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A Removable Derivatization HPLC for Analysis of Methanol in Chinese Liquor Medicine

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ABSTRACT

A high performance liquid chromatography (HPLC) method for the determination of trace methanol in high water content sample has been established. Using 4-[N-methyl, N-(1-naphthylmethyl)]-amino-4-oxobutanoic acid (NAOB), 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) under a mild condition, the derivatization of methanol in aqueous solution was smoothly carried out before HPLC analysis. After derivatization, acidic or basic interfering substances and excess reagents can be easily removed from organic layer by simple acid-base shake-out. The condition for optimal derivatization of methanol in aqueous solution was established and applicable to the analysis of commercial Chinese liquor medicine (ethanol content 8~55%, v/v), the range of trace methanol in the above samples was 2.20×10^{-4}~ 3.28×10^{-3}% (v/v). The result showed that methanol contents in all samples were below regulation standard (< 0.1%, v/v).

Key words: removable derivatization, methanol, HPLC, Chinese liquor medicine

INTRODUCTION

Methanol is highly toxic, and is widely used as one of the solvent media in the extraction of natural ingredients. Therefore, it is essential to detect the trace methanol residue in herbal medicine. Workers and alcoholics may be poisoned by accidental inhaling or ingesting methanol. Methanol is one of the environmental pollution agents; therefore, the amount of the existing methanol can be used as an indicator of environmental pollution. The trace methanol is also used as one indicator in determining the level of sophistication in making Chinese liquor medicine. So far the analysis methods for methanol in different samples as recorded are Gas Chromatography (GC)[7-11], Colorimetry[12], Enzymatic Assay[13-14], and High Performance Liquid Chromatography (HPLC)[15-17]. Colorimetry is one of the common methods; however, it lacks specificity as it is often affected by the complexity of the sample matrix. Using GC to detect methanol is rather convenient, and the detection is often performed by using flame ionization detector (FID). Convenient as it may be, since methanol does not contain much carbon, the sensitivity of FID, which is mass sensitive, is limited. By using HPLC, we need to derivate methanol into chromophoric derivative to increase the analytic sensitivity, as methanol lacks chromophor. The derivatization agents used in HPLC analysis often require dehydration process or are limited to the amount of water contained in the samples[15,18]. HPLC analysis is also affected by the complexity of crude drug in detecting the trace methanol. Our laboratory has already established an analysis method by using Ipecac fluidextract sample to detect trace methanol in samples that contain high volume of alcohol (ethanol volume at 63~69%, v/v). The principal derivative equation is shown in Figure 1. In order to further adopt the removable derivatization analysis method in liquor samples containing high water content (ethanol volume at 8~55%, v/v), our study further explored ways of optimatization when the methanol is completely solved in water. The method was then used in detecting trace methanol in liquor available in local market.

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MATERIALS AND METHODS

I. *Reagents*

4-[N-methyl, N-(1-naphthylmethyl)]-amino-4-oxobutanoic acid (NAOB) was synthesized in the laboratory. 4-Dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-methyl, N-naphthylmethyl amine hydrochloride were purchased from TCI, Japan; flavanone was purchased from Aldrich, USA; pyridine from E. Merck, Germany; succinic anhydride from Showa Chemical, Japan; methanol, dichloromethane, and acetonitrile from Tedia, USA; and various brands of liquor medicine were purchased in Taiwan market.

II. *Synthesis of NAOB*

Took one 200 mL reaction flask, solved N-methyl-N-naphthylmethylamine hydrochloride (5.00 g, 24.1 mmol), DMAP (0.290 g, 2.41 mmol) and pyridine (3.90 mL, 48.1

