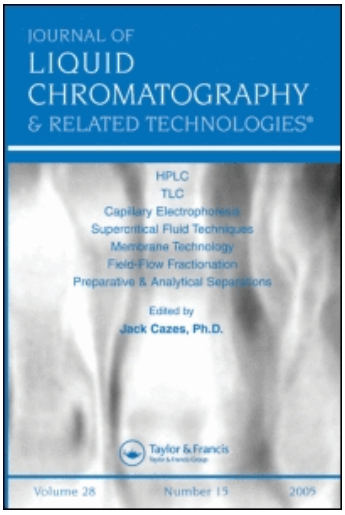

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DERIVATIZATION–HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF METHANOL IN HUMAN PLASMA

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ABSTRACT

A simple and sensitive high performance liquid chromatographic method is described for the determination of methanol in human plasma, as a highly sensitive derivative. The methanol, spiked in plasma, after simple ultrafiltration treatment, was derivatized with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one in a heterogeneous system, using benzalkonium chloride as phase transfer catalyst. The resulting derivative was chromatographed on a LiChrospher diol column with n-hexane:dichloromethane (9:1, v/v) as the mobile phase and 1-nitronaphthalene as the internal standard. The HPLC system showed good selectivity for methanol determination. Several parameters affecting the derivatization of methanol extracted from spiked plasma were investigated. The linear range for the determination of methanol in spiked human plasma was over 1-10 μmol/mL; the detection limit (signal to noise = 5, sample size 10 μL) of methanol was about 0.06 ± 0.02 μmol/mL.

The intraday relative standard deviation (n = 6) and the interday relative standard deviation (n = 8) were all below 5%. Recovery of methanol in spiked human plasma was greater than 98%.

INTRODUCTION

Methanol is one of the most popular solvents and finds extensive application in various industries. Consequently, the potential hazard of human exposure to methanol should not be neglected, especially that by inhalation[1-3] or consuming the methanol-contaminated alcoholic drinks. Methanol is rapidly and well absorbed by inhalation, oral and topical exposure routes. It is primarily metabolized in humans by an alcohol dehydrogenase system to formic acid.

Although formic acid accumulation is now reputed to be the cause of metabolic acidosis and ocular toxicity of methanol poisoning in humans, intraretinal metabolism of methanol, rather than elevated blood formic acid level, was suggested to be responsible for the retinal damage.[4] An accurate, sensitive method for determination of methanol in human plasma is very essential in clinical and forensic toxicology.

Many methods, including gas chromatographic (GC),[5-13] enzymatic,[14,15] spectrophotometric,[16,17] and high performance liquid chromatographic (HPLC)[18-21] methods have been described for the determination of methanol. Among these methods, a direct GC method, coupled with flame ionization detection and a capillary column, is the most widely used technique for determination of methanol in various matrices. The procedure of the direct GC method is simple, but its sensitivity is limited. Comparing with GC, relatively few HPLC methods have been described for the determination of methanol in plasma. Methanol is very simple in structure and transparent to UV detection; thus, the direct HPLC method usually has to be coupled with refractive index detection.

Therefore, with an attempt to increase the detection sensitivity, an analytical derivatization HPLC method, coupled with a UV detector is devised. The method is based on the chemical derivatization of methanol extracted from spiked plasma with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MBX) in a heterogeneous system, using benzalkonium chloride (BAC) as phase transfer catalyst.

EXPERIMENTAL

Chemicals and Reagents

3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MBX) and benzalkonium chloride (benzyl dimethyl n-tetradecylammonium chloride, BAC) (TCI, Tokyo, Japan), potassium hydroxide, 1-nitronaphthalene, methanol and ethanol (E. Merck, Darmstadt, Germany), dichloromethane, n-hexane and other reagents were of analytical reagent grade. Solutions of 1-nitronaphthalene and Br-MBX were prepared in dichloromethane as internal standard and derivatizing agent, respectively. Solutions of methanol and BAC, at various concentrations, were prepared by dissolving a suitable amount of methanol or BAC, respectively, in deionized water.

HPLC Conditions

A Waters-Millipore LC system with Model 510 LC pump, U6K injector, 746 integrator and a model 484 UV-VIS detector was used (Waters Chromatography Div., Millipore Corp., Milford, MA, USA). A LiChrospher diol column (250 x 4 mm I.D., 5 μm) and a mixed solvent of n-hexane:dichloromethane (9:1, v/v) as a mobile phase at a flow-rate of 1.2 mL/min were used.

The column eluate was monitored at 350 nm. The mobile phase was degassed with a vacuum filter before use.

Sample Preparation

A 270 μL volume of plasma was pipetted into a 10 mL glass-stoppered test tube, and 30 μL of aqueous solutions containing various amounts of methanol were added to each tube. The tubes were mixed for 10s. Then, 0.3 mL of 0.05 M potassium hydroxide solution was added and mixed by vortexing for 30s.

A 0.4 mL aliquot of spiked plasma sample was transferred into a Ultrafree-MC filter unit (30,000 NWML polysulfone PTTK membrane, Millipore, Bedford, MA, USA), then centrifuged at 2900g for 1h. A 0.2 mL aliquot of the ultrafiltrate was directly used for the derivatization as described under Derivatization Procedure.

Derivatization Procedure

A 0.2 mL portion of the plasma sample solution (after ultrafiltration) was pipetted into a 10 mL glass-stoppered test tube containing 0.1 mL of 0.5 M BAC solution. Then, 0.3 mL of 1-nitronaphthalene (80 μM) dichloromethane solution and 0.2 mL of Br-MBX dichloromethane solution (20 mM) were added. The reaction mixture was mechanically shaken for 2 h at 30°C in a thermostated water bath. At the end of the reaction, 3.0 mL of water was added to the reaction vessel, with slight shaking, to stop the reaction. After the separation of the organic phase, a 10 μL aliquot of the dichloromethane layer was injected for HPLC determination.

RESULTS AND DISCUSSION

The analytical derivatization of the extracted methanol (after simple ultrafiltration) with Br-MBX in a heterogeneous reaction system, using BAC as phase transfer catalyst, was studied. For the optimization of the extraction conditions of methanol from spiked plasma (2μmol methanol), and conditions for the derivatization of the extracted methanol, several related parameters, including the concentration of KOH, amount of phase transfer catalyst, derivatizing agent, and reaction time, were investigated. The effects of the tested parameters on the extraction/derivatization of methanol were evaluated by the peak-area ratio of resulting derivative to the 1-nitronaphthalene. Because of the high volatility of methanol, the possible loss of methanol in a plasma sample under ultrafiltration conditions should be considered. Before ultrafiltration, 0.3 mL of KOH at various concentrations were added to spiked plasma to study the effect on the extraction/derivatization of methanol. The yield of methanol derivative, evaluated by peak-area ratio, obviously increased at higher concentration of base, but the interference peak of the chromatogram was observed when the concentration of the added KOH was beyond 0.05 M. Therefore, 0.3 mL of 0.05 M KOH was selected as optimum alkali for determination of methanol extracted from plasma. Among the commonly used organic solvents, dichloromethane was found to be the optimum one for the derivatization of methanol in the heterogeneous system.

Effect of the Amount of Phase Transfer Catalyst

The effect of the amount of BAC on the formation of methanol derivative in the heterogeneous system was briefly examined. The results are shown in Figure 1. In the absence of BAC, no derivative was detected.

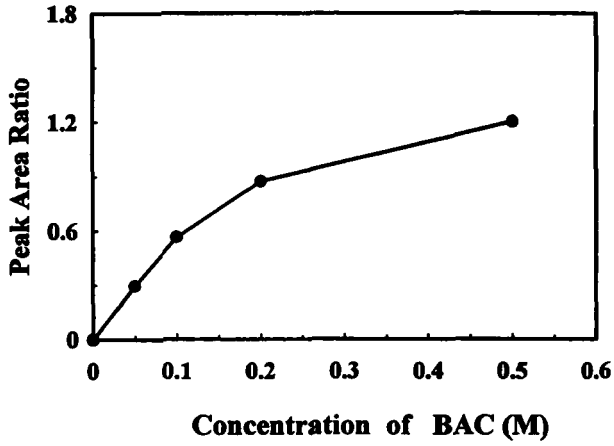

Figure 1. Effect of amount of phase transfer catalyst on the formation of the derivative of methanol extracted from human plasma.

As the concentration of BAC increased, the yield of derivatization of methanol increased. The solubility of BAC in water is limited to 0.5 M. So, 0.5 M BAC was selected as optimum phase transfer catalyst for determination of methanol.

Effect of the Amount of Derivatizing Agent

More than 4 μmol of Br-MBX was needed for derivatizing the methanol isolated from spiked plasma (2 μmol methanol) to a plateau formation of the derivative as shown in Figure 2. In this study, 4 μmol of Br-MBX was used for derivatization of methanol extracted from plasma.

Effect of Reaction Time

The effect of reaction time, at 30°C, on the derivatization of methanol extracted from plasma is shown in Figure 3. The results indicate that plateau formation of the derivative is not attainable in 3 h. That a long reaction time was required for the derivatization of methanol to reach the steady state is probably due, in part, to the high water solvation of methanol and ensuing poor phase partition.

