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A Spectrofluorometric Method for Detection of Low Methanol Levels in Plasma

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Summary: *An easy, cheap and reliable spectrofluorometric method has been developed by modifying the method described by Makar et al. (Biochem. Med. 13: 117-126, 1975) for formic acid, in order to detect and monitor especially low methanol levels in blood due to exposure to methanol through inhalation, dermal route and ingestion at low concentrations, or due to intake of some xenobiotics such as aspartame, which can form methanol after its biotransformation in the body, or due to consumption of large amounts of alcohol.*

The calibration curve is linear between 0-1.5 mg/dL, (y=33.0 x + 1.58, r = 0.996). The detection limit is 0.1 mg/dL. The coefficients of variation at 1 mg/dL concentration are 4.27% ± 0.04 (mean ± SD) and 5.18% ± 0.07 (mean ± SD) for within-run and between-run precisions, respectively. For supplementation of 0.5 mg/dL and 1 mg/dL of methanol on six occasions, the average recovery values are 91.6 % ± 9.4 (mean ± SD) and 100.0 % ± 4.3 (mean ± SD), respectively.

Keywords : *Spectrofluorometric detection, methanol, resazurin.*

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Plazmada Düşük Metanol Düzeyinin Saptanması için Spektrofluorometrik Bir Metot

Özet: *Makar ve arkadaşlarının (Biochem. Med. 13: 117-126, 1975) formik asit için tanımladıkları bir metodun modifikasyonu ile düşük konsantrasyonlarda metanole inhalasyon, dermal yol ile temas ve oral alımı takiben, veya aspartam gibi vücutta biyotransformasyonuyla metanol oluşturan bazı ksenobiyotiklerin alınmasıyla oluşan, veya fazla miktarda alkol tüketimine bağlı, özellikle düşük metanol kan düzeylerinin saptanması ve izlenmesi için kolay, ucuz ve güvenilir bir yöntem geliştirilmiştir.*

Kalibrasyon eğrisi 0-1.5 mg/dL arasında doğrusaldır (y= 33.0 x + 1.58, r = 0.996). Belirlenebilen metanol konsantrasyon alt limiti 0.1 mg/dL'dir. 1 mg/dL konsantrasyon için deney içi ve deneylerarası varyasyon katsayısı sırasıyla % 4.27 ± 0.04 (ort. ± SS) ve % 5.18 ± 0.07 (ort. ± SS)'dur. Altı deneme sonucunda 0.5 mg/dL ve 1 mg/dL konsantrasyonlar için ortalama geri elde etme yüzdesi sırasıyla % 91.6 ± 9.4 (ort. ± SS) ve % 100.0 ± 4.3 (ort. ± SS) olarak saptanmı tır.

Anahtar kelimeler : *Spektrofluorometrik tayin, metanol, resazurin.*

Introduction

Exposure to methanol can occur through inhalation, dermal route or by ingestion, because methanol is a widely used solvent in industry and in a variety of products such as antifreeze, fuels, duplicating machine fluids, or in gasoline as a fuel extender, and found in alcoholic drinks as an impurity. Thus, it is consumed by alcoholics within the alcoholic drinks deliberately or accidentally[1-4]. Methanol is a congener of most alcoholic beverages and some methanol is also formed endogenously. Ethanol, which has a 10-fold higher affinity for alcohol dehydrogenase as compared to methanol, inhibits the oxidation of methanol and thus the presence of ethanol leads to accumulation of methanol in blood. Therefore alcoholics and heavy drinkers are likely to have higher blood-methanol concentration than nonalcoholics[5]. Base on this, blood methanol concentrations have been suggested as a possible indicator in the screening of alcohol-abuse of, e.g., drunken drivers or intoxicated hospital emergency room patients[5-7]. Although the origin of increased blood methanol in alcoholics is not fully known, some methanol may be derived from the beverages consumed, but the most important contributor seems to be the accumulation of endogenous methanol. Possible sources for this endogenous methanol are dietary factors, formation of methanol by intestinal bacteria, metabolic processes involving either the formation of methanol by in-

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testinal bacteria, metabolic processes involving either the formation of methanol by the action of the methanol-forming enzyme on S-adenosylmethionine in the pituitary gland, or the reduction of formaldehyde from demethylation of some xenobiotics such as aminopyrine to methanol[5,8,9], or the biotransformation of other xenobiotics such as aspartame about 10 % of which can form methanol in the body[10]. At present, the involvement of a metabolic process seems the most likely explanation for formation of endogenous methanol[10,11]. The formation may be changed in pathological conditions such as liver diseases.

Sensitive, rapid, and reliable analysis of methanol in blood is very useful and important for human health in the screening of excessive drinking, and also in the detection and monitoring of low methanol levels in blood due to the chronic use of some xenobiotics, which can produce methanol via metabolic processes, or due to exposure to methanol through inhalation, dermal route and by ingestion, because methanol causes toxic manifestations in man [1-4].

Therefore, the aim of the present study is to develop a rapid, sensitive, easy and valid method in order to detect and monitor very low methanol levels in plasma.

Materials and Methods

Reagents

All chemicals used in the study were of analytical grade.

Solution-I: It is made up with the phosphate buffer (0.1 M, pH 7.6), contains ß-nicotinamide adenine dinucleotide(NAD^+, Sigma N-7004) and formaldehyde dehydrogenase(Sigma F-1879, E.C. 1.2.1.46) at $2.56x10^{-3}$ mmol/mL and 0.5 U/mL, respectively. The resulting solution must be freshly prepared for each assay.

Solution-II: Alcohol oxidase(Sigma A-0763, E.C. 1.1.3.13) is dissolved in the phosphatase buffer (0.2 M, pH 7.6) to give a final concentration of 2.5 U/mL. The solution is freshly prepared.

Solution-III: To prepare 13 mL of solution-III 1 mL of resazurin solution (0.17 mg/mL in water) and 2 mL diaphorase solution(Sigma D-5540, E. C. 1.8.1.4., 5U/mL in water) are mixed and the volume is completed to 13 mL with 0.2 M phosphate buffer at pH 6.0.

Standard Solutions of Methanol: Stock solution of methanol is prepared by dissolving 1 gram methanol per dL of water. The proper volume of the stock solution is diluted with distilled water to obtain final methanol concentrations of 0.1, 0.2, 0.5, 1, 1.5, 2 mg/dL.

Sampling and Protein Denaturation Procedure

The following procedure was applied to rat blood samples. Rat blood drained from the trunk after decapitation was transferred into 5 mL-polypropylene tubes containing heparin as an anticoagulant. After mixing, 1 mL of the sample was transferred to 5 mL-polypropylene duplicate tubes with tight-fitting caps. 1.4 mL of distilled water or the methanol standard was added to each of the duplicate tube. Leaving the tubes at room temperature for five minutes, 0.3 mL of 7.5% (% w/v) $ZnSO_4.7H_2O$ (Merck) and 0.3 mL 0.4 N NaOH (Merck) were added into each tube for denaturation of protein in blood[12]. After mixing well, each sample was centrifuged at 8000 rpm (Hettich Zentrifugen, Universal 30 RF) for 20 minutes. Supernatants were collected into tubes with tight-fitting caps, analyzed immediately or stored at 4°C until the day of assay.

The blood standard curve for methanol was also plotted by addition of the methanol standards at 0.1-2 mg/dL to the rat blood sample.

Method of Methanol Detection in Blood

Principle of the Method

The method is based on the following reactions:

$$CH_3OH+O_2 \xrightarrow{\text{Alcohol Oxidase}} HCHO+H_2O_2$$

$$HCHO+H_2O+NAD^+ \xrightarrow{\text{Formaldehyde dehydrogenase}} HCOOH+NADH+H^+$$

$$H^+ + NADH + Resazurin \xrightarrow{Diaphorase} NAD^+ + Resorufin$$

(nonfluorescent) (fluorescent)

In order to detect the methanol level in blood, the coupled enzymatic system proposed for the analysis of formate was employed with a slight modification[12,13]; substituting alcohol oxidase and formaldehyde dehydrogenase for formate dehydrogenase.

Methanol is oxidized by alcohol oxidase which is not specific for methanol: It converts all lower alcohols to their corresponding aldehydes, whereas formaldehyde dehydrogenase is specific. Thus the transformation of NAD^+ to NADH proceeds only if methanol is originally present in the sample. While the formaldehyde is oxidized by formaldehyde dehydrogenase, so NADH is produced[12,14]. NADH reduces resazurin (nonfluorescent) via the coupling enzyme diaphorase to a fluorescent product, resorufin[12,13]. The fluorescence can be measured using a spectrofluoremeter at excitation wavelength 565 nm and emission wavelength 590 nm. The method was applied for methanol as follows: Supernatants (250 µL) were added to 5 mL-culture tubes containing 600 µL solution-I. After being stirred and stored in an ice-bath, 100 µL solution-II was added to the tubes. All tubes were vortexed, incubated at 30°C for 15 min, then were placed in an ice-bath for 10 min to stop the reaction at the end of incubation period, then 1050 µL solution-III was added. The final volume of each tube was 2000 µL. These tubes were vortexed and incubated at 37°C for 5 min, then were placed in boiling water for 3 min to stop the reaction after second incubation period, and the fluorescence was determined by HITACHI, Model 650-40 spectrophotofluorometer at exicitation wavelength 565 nm, and emission wavelength 590 nm.

The concentration-fluorescence intensity relationships were assessed by linear regression.

To assess recovery, additional methanol was added to plasma from healthy volunteers (leftover blood from volunteers, submitted for other clinical chemistry, analysed and known not to contain methanol) to increase the concentration by 0.5 mg/dL and 1 mg/dL of methanol on six occasions.

The plasma samples supplemented with 1 mg/dL of methanol were assayed after denaturation of protein for within-run precision, and were also assayed for over a week for between-run precision.

The method was tested for interference from ethanol because it may be present in blood at the same time. Therefore the samples were supplemented with known amounts (0.5 and 2 mg/dL) of methanol and also ethanol at 100 mg/dL. The dose tested for interference corresponds to a toxicologically significant concentration of ethanol in blood. Formaldehyde and formic acid are two metabolites of methanol[1,14]. For this reason, the method was also tested for interference from the metabolites that may be present in blood. The samples were supplemented with known methanol (0.5 mg/dL and 2 mg/dL) and also formaldehyde and formic acid at 0.5 mg/dL. The low concentration of formaldehyde and formic acid were assayed for interference, because the range of methanol concentrations was narrow in this study. The measurements of methanol were repeated before and after supplementation by the substances tested.

Results

After applying protein denaturation procedure, a linear concentration-fluorescence intensity relationships in blood samples supplemented with methanol standard (0.1-1.5 mg/dL) were obtained ($y = 33.0x + 1.58$, $r = 0.996$). Fig-1 shows a typical standard curve for blood methanol.

Recovery averaged 91.6% ± 9.4 (mean ± SD) and 100.0% ± 4.3 (mean ± SD) on six occasions for supplementation with 0.5 mg/dL and 1 mg/dL of methanol, respectively.

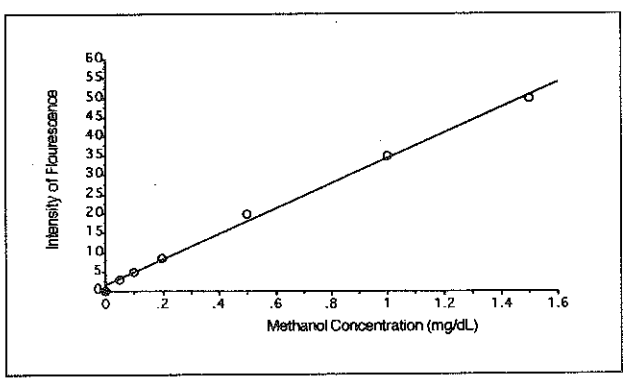

Figure 1. A standard curve for blood methanol.

The CVs at 1 mg/dL concentration of methanol were 4.27% ± 0.04 (mean ± SD) and 5.18% ± 0.07 (mean ± SD) for within-run and between-run precisions, respectively.

Added ethanol did not interfere at the concentrations tested. The concentration of ethanol tested for interference would correspond to toxic concentration in blood (at 100 mg/dL). The major metabolites of methanol (formaldehyde and formic acid) did not also interfere at the concentration tested.

In the present study, zinc sulphate: sodium hydroxide was used to precipitate protein in blood prior to methanol analysis. The concentration of zinc sulphate:sodium hydroxide carried over to reaction mixtures did not influence reaction rates [12].

Discussion

The method used for the detection and monitoring of methanol must be highly specific and sensitive, and unaffected by ethanol and the metabolites of methanol which may be present in biological fluids. More commonly, determination of methanol and other alcohols can be done using gas chromatography, and capillary column chromatography. The methods are highly sensitive (280 ng/L)[6,9,11,15,16], but unfortunately, they are not available in all clinical laboratories, and need a multiple-step process, which requires chromatographic expertise, and are more time-consuming.

Microdiffusion method for alcohols is nonspecific[14]. In a colorimetric method, methanol is oxidized with permangenate to formadehyde and then it is measured colorimetrically after being reacted with chromatograpic acid in the presence of concentrated sulfuric acid[17]. The colorimetric method is not specific and sensitive, and additionally, the use of highly concentrated sulfuric acid is not convenient.

Another enzymic assay for specific determination of methanol in serum (1-12.5 mmol/L) has been also described, but the method requires continous monitoring of absorbance at 340 nm in thermostated flow cell[14]. The accessory (thermostated flow cell) is not available in all clinical laboratories.

