Research Paper

# Quantification of ethanol in whole blood by extraction using NeedlEx® and gas chromatography/mass spectrometry

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**Abstract** In this study, a new quantification method was developed for analysis of ethanol in whole blood samples by needle extraction (NeedlEx®) and gas chromatography/mass spectrometry (GC/MS). Whole blood samples (0.1 mL) containing ethanol and ethanol-$d_6$ (internal standard) were incubated at room temperature in a 10-mL headspace vial. The NeedlEx® for alcohols with a gas aspirating pump was exposed to the headspace of vial to allow adsorption alcohols before GC/MS. The GC separation of the compounds was achieved on a fused-silica capillary column Rtx-5 ms (30 m × 0.25 mm i.d.; 0.25-$\mu$m film thickness) with MS detection operated in electron impact ionization ion source mode. The regression equations showed good linearity ($r$>0.998) from 0.1 to 5.0 mg/mL for whole blood. The accuracies and precisions were 99.0−112% and 3.0−7.0%, respectively. The method was successfully applied to actual analyses of autopsy samples. The present results on the analysis of ethanol by NeedlEx®-GC/MS suggest its applicability to a number of other volatile compounds in clinical and forensic toxicology.

**Key words:** ethanol, NeedlEx®, whole blood, gas chromatography/mass spectrometry

## Introduction

Ethanol is a most popular beverage, but its excessive consumption causes poisonings, fatal accidents, and many violent crimes[1]. Blood ethanol quantifications are mandatory in clinical and forensic toxicological investigations[2,3]. Headspace gas chromatography with flame-ionization detection (GC-FID) has been the gold standard for ethanol analysis, because of its easiness, sensitivity, accuracy, and relative specificity[4]. However, this method has inherent drawbacks in that it depends on only the retention time of the target compound for identification[5−7]. Gas chromatography/mass spectrometry (GC/MS) is a powerful analytical technique that combines the chromatographic separation power of gas chromatography with the high detection specificity power of mass spectrometry. Thus, this approach has been widely used in various analytical works[8−12].

NeedlEx®, a new extraction needle, is a device that concentrates volatile organic compounds (VOCs), such as alcohols, organic solvents, amines, and fatty acids. Several reports have described the application of NeedlEx® in the analysis of VOCs in biological samples[13−16]. However, to the best of our knowledge, there are no reports of quantification of ethanol in whole blood by NeedlEx®-GC/MS. Accordingly, in this study, we developed and validated a new quantification method for ethanol in whole blood. This method was successfully applied for the quantification of ethanol in whole blood samples collected from autopsy cases.

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## Materials and Methods

### *Reagents and chemicals*

NeedlEx® for alcohols, which adsorbs monohydric alcohols with up to 3 carbon atoms, and solvents, such as acetone and diethyl ether, with boiling points below 100°C,

were purchased from Shinwa Chemical Industries (Kyoto, Japan). Ethanol and ethanol-$d_6$ [internal standard (IS)] were purchased from Wako Pure Chemicals (Osaka, Japan) and Merck KGaA (Darmstadt, Germany), respectively. All other reagents were of analytical grade. Laboratory distilled water was purified using a Synergy UV (Millipore, Molsheim, France). Other common chemicals used in this study were of the highest purity commercially available. Human whole blood to be used as blank samples was obtained from Tennessee Blood Services (Memphis, TN, USA).

*Calibration standards and validation samples*

The IS solution was prepared in water at a concentration of 1.0 mg/mL. The concentrations of calibration standard solutions were 0.1, 0.2, 0.5, 1.0, 2.5, and 5.0 mg/mL in blank whole blood. Validation samples were adjusted to 0.1, 0.3, 2.0, and 4.0 mg/mL.

*Extraction procedure*

Whole blood samples (0.1 mL) and 0.1 mL IS solution (1.0 mg/mL ethanol-$d_6$) were pipetted into the 10-mL headspace vial; the vial was covered with a rubber stopper, and an aluminum cap was pressurized with a crimper and seal. The samples were incubated at room temperature for 15 min. A NeedlEx® for alcohols was then connected to a gas aspirating pump (Model AP-20 KITAGAWA; Komyo Rikagaku Kogyo K. K., Kawasaki, Japan) to draw the gas present in the headspace of the vial. After drawing the 10 mL gas, the NeedlEx® was attached to a Luer-lock type gas-tight syringe. The NeedlEx® was then inserted into the injection port of the GC/MS, and helium gas (0.2 mL) was drawn through the NeedlEx® by pulling the plunger of the gas-tight syringe. After 10 s, the helium collected in the gas-tight syringe was injected by pushing the plunger, which resulted in desorption of the analytes from the media in the NeedlEx®.