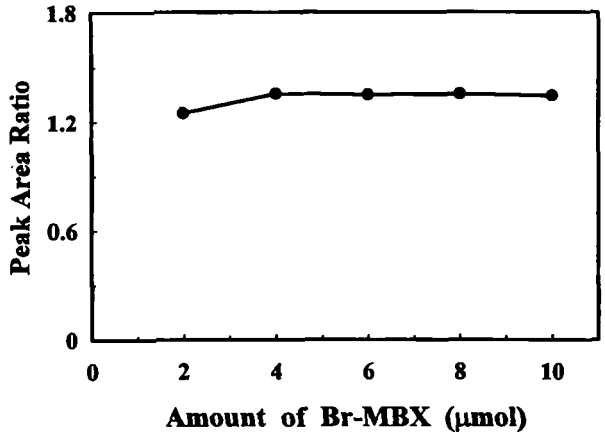

Figure 2. Effect of the amount of Br-MBX on the formation of the derivative of methanol extracted from human plasma.

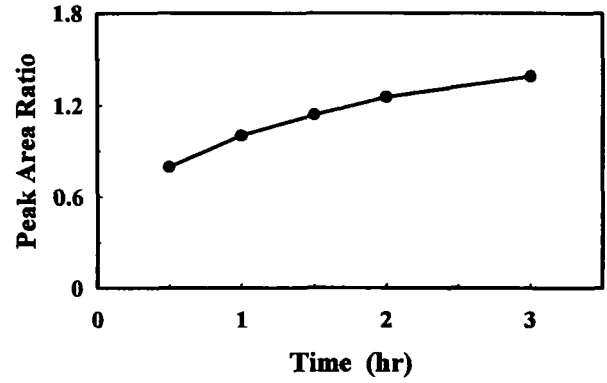

Figure 3. Effect of reaction time on the formation of the derivative of methanol extracted from human plasma.

In the light of the fact that in the reaction time of 2 h, the derivatization yield of methanol, which was evaluated by peak-area ratio, can reach more than 90% of that obtained from 3 h of reaction. The reaction time for determination of methanol in this study was set at 2 h.

Analytical Calibration

On the basis of the optimum extraction/derivatization conditions, we formulated the analytical procedure for methanol extracted from spiked plasma as described under the Methods section. The quantitative application of the method to the determination of methanol was evaluated at five different levels of methanol in the range of 1-10 μmol/mL spiked in plasma. The calibration graph was established with the peak-area ratio of the derivative to 1-nitronaphthalene as ordinate (y) vs. the amount of methanol in μmol/mL as abscissa (x). The linear regression equations obtained were

$$y = (0.042 \pm 0.013) + (0.124 \pm 0.002)x \text{ for intraday assay } (n = 6, r = 0.999);$$

$$y = (0.038 \pm 0.006) + (0.124 \pm 0.001)x \text{ for interday assay } (n = 8, r = 0.999).$$

The data indicate good linearity of the method. The detection limit (signal- to-noise ratio = 5) of methanol extracted from plasma is 0.06 ± 0.02 μmol/mL. The concentration of methanol in the range of 6.2-25 μmol/mL have been reported lethal to human beings.[22] So, the proposed method can be used in the analysis of biological samples of forensic interest.

Mass Spectral Analysis of the Derivative

A typical HPLC chromatogram for the analysis of methanol extracted from plasma is presented in Figure 4. Peaks 1 and 3 of the internal standard and the methanol derivative, respectively, in Fig. 4 did not receive interference from the reagent blank. The structure of the methanol derivative of peak 3 in Fig. 4 was briefly identified as 3-methoxymethyl-7-methoxy-1,4-benzoxazin-2-one by comparing the retention time with that of an authentic sample, which was synthesized and confirmed by electron ionization-mass spectrometry and a molecular ion at m/z = 221 corresponding to the methanol derivative was given. The stability of the methanol derivative, after derivatization, was studied over a period of 7 h; no significant change of the peak-area ratio was found. This indicates the favorable stability of the derivative for methanol analysis. The specificity of the method was studied by similar extraction /derivatization of ethanol, which usually coexists with methanol, from plasma spiked with 8 μmol of ethanol and 2 μmol of methanol, simultaneously.

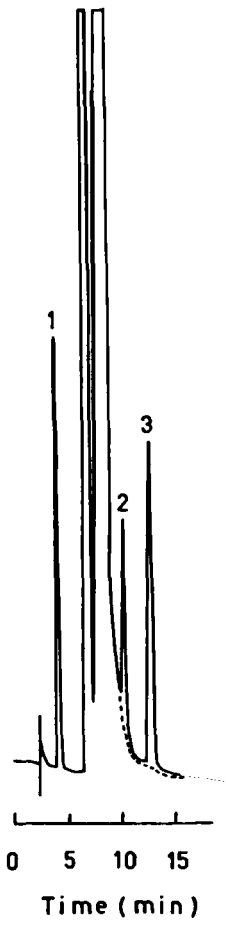

Figure 4. Composite HPLC chromatogram for the determination of methanol (solid line) in human plasma and reagent blank (dotted line). Peaks: 1, 1-nitronaphthalene (I.S.); 2, the derivative of ethanol; 3, the derivative of methanol. HPLC conditions: LiChrospher diol column (250 x 4 mm I.D.; 5 μm); mobile phase, n-hexane:dichloromethane (9: 1, v/v); flow rate, 1.2 mL/min; UV detection, 350 nm.

Under the present HPLC conditions, a composite chromatogram was obtained as shown in Fig. 4, indicating good separation of methanol derivative from the ethanol derivative (peak 2). The structure of the ethanol derivative was also tentatively identified as 3-ethyoxymethyl-7-methoxy-1,4-benzoxazin-2-one by electron ionization-mass spectrometry. The proposed method also revealed the potential for the analysis of ethanol in human plasma.

Table 1

Precision and Accuracy for the Analysis of Methanol from Spiked Plasma

Concentration Spiked (μmol/mL)	Concentration Found (μmol/mL)	R.S.D. (%)	Recovery (%)
Intraday* (n = 6)			
2.00	1.94 ± 0.08	4.12	97.0
5.00	5.13 ± 0.15	2.92	102.6
10.00	9.98 ± 0.11	1.10	99.8
Interday* (n = 8)			
2.00	1.95 ± 0.08	4.10	97.5
5.00	5.09 ± 0.14	2.75	101.8
10.00	9.92 ± 0.18	1.81	99.2

* Intraday data was based on six replicate analyses and interday data was from eight consecutive days.

Reproducibility and Recovery

The precision (relative standard deviation, R.S.D.) of the proposed method for analysis of methanol in human plasma at 10.0, 5.0 and 2.0 μmol/mL was studied. The results, presented in Table 1, show that the intraday and interday variances at the three concentrations were all below 3.0%, indicating satisfactory precision of the method. The relative recovery of the methanol, as shown in Table 2, is more than 97%, which was obtained from the calibration graph constructed from plasma spiked with different amounts of methanol over the range of 1-10 μmol/mL.

Stability of Methanol in Spiked Plasma

Methanol is a volatile substance and its stability in plasma under storage has to be considered. Two different concentrations of methanol at 5 and 10 μmol/mL of spiked plasma were studied to evaluate the stability of the methanol stored at -40 ± 5°C.

Table 2

Recoveries of the Analyses of Methanol from Spiked Human Plasma

Sample No.	Concentration Spiked (μmol/mL)	Concentration Found* (μmol/mL)	R.S.D. (%)	Recovery (%)
1	-	-	-	-
	5.00	5.18 ± 0.20	3.86	103.60
	10.00	10.02 ± 0.17	1.70	100.20
2	-	-	-	-
	5.00	5.08 ± 0.11	2.16	101.60
	10.00	9.93 ± 0.29	2.92	99.30
3	-	-	-	-
	5.00	5.17 ± 0.13	2.51	103.40
	10.00	9.93 ± 0.16	1.61	99.30
4	-	-	-	-
	5.00	5.04 ± 0.11	2.18	100.80
	10.00	9.89 ± 0.24	2.43	98.90
5	-	-	-	-
	5.00	5.02 ± 0.10	1.99	100.40
	10.00	9.88 ± 0.11	1.11	98.80
6	-	-	-	-
	5.00	5.07 ± 0.18	3.55	101.40
	10.00	9.92 ± 0.24	2.42	99.20
7	-	-	-	-
	5.00	5.04 ± 0.16	3.17	100.80
	10.00	9.86 ± 0.17	1.72	98.60
8	-	-	-	-
	5.00	5.08 ± 0.20	3.94	101.60
	10.00	9.90 ± 0.17	1.72	99.00

* Mean ± S.D. of three replicate analyses.

For each sample, determination of plasma methanol was performed on day 0, 3, 7, 14, 21, and 28. Statistical analysis of the results did not show a significant difference; therefore, methanol is stable in plasma samples stored at -40 ± 5°C for periods up to 28 days.

In conclusion, a simple and sensitive method based on the derivatization of methanol extracted from plasma with Br-MBX is developed. Separation of methanol from the spiked plasma by ultrafiltration, for the analysis, is very simple. The proposed method is expected to be feasible for the analysis of methanol in plasma related to the case of methanol poisoning.

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