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Determination of tropane alkaloids by heart cutting reversed phase – Strong cation exchange two dimensional liquid chromatography

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ABSTRACT

Current Chinese Pharmacopoeia (ChP) standards apply liquid extraction combined with one dimensional liquid chromatography (1DLC) method for determining alkaloids in herbal medicines. The complex pretreatments lead to a low analytical efficiency and possible component loss. In this study, a heart cutting reversed phase – strong cation exchange two dimensional liquid chromatography (RP – SCX 2DLC) approach was optimized for simultaneously quantifying tropane alkaloids (anisodine, scopolamine and hyoscyamine) in herbal medicines and herbal medicine tablets without further treatment of the filtered extract. The chromatographic conditions were systematically optimized in terms of column type, mobile phase composition and flow rate. To improve peak capacity and obtain symmetric peak shape of alkaloids, a polar group embedded C18 column combined with chaotropic salts was used in the first dimension. To remove the disturbance of non-alkaloids, achieve unique selectivity and acquire symmetric peak shape of alkaloids, an SCX column combined with phosphate buffer was used in the second dimension. Method validation was performed in terms of linearity, precision (0.54–0.82%), recovery (94.1–105.2%), limit of detection (LOD) and limit of quantification (LOQ) of the three analytes varied between 0.067–0.115 mg L^{-1} and 0.195–0.268 mg L^{-1}, respectively. The method demonstrated superiority over 1DLC method in respect of resolution (less alkaloid co-eluted), sample preparation (no pretreatment procedure) and transfer rate (minimum component loss). The optimized RP – SCX 2DLC approach was subsequently applied to quantify target alkaloids in five herbal medicines and herbal medicine tablets from three different manufactures. The results demonstrated that the developed heart cutting RP – SCX 2DLC approach represented a new, strategically significant methodology for the quality evaluation of tropane alkaloid in related herbal medicines that involve complex chemical matrix.

1. Introduction

Tropane alkaloids [1] widely occur in *Atropa belladonna* L., *Datura metel* L and the seeds of *Hyoscyamus niger* L. Some of these compounds, such as hyoscyamine, scopolamine and anisodine, exhibit anticholinergic and central nervous system activities [2,3]. This has led to their use as anticonvulsants in the form of herbal medicines and their tablets (e.g., Dianqie tablet) [4]. To evaluate the quality of these anticonvulsant drugs, accurate measurement of tropane alkaloids is critically important. However, their determination by conventional one-dimensional liquid chromatography (1DLC) is very difficult due to the complex matrix of these medicines, which includes the presence of many non-alkaloid species [2] and common occurrence of peak tailing, thereby limiting resolution [5,6]. Significant effort during the last decade has been aimed at overcoming these difficulties, including development of matrix clean-up techniques [2] and development of new stationary phases to improve peak symmetry [7]. However, currently available methods for quantitative analysis of tropane alkaloids in herbal medicines are still limited in terms of overall applicability and robustness [1,2,8,9].

Two-dimensional liquid chromatography (2DLC) can provide significantly higher resolving power compared to conventional 1DLC. 2DLC is widely used for the separation of very complex samples, such as in metabolomics [10] and proteome [11]. We have previously reported an offline 2D reversed phase (RP) – strong cation exchange (SCX) method [3,9], which was successfully used for alkaloid purification and shown to

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provide greater peak symmetry and more orthogonal separation than other 2DLC methods. However, as other offline 2DLC methods [9,12–14], this method used for quantitative analysis was found to be time-consuming, operationally intensive, difficult to automate and characterized by low analytical reproducibility [15]. Furthermore, sample contamination or formation of artifacts can occur [16]. Online 2DLC may be used to overcome some of the problems with offline approaches and offer advantages of ease of automation and greater reproducibility in a shorter analysis time. Generally, there are two kinds of online 2DLC including comprehensive and heart cutting techniques. Heart cutting 2DLC involves the transfer of only selected eluent fractions from the first dimension for further separation in the second dimension. While this technique can be very powerful for determining target compounds in complex matrices, such as herbal medicines [17], there are limited reports of its use for alkaloid determination.

In this study, a heart cutting RP – SCX 2DLC method was developed for simultaneously determining tropane alkaloids in herbal medicines and herbal medicine tablets. The effects of mobile phase composition, column type and flow rate was studied and optimized. The developed heart cutting RP-SCX 2DLC method was validated in terms of linearity, sensitivity, precision and recovery. With this method, herbal medicine extract can be analyzed without further pretreatment, since non-alkaloids are removed and alkaloids enriched on the second dimension SCX column. The described technique was finally shown to be broadly applicable with the potential for the quantitative assessment of tropane alkaloids in herbal medicines and herbal medicine tablets.