*GC/MS conditions*

GC/MS analysis was performed on a Shimadzu GCMS QP2020 (Shimadzu, Kyoto, Japan). A 30-m Rtx-5 ms column (Restek, PA, USA) with 0.25 mm internal diameter and 0.25 $\mu$m film thickness was used. The initial oven temperature was 40°C. After holding at the initial temperature for 10 min, the temperature was increased up to 250°C at a rate of 20°C/min. Helium was used as a carrier gas at a flow-rate of 1.5 mL/min with a 40 : 1 split ratio. The ion source and interface temperatures were 200 and 240°C, respectively, and electron ionization at 70 eV was used. The injection port was kept at 200°C. The analysis was carried out with a scanning speed of 833 u/s. For single-scan analysis, the scan range was *m/z* 20 to 250. The MS ion source and interface temperatures were maintained at 200°C and 280°C, respectively. The data were acquired by using GCMS solution software (Shimadzu, Kyoto, Japan). The ions observed as the base peak (ethanol: *m/z* 31, ethanol-$d_6$: *m/z* 33) were used for quantification, and the second and third most abundant ions (ethanol: *m/z* 45, 46, ethanol-$d_6$: *m/z* 49, 51) were used as qualifiers.

*Method validation*

The method has been validated according to the US FDA guidelines on bioanalytical method validation[17]. Calibration curves were constructed by plotting the peak area ratio of each analyte to the IS (*y*-axis) versus the concentration of the corresponding analytes (*x*-axis). The slope and *y*-intercept of the regression line were obtained by six different injections of each calibrator. The lower limit of quantitation (LOQ) was defined as the lowest quantitative concentration. The intra- and interday accuracies and precisions were determined by conducting 5 experiments in the course of a single day and by conducting an experiment on 5 different days, respectively. The precisions were expressed as percent coefficients of variation. The accuracies and precisions should be within 20%.

*Forensic autopsy samples*

Femoral vein and right heart blood samples were obtained from autopsy cadavers at Aichi Medical University in 2017. Whole blood samples were collected in 5- or 15-mL tubes and stored at −80°C until analysis. The alcohol content examinations of these samples were officially determined by judicial authorities.

## Results and Discussion

*GC/MS analysis*

GC separation was optimized and carried out as described in the "Materials and Methods" section. MS ionization parameters were optimized by injecting ethanol and ethanol-$d_6$ at a concentration of 1.0 mg/mL. The fragment ion at *m/z* 31 observed as the base peak of ethanol corresponded $[M-CH_3]^+$, and the second and third most abundant ions at *m/z* 45 and 46 were $[M-H]^+$ and $[M]^+$, respec-

tively.

Selected ion chromatograms of blank human whole blood samples without spiking (Fig. 1a) and a blank human whole blood sample spiked with 1.0 mg/mL ethanol and ethanol-$d_6$ (Fig. 1b) are shown. The peaks of ethanol and ethanol-$d_6$ in Fig. 1b were clearly visible, with retention times of 1.0 min, and there were no peaks that interfered with the measurements. It should be mentioned that the ethanol-$d_6$ gave peaks at *m/z* 33, 49 and 51, despite the fact that six deuterium atoms are present in the molecule. This is due to the rapid exchange of a deuterium at the hydroxy moiety for a hydrogen atom immediately after the contact with water[18].

*Method validation*

The method for ethanol quantification was validated by characterizing a series of parameters, such as linearity, accuracy, and precision (intra- and interday). The calibration curve showed a good linear relationship in the range from 0.1 to 5.0 mg/mL, with a correlation coefficient of 0.998 (Fig. 2), thus establishing a high degree of linearity within this range. The LOQ was set at 0.1 mg/mL, as indicated that blood alcohol concentration less than 0.1 mg/mL should be reported negative in postmortem toxicology[1].

The intra- and interday accuracies and precisions were evaluated at four levels, as summarized in Table 1. The accuracies and precisions were 99.0−112% and 3.0−7.0%, respectively. Their values were acceptable at all concentrations. Overall efficiencies including both extraction recovery and matrix effect were determined at the low (0.3 mg/mL) and high concentrations (4.0 mg/mL) by comparing whole blood samples with solutions of reference standard ethanol dissolved in water (n=5). They were 108% for 0.3 mg/mL and 104% for 4.0 mg/mL, which showed almost the same results as described in previous reports by headspace GC/MS[12,19].