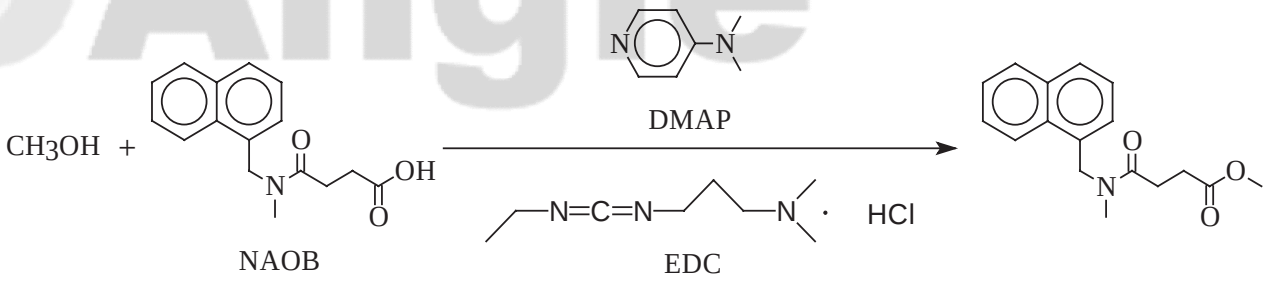

Figure 1. Derivatization scheme for methanol.

mmol) with 60 mL dichloromethane. Slowly added in succinic anhydride (7.23 g, 72.2 mmol), stirred under room temperature for two hours. Then moved the solution to a 250 mL separatory funnel, washed with 1N sulphuric acid (20 mL × 3) and water (40 mL × 2) respectively. Collected dichloromethane layer, and concentrated until dry. Added in 60 mL of acetonitrile-water (5/7, v/v) to the residue and stirred under room temperature for two hours, then condensed the solution. When the solution was condensed to half of its original volume, added water (160 mL) then white precipitate appeared and continuously stirred for one hour. Filtered the solid white matter with Büchner funnel, dehydrated it, and NAOB was synthesized (5.96 g, yield 91.3%, m.p. 122.0-122.8˚C).

III. *HPLC*

We used Waters Associates 510 LC pump, Basic-Marathon automatic sample injector and Applied Biosystems 785A programmable absorbance detector (λ 281 nm). The precolumn was NovaPak C_{18} (150 × 3.9 mm I.D., 4 μm). The mobile phase was acetonitrile-water 40:60 (v/v) and flow rate was 1.0 mL/min.

IV. *Optimal Conditions for Methanol Derivatization in Aqueous Solution*

Except for methanol in water solution, the rest of the reagents were dissolved in acetonitrile. Added methanol aqueous solution 50 μL, 200 μL NAOB as the derivatization reagent, 50 μL DMAP as the catalyst and 50 μL flavanone as the internal standard in a 10 mL reaction test tube with a glass cap, mixed well and added 825 μL dichloromethane and 200 μL EDC as the coupling reagent to be reacted under 30˚C. When derivatization occurred, washed the reaction solution with 2 mL 1N sodium hydroxide, and washed again with 2 mL 1N sulphuric acid. Aspirated dichloromethane 500 μLand blew dry with nitrogen, solved the residue with 500 μL acetonitrile, and took 10 μL to be injected into HPLC for analysis.

V. *Determination of Methanol in Over-the-counter Liquor Medicine*

Used the standard addition method to detect methanol in over-the-counter liquor medicine. Added five different concentrations of methanol (1×10^{-3}%, 2×10^{-3}%, 4×10^{-3}%, 6×10^{-3}%, 8×10^{-3}%(v/v)) to the liquor medicine for derivatization reaction to plot the calibration curve, with methanol concentration (%, v/v) as the X axis, and peak area ratio between methanol derivative and internal standard as the Y axis. The methanol content in the liquor medicine was the extrapolation along X axis, calculated by linear regression.

DISCUSSION

In order to detect trace methanol in over-the-counter liquor medicine with high water volume (alcohol at 8%), we explored the optimal methanol derivatization condition in aqueous solution. After establishing the best derivatization condition for methanol solution, we then used standard addition to detect the methanol content in our samples.

I. *The Optimal Derivatization Condition for Methanol Solution*

We used the 50 μL methanol solution (containing 0.125 μmol methanol) to explore the optimal conditions in derivating each required parameter. When the derived EDC quantity (7.50~67.5 μmol) was at 48.8 μmol, the quantity of the derivative could reach plateau, and the molar ratio between EDC and methanol was at 390. In order to ensure a complete reaction, we chose 56.3 μmol EDC as the optimal derivatization condition. The production of the derivative reached plateau when NAOB quantity (5.00~27.0 μmol) was at 17.5 μmol, and the molar ratio between NAOB and methanol was at 140. 20.0 μmol was chosen as the optimal condition to ensure a complete reaction. DMAP (10.0-48.0 μmol) at 30.0 μmol could make the derivative reach plateau, the molar ratio with methanol was 240, and 37.5 μmol was chosen as the optimal derivatization condition in order to ensure a complete reaction. The production of the derivative reached plateau at two hours into the required derivatization duration (5 min – 180 min), the reaction time was then set at two hours. Using the above conditions, we used five different concentrations of methanol to certify the reliability of this analysis method in quantitating methanol. The range investigated was 1×10^{-3}%~1×10^{-4}%, and the liner regression equation was $Y = (740 \pm 5) X + (0.05 \pm 0.007)$ (n = 6). X axis was the concentration of methanol (%, v/v) and Y axis was

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the peak area ratio between methanol derivative and the benchmark internal standard sample. The relative standard deviation (R.S.D.) of slope of the calibration curve was 0.68%, with a correlation factor of r = 0.999, thus we learned that this analysis method has a good reproducibility to methanol. In order to evaluate the accuracy and the precision of the calibration curve, we further inspected with three different concentrations of methanol 1×10^{-4}%, 5×10^{-4}%, and 1×10^{-3}% , and the results are shown in Table 1, which indicated the high accuracy of this analysis method. The derivatization results of added 2.50 mM methanol, 5.00 mM ethanol, 6.00 mM n-propanol, 10.0 mM isopropanol, and 8.00 mM a n-butyl alcohol were shown in Figure 2. The analysis method proved to have good selectivity.

II. *Trace Methanol in Over-the-counter Liquor Medicine*

Methanol content in liquor medicine samples was detected by the conditions established above. Due to interfering peaks, the analysis was conducted by standard addition method, the chromatogram is shown as Figure 3. Table 2 showed the liquor medicine declared compositions and the ethanol volume; and the methanol analysis results are shown in Table 3. The results indicated that the methanol content in the above liquor medicine was within standard (<0.1%, v/v). However, the level of methanol contamination during the production of brand A liquor medicine was the lowest, this meant that there was less methanol in the material than in the production environment. Brands A, B and C are manufactured by pharmaceutical companies; whereas brands D and E were manufactured by food companies. The analysis results showed that the level of trace methanol contamination in the products produced by pharmaceutical factories was lower than that by food factories. This was due to the high quality control requirement in pharmaceutical factories. Also, there were at least 25 types of Chinese medicine ingredients contained in the sample liquor medicine A and our method was able to rule out the interference created by most other ingredients to detect the trace methanol with HPLC (Figure 3), this indicated that the removable derivatization analysis could be used in samples with a complex matrix to detect the trace methanol.

CONCLUSION

Our study used NAOB with DMAP and EDC reagents to form a removable derivatization. This derivatization method could be used with methanol samples containing higher water volume (around 92%) and of complicated matrix without going through dehydration or pretreatments. With HPLC, we successfully established the analysis method that could detect trace methanol from the samples that contain multiple ingredients. This method could engage derivatization reaction with methanol under 30˚C. With simple water wash to remove interfering materials and reagents in the sample, the trace of methanol could be detected from the methanol derivative by using a C_8 column. Using acetonitrile and water 40:60 (v/v) as the mobile phase under the ultra violet detector wavelength at 281 nm, the trace methanol quanti-

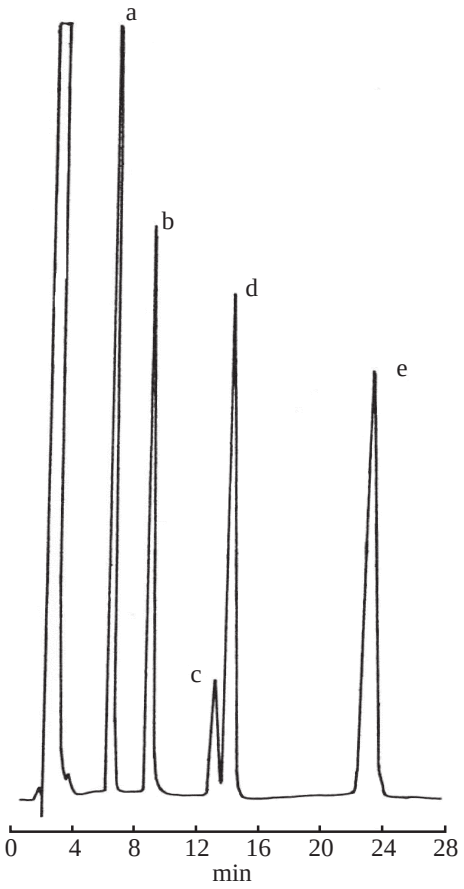

Figure 2. The chromatogram of a standard mixture of alcohols derivatized with NAOB. Peaks: a. methanol (2.50 mM) ; b. ethanol (5.00 mM); c. isopropanol (6.00 mM); d. n-propanol (10.0 mM); e. n-butanol (8.00 mM).

Table 1. Precision and accuracy for the determination of methanol

Concentration known(v/v, $\times 10^{-4}$%)	Concentration found (v/v, $\times 10^{-4}$%)	R.S.D.(%)	R.E.(%)
Intraday(n=6)			
1.00	1.01 ± 0.03	2.97	1.00
5.00	4.96 ± 0.06	1.21	–0.80
10.00	10.05 ± 0.11	1.09	0.50
Interday(n=7)			
1.00	1.02 ± 0.04	3.92	2.00
5.00	5.03 ± 0.12	2.39	0.60
10.00	9.98 ± 0.19	1.90	–0.20

