Determination of $dl$-tetrahydropalmatine in Corydalis yanhusuo by $l$-tetrahydropalmatine imprinted monolithic column coupling with reversed-phase high performance liquid chromatography

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Abstract

A method for direct determination of $dl$-tetrahydropalmatine ($dl$-THP) in Corydalis yanhusuo, a traditional Chinese herb, by $l$-THP imprinted monolithic precolumn on-line/off-line coupling with reversed-phase high performance liquid chromatography (RP-HPLC) was developed. The $l$-THP imprinted monolithic column has been prepared by in situ polymerization using methacrylic acid (MAA) and ethylene dimethacrylate (EDMA) as functional monomer and cross-linker, respectively. With the optimization of chromatographic conditions, such as mobile phase composition, flow rate, column temperature and sample loading, for the separation of enantiomer, $dl$-THP was base-line separated on the MIP. The imprinted monolithic column was used as a precolumn for fractionation of the C. yanhusuo extract. Both the non-retained and retained fractions were separated by RP-HPLC. Meanwhile, the $d$-THP and $l$-THP can be detected in the non-retained and retained fractions, respectively. Additionally, direct determination of $l$-THP using molecularly imprinted monolith on-line coupling with a reversed-phase column was acquired.

Keywords: Molecularly imprinted polymer; Monolithic column; Tetrahydropalmatine; Chiral separation; On-line; Solid-phase extraction; Corydalis yanhusuo

1. Introduction

Corydalis yanhusuo is well known as a traditional Chinese medicine (TCM). It has been used to promote blood circulation, reinforce vital energy and alleviate pain such as headache, chest pain. One of the active ingredients isolated from it is $dl$-tetrahydropalmatine ($dl$-THP), which is a very effective monoamine depletory in brain [1,2]. Xu et al. [3] have reported that the two enantiomers of $dl$-THP act on different targets. Thus, enantioseparation and extraction of $dl$-THP from TCM is very important. Derived from the tetrahydroprotoberberine backbone structure, $dl$-THP belongs to the isoquinoline alkaloid family. There are some structural analogues such as corybulbine only exists $d$-conformation in C. yanhusuo, but the canadine and tetrahydrocoptisine only exists $l$-conformation (Fig. 1). Because of the complexity of the herb composition, tedious procedures involving several liquid–liquid extractions are generally performed in the traditional method for the extraction of the isoquinoline alkaloid including both $d$- and $l$-conformation of THP.

Molecular imprinting polymer (MIP) is a class of tailor-made material with predetermined selectivity for analytical field, such as chiral separation, biosensors, and so on [5–11]. In addition, the application of solid-phase extraction (SPE) procedures involving molecularly imprinted polymers, called MISPE, has received increasing attention over the past decade as an attractive alternative for the analysis of complex sample, particularly in complex pharmaceutical and environmental samples. To date, successful applications of MIPs to SPE have been described in refs. [12–16]. Most of application of MIP for SPE was investigated in an off-line mode; in other words, the MIP was used as the sorbent for extraction followed by analysis and determination. In this study, $l$-THP imprinted polymer has been directly prepared in a stainless-steel column by in situ polymerization.
The enantioseparation of \( \text{dl-THP} \) was obtained on the prepared monolith by HPLC. Further, the monolith as the precolumn was on-line coupled with C18 column for extraction of \( \text{l-THP} \) and its analogues, thus the \( \text{l-THP} \) was directly determined by RP-HPLC.

2. Experimental

2.1. Materials

Racemic \( \text{dl-THP} \) and enantiomer \( \text{l-THP} \) (98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Ethylene dimethacrylate (EDMA) from Sigma (St. Louis, MO, USA) was extracted with 10% aqueous sodium hydroxide and dried over anhydrous magnesium sulfate. Toluene (ACN) and other solvents were HPLC or analytical grade.

2.2. Preparation of molecularly imprinted monolithic column

The \( \text{l-THP} \) imprinted monolithic column was prepared by in situ polymerization within the confines of a stainless-steel column tube of 30 mm × 4 mm I.D. The template molecule, functional monomer (MAA), cross-linker (EDMA) and initiator (AIBN) were dissolved in porogenic solvents (1.5 mmol toluene and 6.0 mmol dodecanol) to form homogenous solution, in the compositions as indicated in Table 1, which was sonicated for 10 min and purged with dry nitrogen for 15 min to remove oxygen.

\[
\text{P1} \quad 0.30 \quad 0.40 \quad 4.0 \quad 0.96 \quad 3.08 \quad 3.21 \quad 0.78 \\
\text{P2} \quad 0.30 \quad 0.60 \quad 4.0 \quad 1.12 \quad 4.80 \quad 4.28 \quad 1.02 \\
\text{P3} \quad 0.30 \quad 0.80 \quad 4.0 \quad 1.30 \quad 5.04 \quad 3.88 \quad 0.90 \\
\text{P4} \quad 0.20 \quad 0.40 \quad 4.0 \quad 0.86 \quad 2.72 \quad 3.16 \quad 0.75 \\
\text{P5} \quad 0.35 \quad 0.70 \quad 4.0 \quad 1.24 \quad 4.67 \quad 3.77 \quad 0.82
\]

HPLC conditions: column size, 50 mm × 4.0 mm I.D.; column temperature, 20 °C; flow rate, 0.3 mL/min; detection wavelength, 280 nm. The mobile phase was pure ACN with addition of 1.2% acetic acid (v/v).

Similarly, the non-imprinted blank monolithic column (NIP) without the imprinted molecule was prepared in the same way.

2.3. Chromatographic evaluation

The enantiomer separation of \( \text{l-THP} \) was directly determined by RP-HPLC. Further, the monolith as the precolumn was on-line coupled with C18 column for extraction of \( \text{l-THP} \) and its analogues, thus the \( \text{l-THP} \) was directly determined by RP-HPLC.

2.4. Breakthrough curves of \( \text{l-THP} \) on the MIP and NIP monolith

Firstly, the MIP and NIP monoliths, respectively, were thoroughly flushed with pure ACN until a stable baseline was observed at 280 nm. Secondly, the monoliths were shortly removed from the LC system, and the tube from the reservoir to the inlet of the monolithic column was filled with a solution of the \( \text{l-THP} \) in ACN (0.15 mg/mL). In the following, the columns were again connected with the lines, and by starting the pump.
2.5. Preparation of C. yanhusuo extraction

The powdered root (1.0 g) of C. yanhusuo purchased from a local store (Dalian, China) was extracted with 1.0 mL methanol for 30 min in an ultrasonic bath. The extraction was repeated three times. The extracts were combined and diluted with methanol to 5.0 mL. The solution was passed through a 0.45 μm filter and stored at 4 °C for further experiments.

2.6. On-line determination of l-THP

The HPLC analysis was performed on a column (200 mm × 4.6 mm I.D.) packed with Kromasil ODS (5 μm) purchased from Eka Chemicals (Bohus, Sweden). For the on-line molecularly imprinted SPE (MISPE), a six-port column-switching valve (Valco Instruments Co. Inc., USA) and an Elite P230 pump (Elite, Dalian, China) were used. The monolithic column was coupled to a Shimadzu LC-10A liquid chromatographic system mentioned above. The schematic diagram of on-line SPE-HPLC system is similar to that previously reported by Caro et al. [28].

Firstly, the precolumn was conditioned with ACN at the flow rate of 0.3 mL/min. Secondly, the 20 μL extraction solution of C. yanhusuo was applied to the conditioned precolumn, and the precolumn then washed with different volume of ACN to elute the non-retained compounds. Thirdly, the compounds retained on the precolumn were eluted by the mobile phase B, organic solvent (ACN/acetic acid, 98/2, v/v), and transferred into the analytical column in the back-flush mode. Finally, the mobile phase B was mixed with the mobile phase A, aqueous solvent (water/ACN/acetic acid/SDS, 90/10/2/0.3, v/v/v/w, pH 3.0), prior to reaching the analytical column. The analysis was performed on an ODS column using gradient elution at the flow rate of 1.0 mL/min.