*Applications*

We analyzed the 17 blood samples collected at autopsies in 2017. These samples were taken from femoral veins (n=4) and right hearts (n=13) and were previously checked to contain ethanol by GC-FID. Figure 3 shows representative

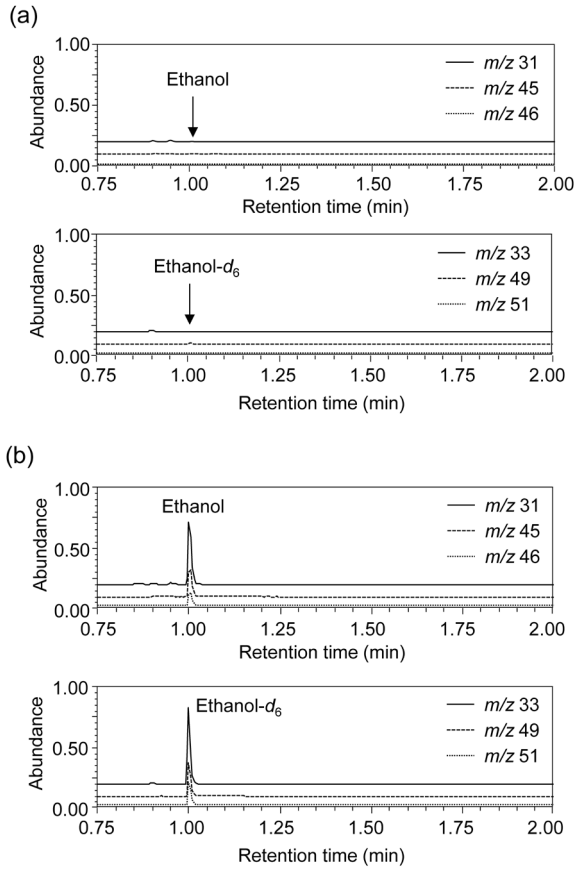


**Fig. 1. SIM chromatograms of blank human whole blood samples spiked without (a) and with (b) 1.0 mg/mL ethanol and 1.0 mg/mL ethanol-$d_6$.**

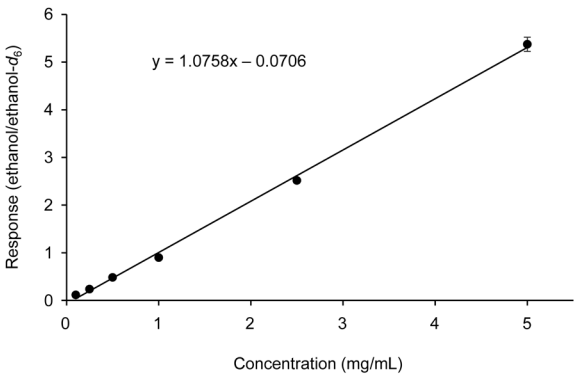


**Fig. 2. Calibration curve of ethanol in whole blood. Results are means of six different injections for each calibrator.**

**Table 1. Intra- and interday accuracy and precision data for ethanol in whole blood**

| Spiked concentration (mg/mL) | Intraday[a] | | Interday[a] | |
|---|---|---|---|---|
| | Accuracy (%) | Precision (%CV) | Accuracy (%) | Precision (%CV) |
| 0.1 | 111 | 3.0 | 112 | 3.9 |
| 0.3 | 104 | 4.0 | 99.6 | 6.3 |
| 2.0 | 105 | 3.7 | 103 | 5.7 |
| 4.0 | 103 | 6.4 | 99.0 | 7.0 |

[a] Values are the means of five replicate determinations.

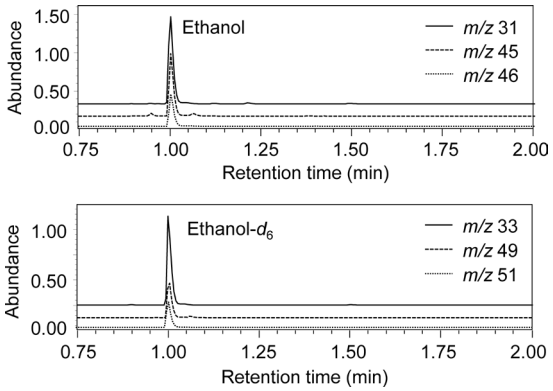


**Fig. 3. SIM chromatograms obtained from an autopsy blood sample.**

**Table 2. Quantified concentrations of ethanol in blood samples collected from autopsies in 2017 (*n*=17)**

| Blood sample | Quantified concentration (mg/mL) | |
|---|---|---|
| | Median | Range |
| Femoral vein (*n*=4) | 0.72 | 0.28−2.24 |
| