in the blank with standard and spiked samples. The specificity of the method was futher guaranted by a confirmation method based on the IP (identification point) and ion ratio according to the regulations of the Commission Implementing Regulation (EU) 2021/808 [8]. For the LC-MS/MS, the method is specific when the IP score was 4.

2.5.2. Linearity

Prepared a CP standard solution at 8 concentraions: 0,1 ng/mL; 0,2 ng/mL; 0,5 ng/mL; 1 ng/mL; 2ng/mL; 5 ng/mL; 20 ng/mL and 50 ng/mL. The calibration curve was constructed based on the relation between the concentration and the peak area of the corresponding standards. The calibration curve is linear when the correlation coefficient R^2 was higher than 0.99

2.5.3.Limit of detection and limit of quantification

Limit of detection (LOD) is the lowest CP concentration in a sample that can be detected from the background noise but can not be quantitated. Limit of quantification (LOQ) is the lowest concentration of an analyte that can be determined with acceptable precision and accuracy. LOD and LOQ are calculated following the formular: LOD = x_o + 3 x SD and LOQ = x_o + 10 x SD

2.5.4. Repeatability and recovery

The method repeatability (intra-day precision) and the recovery were determined at three diffirent concentrations. Blank samples were prepared by spiking stock standard solutions to give additional concentrations of by measuring the spiked 5, 100, 1000 ng/g for CP. The repeatability and the recovery were performed by seven replicate analyses for each concentration on the same day (n=7). The repeatability is expressed as % RSD and the recovery is expressed as the percentage recovery of the added CP.

2.6. Data analysis

Using the software supplied with the LC-MS/MS to obtain the chromatograms, peak areas and retetion times. Linearity, recovery rate, standard deviation were determined by Excel software. The results of the validation method were evaluated according to the AOAC 2016 [9].

III. RESULTS AND DISCUSSION

3.1. Method development

The mass spectrometric conditions were optimised to obtain the maximum signal intensity for CP using standard solution of 100 ng/mL. These molecules were easily ionizable in positive mode, using an electrospray ionization source (ESI). The optimization in source parameters were as following: curtain gas (CUR), 25 psi; ion spray voltage (IS), 5.500 V; ion source gas 1 (GS 1), 20 psi; ion source gas 2 (GS 2), 20 psi; temperature (TEM), 400ºC. The analyte was quantified in multiple reaction-monitoring (MRM) mode. For optimal MS parameters, the highest intensity ion was used as quantitative ion and lower intensity ion was used as confirmation ion. The MS/MS conditional results for CP analysis were presented in Table 1.

Table 1. *MS/MS conditions for analysis of CP*

Analyte	Precursor ion *(m/z)*	Product ion *(m/z)*	Declustering potential (V)	Collision energy (V)	Collision Cell Exit Potential (V)
CP	288.2	191.1 [(a)]	16	45	22
		96.0 [(b)]	11	31	14

(a): using for quantifier; (b): using for qualifier

The chromatographic separation was achieved using a Eclipse Plus C18 column. Good efficiency and peak shape were obtained in a 3 min analysis time. Figure 2 shows the chromatogram of the acquisition window for the two MRM transitions for CP: 228.2>191.1 and 228.2>96.0

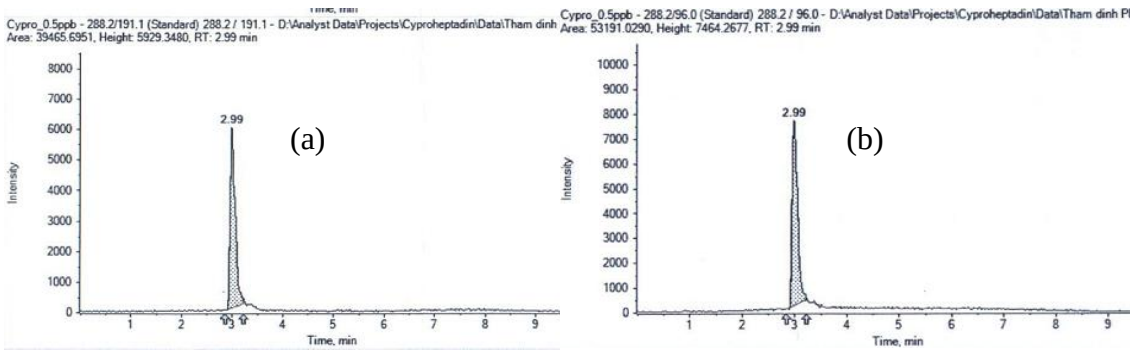

Figure 2. *Chromatogram of the acquisition window for the two MRM transitions for CP: 288.2>191.1 (a) and 288.2>96.0 (b)*

3.2. Method validation

3.2.1. Specificity

The chromatogram of the analytes in the blank with standard and spiked blank sample are shown in Figure 3. The blank sample did not shown the signal of the CP, while the the standard solution and the spiked blank sample with standard have peak of CP with retetion time similar to that of corresponding standard.

Under the chromatographic conditions described in Section 2.5, a complete separation of the CP in the sample was possible. This confirmed that the method was specfic for the detection of cyproheptadine in dietary supplements.

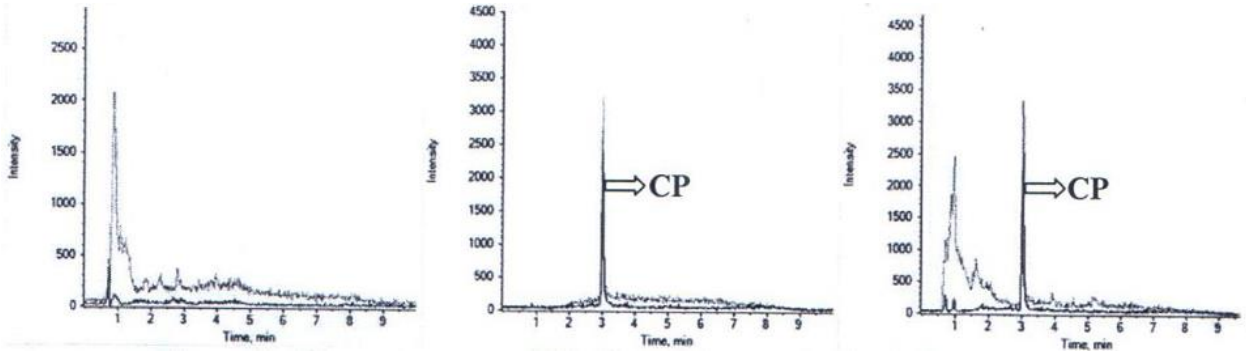

Figure 3. *Chromatograms of blank sample, standard solution and spiked blank sample*

Table 1 showed that the CP precursor ion was bombarded into 2 daughter ions, so the total number of identification points of the method corresponding to each substance was 4 conformable with Commission Implementing Regulation (EU) 2021/808 [8], which confirmed the LC-MS/MS method in this study was specific for determination of CP in dietary supplement.

3.2.2. Linearity and calibration curves

The standard solution of CP from 0.1 to 50 ng/mL was analyzed to determine the linearity. Correlation coeficient of calibration was higher than 0.990, bias was smaller than 15%, which met the criteria of AOAC requirements and proved the high linearity between the peak area and concentration of analyte (Figure 4).

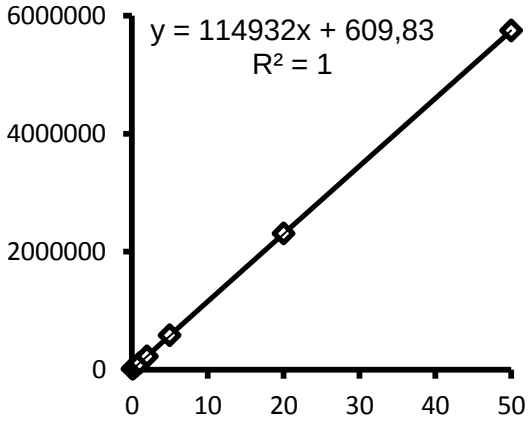

Figure 4. *Calibration curve of CP*

3.2.3. Limit of detection and limit of quatification

Table 2: *Limit of detection (LOD) and limit of quantification (LOQ)*

Matrix	Concentration of CP (*n*=7) (ng/g, ng/mL)	SD	R	LOD (ng/g, ng/mL)	LOQ (ng/g, ng/mL)
Solid dietary supplement	4.89	0.35	4.68	1.04	3.48
Hydrophilic dietary supplement	5.58	0.47	4.00	1.42	4.72
Lipophilic dietary supplement	5.40	0.44	4.11	1.31	4.38

The limit of detection (LOD) and limit of quantification (LOQ) of three kind of matrix were showed in Table 2. The method's LOQs of CP for three kind of matrix were below 5 (ng/g, ng/mL). These results indicate that the method provided adequate sensitivity. Previous studies detemine LOD, LOQ based on the ratio of peak signal of analyte to noise (S/N) [6] [7], this method is only applicable to analytical procedures using tools with backgraound noise and R value is not determined to evaluate the reliability of LOD. Our study uses the calculation of LOD, LOQ based on the standard deviation of 7 blank samples with spiked standards and the R value is between 4 and 10, showing that the concentration of solution in the sample is suitable and the LOD is determined as reliable [9].

3.2.4. Repeatability and recovery

Table 3. *Repeatability of CP at three concentration in three matrices*

Matrix	Repeability (RSD, %)		
	5 (ng/g, ng/mL), *n*=7	100 (ng/g, ng/mL), *n*=7	1000 (ng/g, ng/mL), *n*=7
Solid dietary supplement	2.7	3.1	2.1
Hydrophilic dietary supplement	2.0	4.4	1.3
Lipophilic dietary supplement	5.9	2.4	1.5

The repeability and recovery are given in Table 3 and Figure 5. The %RSD values were 2.0-5.9, 2.4-4.4, 1.3-2.1 for three different concentration, respectively. The percentage recoveries were 95-98%, 95-99%, 92-94% for three diffirent concentration, respectively. The repeability and %RSD meet the requirements of AOAC 2016 (recovery of 80-110%, RSD≤11% at 1 mg/kg concentration) [9].

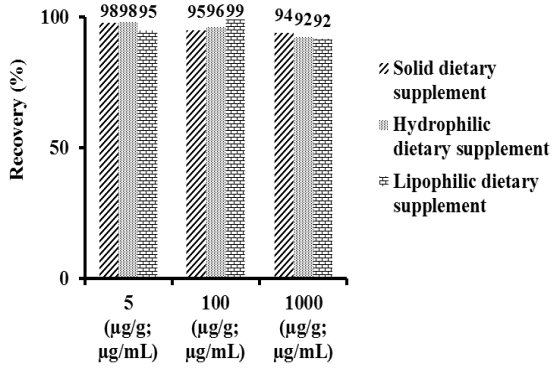

Figure 5. *Recovery of CP at three concentration in three matrices*

IV. CONCLUSION

An analytical method using LC-MS/MS for determination of CP in dietary supplement was fully validated. All the parameters meet the acceptance criteria for method validation according to the AOAC 2016. The method showed good specificity and linearity. The developed method is rapid, sensitive and can be used for the quantification of CP. Moreover, this validated method can be transferred to laboratories equipped with mass spectrometry liquid chromatography instrument. The method helps to alert the authorities and consumers of the CP occurrences in dietary supplement.

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