In our previous study, we have developed an enzymatic method to determine methanol levels in blood. The spectrophotometric method can also be used to detect methanol concentrations in methanol overdose (0.5-20 mg/dL)[18].

In the present study, a rapid, sensitive, easy, and valid method has been developed in order to detect and monitor very low methanol levels in blood. It is believed that this method fulfills the need of laboratories to detect and monitor the possible hazards arising from exposure to methanol even at very low levels.

In summary, the method can be used for a variety of purposes in laboratories interested in toxicology and drug metabolism. The lowest level of blood methanol can be accurately determined by this method. Moreover, it can be also used in the screening of alcohol abuse.

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References

1. Ellenhorn, M. J., Barceloux, D. G. (eds), *Medical Toxicology. Diagnosis and Treatment of Human Poisoning*. New York, Elsevier Science Publishing Company Inc., pp. 782-805, 1988.

2. Tephly, T. R., McMartin, K. E., "Methanol Metabolism and Toxicity", in Stegink, L. D., Filer, L. J., (eds), *Aspartame: Physiology and Biochemistry*, New York and Basel, Marcel Dekker Inc., pp. 111-140, 1984.

3. Tephly, T. R., "The Toxicity of Methanol", *Life Sci.*, 48, 1031-1041, 1991.

4. Tephly, T. R., Makar, A. B., McMartin, K. E., Hayreh, S. S., Martin-Amat, G., "Methanol: Its Metabolism and Toxicity", in Majchrowicz, E., Noble, E. P., (eds), *Biochemistry and Pharmacology of Ethanol.*, New York, Plenum Publishing Corporation, pp. 145-164, 1979.

5. Roine, R. P., Eriksson, C. J. P., Ylikahri, R., Penttilä, A., Salaspuro, M., "Methanol as a Marker of Alcohol Abuse", *Alcohol. Clin. Exp. Res.*, 13, 172-175, 1989.

6. Jones, A. W., "Abnormally High Concentrations of Methanol in Breath: A Useful Biochemical Marker of Recent Heavy Drinking", *Clin. Chem.*, 32, 1241-1242, 1986.

7. Majchrowicz, E., Mendelson, J. H., "Blood Methanol Concenrations During Experimentally Induced Ethanol Intoxication in Alcoholics", *J. Pharm. Exp. Ther.*, 179, 293-300, 1971.

8. Axelrod, J., Daly, J., "Pituitary Gland: Enzymic Formation of Methanol from S-Adenosylmethionine", *Science*, 150, 892-893, 1965.

9. Eriksen, S. P., Kulkarni, A. B., "Methanol in Normal Human Breath", *Science*, 141, 639-640, 1963.

10. Stegink, L. D., Brummel, M. C., McMartin, K., Martin-Amat G., Filer L. J., Baker, G. L., Tephly, T. R., "Blood Methanol Concentrations in Normal Adult Subjects Administered Abuse Doses of Aspartame", *J. Toxicol. Environ. Health.*, 7, 281-290, 1981.

11. Lee, E. W., Terzo, T. S., D'Arcy, J. b., Gross, K. B., Schreck, R. M., "Lack of Blood Formate Accumulation in Humans Following Exposure to Methanol Vapor at the Current Permissible Exposure Limit of 200 ppm", *Am. Ind. Hyg. Assoc. J.*, 53, 99-104, 1992.

12. Makar, A. B., McMartin, K. E., Palese, M., Tephly, T. R., "Formate Assay in Body Fluids: Application in Methanol Poisoning", *Biochem. Med.*, 13, 117-126, 1975.

13. Makar, A. B., Tephly, T. R., "Improved Estimation for Formate in Body Fluids and Tissues", *Clin. Chem.*, 28, 385, 1982.

14. Vinet, B., "An Enzymic Assay for the Specific Determination of Methanol in Serum", *Clin. Chem.*, 33, 2204-2208, 1987.

15. Wargotz, E. S., Werner, M., "Asymptomatic Blood Methanol in Emergency Room Patients", *Am. J. Clin. Pathol.*, 87, 773-775, 1987.

16. Baker, R. N., Alenty, A. L., Zack, J. F., "Simultaneous Determination of Lower Alcohols, Acetone, and Acetaldehyde in Blood by Gas Chromatography", *J. Chromatog. Sci.*, 7, 312-314, 1969.

17. Hindberg, J., Wieth, J. O., "Quantitative Determination of Methanol in Biologic Fluids", *J. Lab. & Clin. Med.*, 61, 355-362, 1963.

18. Baydar, T., Şahin, G., "A Method for the Specific Determination of Methanol in Plasma", *Pharm. Sci.*, 1995 (in press).