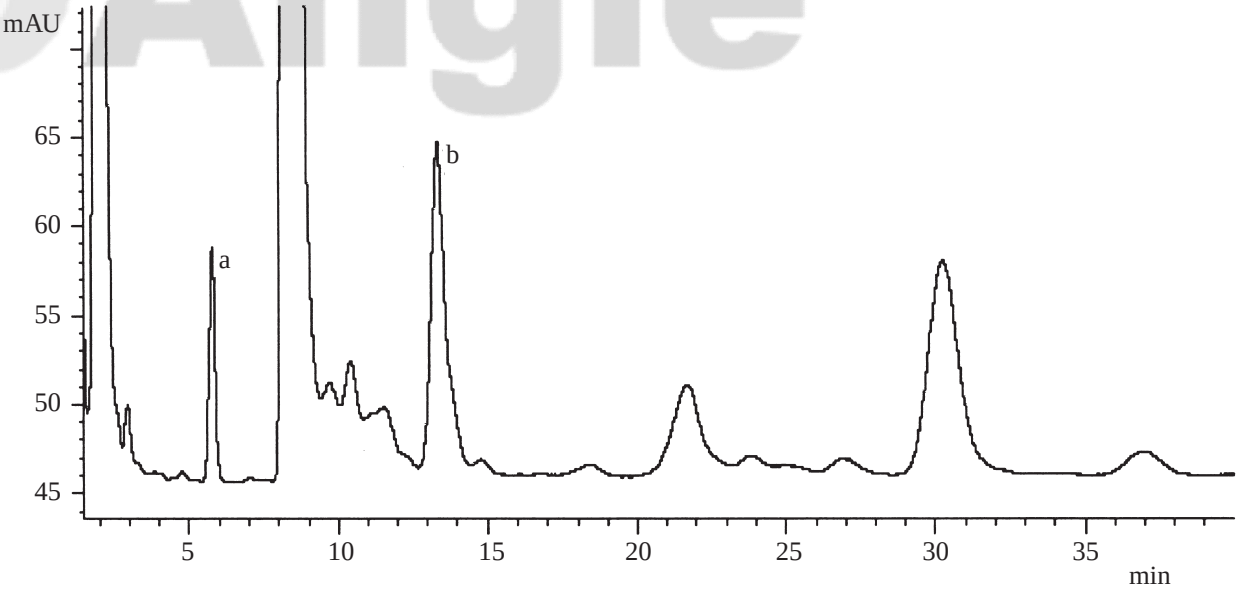

Figure 3. HPLC chromatogram of methanol derivative in brand A Chinese liquor medicine. Peaks: a, methanol derivative; b, internal benchmark standard.

Table 2. The compositions of commercial Chinese liquor medicine and their ethanol content

Brands of Chinese liquor medicine	Listed ingredients	Percentage of ethanol
A	*Panax ginseng, Polygonum multiflorum, Rehmannia glutinosa, Polygala sibirica, Cornus officinalis, Cuscuta japonica, Angelica sinensis, Scrophularia ningpoensis, Astragalus membranaceus, Cistanche salsa, Polygonatum cirrhifolium, Carthamus tinctorius, Rubus parvifolius, Polygonatum odoratum, Euphoria longan, Epimedium spp., Glycyrrhiza uralensis, Eleutheroccus gracilistylis, Cnidium monnieri, Lycium chinense, Ligusticum wallichii, Syzygium aromaticum, Poria cocos, Lindera aggregata, Asparagus lucidus*	55%
B	Ext. angelicae radix, Ext.ginseng radix, Ext.cnidii rhizoma, Glucuronic acid, Thioctamide, L-lysine, Vitamin B_1HCl, VitaminB_2, VitaminB_6, Calcium pantothenate, Nicotinamide, Taurine, Caffeine hydrate	8%
C	Ext. angelicae radix, Ext. ginseng radix, Ext. cnidii rhizoma, Glucuronic acid, Thioctamide, L-lysine, DL-methionine, Vitamin B_1HCl, Vitamin B_2, Vitamin B_6, Calcium pantothenate, Nicotinamide, Taurine, Caffeine hydrate, Invert sugar	8%
D	rice liquor, sugar, spice	30%
E	kaoliang liquor, sugar, spice	30%

Table 3. The trace amount of methanol detected in commercial Chinese liquor medicine

Brands	A	B	C	D	E
Amount of methanol (v/v) %	2.20×10^{-4}	6.20×10^{-4}	1.21×10^{-3}	3.27×10^{-3}	3.28×10^{-3}

ty could easily be determined in over-the-counter liquor medicine with high water content and multiple natural ingredients.

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可移除性衍生之液相層析法分析藥酒中微量甲醇含量

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摘　要

本研究建立了一個可直接在高含水量且成分複雜之檢品中進行衍生而偵測微量甲醇的高效能液相層析法（HPLC）。以4-[N-methyl, N-(1-naphthylmethyl)]-amino-4-oxobutanoic acid（NAOB），4-dimethylaminopyridine（DMAP）和1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride（EDC）在溫和的條件下，將水溶液檢品之甲醇順利地進行衍生反應。反應液衍生後可用鹼洗、酸洗來移除有機層中過量的試劑及其他可被酸鹼移除之干擾物質。並對影響衍生反應之諸多因素皆詳加探討，找出最適當的衍生條件。應用此法於五種市售藥酒（乙醇含量8 ~ 55%，v/v）中甲醇含量之檢測，其結果為2.20×10^{-4} ~ 3.28×10^{-3}%（v/v），皆合乎甲醇含量之衛生標準（<0.1%，v/v）。

關鍵詞：可移除性衍生，甲醇，高效能液相層析，藥酒