2. Materials and methods

2.1. Chemical and materials

The reference compounds of anisodine, scopolamine and hyoscyamine and the root of *scopolia tangutica* Maxim. were prepared by Dalian Institute of Chemical Physics, Chinese Academy of Sciences (DICP, ACS) according to reference method [3]. The reference compounds of 2-amino-4,6-dimethoxypyrimidine, 5-amino-2-methoxyphenol, 3-aminobenzyl alcohol, 2-amino-4,6-dimethylpyrimidine, 4-amino-3-methylphenol, 3-aminophenol, 2-amino-4-thiazoleacetic acid, 3-amino-4-cyanopyrazole, 4-amino-4H-1,2,4-triazole, 6-aminopyridine-2-carboxylic acid, dopamine, cytosine, serotonin, epinephrine and naphthalene were purchased from J&K (Hebei, China). The mixture of 21 kinds of basic compounds which used to evaluate orthogonality of the current 2DLC system was prepared by DICP according to the reference [9]. Two bathes of the flower of *Datura metel* L. were from Bozhou TCM market (Bozhou, China) and Chengdu TCM market (Chengdu, China), respectively. The root of *Datura metel* L. and the seeds of *Hyoscyamus niger* L. were from Zhangshu TCM market (Zhangshu, China). The TCM tablet, Dianqie tablet, which is used for stomach cramps were from Sidashu, Renhe and Qiaoguang. HPLC grade acetonitrile (ACN), methanol, sodium dihydrogenphosphate (NaH_2PO_4) sodium perchlorate ($NaClO_4$) and 85% phosphoric acid (H_3PO_4) were obtained from ThermoFisher (Sunnyvale, CA, USA). Water was prepared by a Barnstead GenPure system (Sunnyvale, CA, USA).

2.2. Sample preparation

Herbal medicines were extracted according to reference [2]. Briefly, the dried samples were powdered to a homogeneous composition. A 5.0-g portion of sample powder was placed into an Erlenmeyer flask, and 100 mL of ethanol: water (95:5, v/v) was added. The mixture was shaken and then ultra-sonicated for 2 h. A 1.0 mL volume of extract was diluted with 1.0 mL of water and filtered through a 0.22 μm membrane before HPLC analysis. One TCM tablet of each brand was ground to a powder and extracted with 2 mL of methanol: water (50:50, v/v) for 2 h using ultra-sonication. The extract was filtered through a 0.22 μm membrane before HPLC analysis.

2.3. Heart cutting RP-SCX 2DLC

Heart cutting RP-SCX 2DLC experiments were performed on an Ultimate 3000 dual gradient system consisting of a DGP-3600RS pump, WPS-3000TRS autosampler, DAD 3000RS detector and TCC-3000RS column oven. A two-position, six-port switching valve equipped with a 250 μL loop was used to capture fractions eluted from the first dimensional (^{1}D) column. The maximum pressure allowed by the system was 1034 bar. The gradient delay volume was 1.1 mL which was measured using a reference method [18]. Data were collected and analyzed using Chromeleon version 7.2. All above instruments and workstation were from ThermoFisher (Sunnyvale, CA, USA). The first dimensional separation was performed on a polar group modified column, Acclaim PA2 column (2.1 × 250 mmm, 2.2 μm) from ThermoFisher (Sunnyvale, CA, USA). The second dimensional (^{2}D) separation was carried out on an SCX column, XCharge SCX (4.6 × 150 mm, 5 μm), which was a kind gift from DICP [7,19,20]. The injection volume was 10 μL. The ^{1}D and ^{2}D column shared the column oven and the column oven temperature was 30 °C. The DAD recorded signals at 210 nm and scanned from 190 to 400 nm to examine the peak purity for validation of specificity. The elution programs for the ^{1}D and ^{2}D separation as well as valve switching information are shown in Table 1. The system and valve configuration is shown in Fig. 1.

2.4. Method validation

The established heart cutting RP – SCX 2DLC method was validated using the reference compounds and the extract of *scopolia tangutica* Maxim., since this sample contained all target alkaloids. Parameters estimated for the current method included limit of detection (LOD), limit of quantification (LOQ), linearity, precision, method recovery and sample recovery. The LOD and LOQ values were defined as the concentration of each analyte determined with signal-to-noise (S/N) above 3 and 10, respectively. The precision was measured by calculating the RSD (%) of the contents of three analytes in three parallel samples at the concentrations of 100% sample concentration. Method recovery (recovery 1) was calculated based on the formula: method recovery (%) = $((A_{SCX}\text{-}A_{RP/SCX})/A_{SCX}) \times 100\%$. $A_{RP/SCX}$ was the peak area of reference standard obtained with heart cutting RP − SCX 2DLC method. A_{SCX} was the peak area of the same concentration of reference standard obtained with SCX column. Chromatographic conditions for the SCX column: mobile phase was acetonitrile: 100 mM NaH_2PO_4 (pH 2.8) (50:50, v/v) and flow rate was 1.2 mL min^{-1}. Reference standards of 100% sample concentration (spiked amount) were added into *scopolia tangutica* Maxim. extract (original amount). The samples

Table 1
Optimized gradient eluting program and valve-switching information for the heart cutting RP – SCX 2DLC method.

Time (min)	1D-LC separation				2D-LC separation			valve
	Flow rate (mL/min)	A	B1	C	Flow rate (mL/min)	A	B2	
0	0.2	10	30	60	0.5	50	50	6 – 1
10	0.2				0.5			
11	0.2				1.2			
11.8	0.2				1.2			1–2
12.5	0.2				1.2			6 – 1
18.8	0.2				1.2			1–2
19.4	0.2				1.2			6 – 1
24.5	0.2				1.2			1–2
25.5	0.2				1.2			6 – 1
30	0.2	30	30	40	1.2			
35	0.2	50	30	20	1.2			
35.1	0.2	10	30	60	0.5	50	50	

Solvent A: acetonitrile; B1: 100 mM $NaClO_4$ + 50 mM NaH_2PO_4 (pH 2.8); B2: 100 mM NaH_2PO_4 (pH 2.8); C: water.