3. Results and discussion

3.1. Separation behaviors of THP enantiomers on the acquired monolithic column

The molar ratio of the functional monomer to template (M/T) has been found to be important with respect to the number and quality of MIP recognition sites [31]. A series of polymers (P1–P5) were prepared using different amounts of the functional monomer and the concentration of the functional monomer on the retention factor, enantioseparation factor, and resolution of THP: The retention factor of both d-THP and l-THP increased with an increase of the amount of functional monomer, MAA, used by keeping the template molecule of l-THP at 0.30 mmol. However, the maximum enantioselectivity and resolution were observed on the P2 when 0.60 mmol of MAA was used. The enantioselectivity and the resolution of THP on the P4 and P5 were not bigger than those on the P2 when the ratio of M/T was kept at 2. Thus, the amounts of EDMA, MAA and l-THP in polymerization solution at 4.0, 0.60 and 0.30 mmol, respectively, are the optimal composition for the preparation of l-THP imprinted polymer. In addition, the batch-to-batch and column-to-column reproducibility for the preparation of the monolith were also evaluated, and the satisfied results were obtained (RSD < 5%, n = 3).

Fig. 2 indicates the chromatogram for the separation of racemic THP on the l-THP imprinted polymer under the isocratic elution. It can be seen that the second eluted peak is seriously broadening, tailing and asymmetry. Although the phenomenon of peak broadening due to the diffusion exists in any chromatographic model, it is particularly severe for MIP columns. The poor peak efficiency could be attributed to many possible reasons. In addition to the non-specific interaction, the heterogeneous population of different energy binding sites formed in the MIP is usually the result of a partially incomplete monomer-template association [32–34]. As the addition of polar substances, such as water, acetic acid into the mobile phase can weaken the interaction between template molecules and MIP, the composition of mobile phase need to be optimized [35].

As shown in Fig. 3a, the l-THP is strongly retained with the retention factor of 13.1 when the concentration of acetic acid is 0.75% (v/v). While the concentration of acetic acid further increased, the retention of l-THP on the MIP decreased more remarkably. The retention factor of the l-THP decreased from 13.1 to 1.8, however, that of d-THP decreased from 1.7 to 0.35 as the concentration of acetic acid ranged from 0.75 to 2.0% (v/v). At 2% (v/v) acetic acid in ACN only a shoulder peak was observed.

To further study the effect of the concentration of polar substances on separation of enantiomers, the experiments with mobile phases of ACN containing different concentration of water were performed. As can be seen from Fig. 3b, the retention of the l-THP is very strong when the concentration of water is...
Fig. 3. Effect of the content of (a) HOAc and (b) water in ACN on the retention of THP on l-THP imprinted polymer. Retention factor of (▲) d-THP, k_d; (■) l-THP, k_l; (▲) enantioseparation factor, α; HPLC conditions as in Table 1.

5% (v/v), the retention factor of the l-THP is 16.6. While the concentration of water increased up to 30% (v/v), the retention factor of l-THP on the MIP decreased up to 2.2, however, that of d-THP decreased from 2.2 to 0.7, thus the enantioseparation factor decreased remarkably. When the content of water in ACN was in the range of 30–70% (v/v), no enantioseparation was obtained. However, the retention factor further increased when the percentage of water reach to 80%. It has been shown that intermolecular hydrogen bonding between solute molecules is weak in aqueous solution because of the competition from the high concentration of water molecules. These results can be explained by considering that at lower water percentages in ACN, water molecules act as competing ligands for the hydrogen bonding sites of the MIP and reduce both the retention and the resolution [36]. However, since the THP and the polymer are relatively hydrophobic, the hydrophobic interaction came to play when the percentage of water was increased, the retention began to increase and the recognition was regained.

Table 2 shows the effect of flow rate on the retention factor, enantioseparation factor and resolution of the enantiomers of THP. With a decrease in the flow rate, the retention time increased and the best resolution for enantioseparation of THP was obtained at the flow rate of 0.3 mL/min. It may be due to the slow mass transfer of the enantiomers on the MIP [37]. The effect of sample loading on the retention factor, enantioseparation factor and chiral resolution of THP was also investigated by injecting of THP at different concentration onto the monolithic column. The results are listed in Table 3. With an increase of sample loading, both enantioseparation factor and resolution gradually decreased.

Table 3 shows the effect of sample loading on the chiral separation of THP enantiomers on the l-THP imprinted polymer. In fact, since the monolithic columns in HPLC have gained significant interest due to their ease of preparation, high reproducibility and fast mass transport, the speed of separation can be increased at a higher flow rate and column temperature, and the peak shape of late-eluting enantiomer can be imposed with gradient elution [38]. Based on the results of Fig. 3, the condition of gradient elution could be easily acquired. Thus, the rapid enantioseparation of THP on the molecularly imprinted monolithic column was obtained with stepwise gradient elution at higher flow rate, as shown in Fig. 4. It can be seen

![Fig. 4. Chromatogram for separation of the racemate of THP on the l-THP imprinted polymer. HPLC conditions as in Table 1 except that stepwise gradient elution by ACN/HOAc (99.5/0.5, v/v) for 0–3 min and ACN/HOAc (98/2, v/v) for 3–6 min at flow rate of 1.0 mL/min.](image-url)
that this chiral compound was successfully discriminated within 5 min.

3.2. Off-line and on-line quantitative determination of \( \text{dl-THP} \) in \( C. \) yanhusuo by the monolithic precolumn hyphenated with RP-HPLC

As mentioned above, it is because of the limited number of recognition sites on the surface of the MIP that the sample loading has an effect on the chiral separation of \( \text{dl-THP} \). Thus, studying on the adsorption capacity of the MIP for the \( l \)-THP is very helpful to verify the workability of the monolithic column to extract the analogues from \( C. \) yanhusuo. Fig. 5 illustrates the breakthrough curves of \( l \)-THP on the \( l \)-THP imprinted and non-imprinted monolithic columns. It can be seen that the breakthrough time is 15.60 min for the MIP and 5.93 min for the NIP. Considering the void volume of the column, the adsorption capacity of both MIP and NIP monoliths for \( l \)-THP were 3.90 and 1.29 mg/g, respectively. As a result, the number of specific affinity sites on the surface of the MIP was reached to

Fig. 5. Breakthrough curves of \( l \)-THP on non-imprinted polymer (NIP) and \( l \)-THP imprinted polymer (MIP) column. Mobile phase, ACN in addition of 0.15 mg/mL \( l \)-THP; flow rate, 0.5 mL/min.

Fig. 6. Chromatograms for (a) the separation of crude extraction of \( C. \) yanhusuo on non-imprinted polymer (NIP) and \( l \)-THP imprinted polymer (MIP) column and the separation of (b) crude extraction of \( C. \) yanhusuo, (c) the non-retained and (d) the retained fractions on MIP column by RP-HPLC. (a) HPLC conditions as in Table 1 except that stepwise gradient elution by pure ACN for 0–14 min, and switching to ACN/HOAc (98/2, v/v) for 14–40 min (b-d) Analytical conditions: C18 column, 200 mm × 4.6 mm I.D.; mobile phase, (A) water/ACN/acetic acid/SDS (90/10/2/0.3, v/v/v/w, pH 3.0), (B) ACN/acetic acid (98/2, v/v); flow rate, 1.0 mL/min; detection wavelength, 280 nm. Linear gradient elution from 35% B to 60% B in 30 min and from 60% B to 90% B in another 10 min, then stepwise gradient elution from 90% B to 100% B in 5 min and lasting for another 5 min.
7.34 μmol/g, which indicates the specific adsorption capacity of the obtained MIP was high enough to use it as the sorbent material for SPE.

The crude extraction of *C. yanhusuo* was directly loaded onto the MIP and NIP monoliths for the following separation by off-line mode, respectively. Because of the complicity of the extraction sample, the separation conditions were optimized again. It can be observed from the chromatogram shown in Fig. 6a that a very high peak was appeared at the void time, and only a small peak was retained longer on the MIP column. However, no retained peak was observed on the NIP column after eluted by 4 mL of ACN. Both the non-retained and the retained fractions on the MIP column corresponding to the peaks 1 and 2, respectively, were collected and evaporated to dryness under vacuum, and redissolved in 100 μL of ACN. Then they were determined by RP-HPLC.

The separation conditions of HPLC for the extraction solution were optimized prior to determining the THP. As indicated in Fig. 6b, n-THP was completely separated with other compounds. The linearity between 0.01 and 0.30 mg mL\(^{-1}\) in Fig. 6b, determined by RP-HPLC, and the good regression coefficient (\(R^2 = 0.9998\)) was obtained. The limit of detection (signal-to-noise, 3) and limit of quantification (signal-to-noise, 10) for THP were 1.5 and 5.0 μg mL\(^{-1}\), respectively. The total THP amount of 0.11 mg mL\(^{-1}\) in *C. yanhusuo* extraction solution was acquired. According to the separation behavior of THP on the MIP mentioned above, the n-THP and l-THP were existed in the collected fractions 1 and 2, respectively. It can be seen from Fig. 6d that there are only a few compounds observed in fraction B. Thus, the concentration of n-THP and l-THP in the extraction were directly determined by HPLC, and were 0.033 and 0.038 mg mL\(^{-1}\), respectively. This difference may be due to the loss of analytes during the sample preparation process with off-line mode. The recovery of l-THP spiked in extraction was 62% (RSD < 10%, n = 3).

The general application of MIP for SPE of the active ingredients from the TCM was reported previously in the off-line mode [17–25]. This method is time-consuming and tedious, however, the on-line mode may overcome some disadvantages. For example, there is no sample manipulation between the fractionation and the analysis steps, which reduces the loss of the analytes and the risk of contamination, thus the detection limit and reproducibility are improved. Furthermore, the whole SPE sample is directly transferred onto the analytical column, so the consumption of solvents is lower, and the automation can be easily realized. In our case, the l-THP-imprinted monolith was on-line coupled with reversed-phase column for extracting and determining active ingredients in *C. yanhusuo*.

The SPE conditions for the extraction solution such as the volume of sample loading, selection and consumption of eluting solvents were systematically optimized prior to on-line analysis. The 20 μL crude extraction of *C. yanhusuo* was injected onto the monolithic precolumn, and eluted by the 4 mL of ACN. After the SPE process, the active ingredients retained on the precolumn were eluted by ACN containing 2% (v/v) acetic acid (mobile phase B) with back-flush mode to reduce band-broadening, and then on-line determination was performed by RP-HPLC [39]. The chromatogram of crude extraction was obtained (Fig. 7), and the content of l-THP in extraction was 0.049 mg mL\(^{-1}\), which demonstrated that the on-line SPE by imprinted monolithic column was very efficient and fast. Meanwhile, the l-THP content in extraction can be indirectly obtained. Standard addition method was used to evaluate the accuracy and precision of this MIP-SPE process. The recovery of l-THP spiked in *C. yanhusuo* extraction was reached to 91% (RSD < 7%, n = 3), which is higher than that obtained with off-line mode. These results indicate that on-line MIP-SPE technique can be successfully applied for extraction of l-THP and other analogues in natural *C. yanhusuo*.

4. Conclusion

The l-THP, an active ingredient of *C. yanhusuo*, imprinted monolithic column has been successfully prepared in stainless-steel column by in situ polymerization method. Compared with bulk polymerization, suspension polymerization and other polymerization methods for preparation of MIP, this method is not only quite simple but also cost-effective because the template molecules are sometimes rare and expensive. The monolithic column with good flow-through property and selectivity was successfully used as the precolumn for solid-phase extraction of l-THP from *C. yanhusuo* with off-line and on-line mode, respectively, and the concentration of n-THP and l-THP in extraction was directly detected by HPLC. The result indicated that on-line MISPE mode has advantages over off-line MISPE, such as fast speed analysis and high efficiency.

Furthermore, the imprinted monolith can efficiently extract not only the template, l-THP, but also its analogues from natural herb. Although the bioactivity evaluation of these analogues has not been performed, it can be deduced that they might...
also relate with biological function of TCM according to the molecular recognition property of the MIP. Thus, the MIP has been recommended as the mimic receptor for bioassay and drug development [40]. It is expected that the MIP monolith is very prospective as a receptor mimic to assay the bioactivities from the complicated natural herbs.

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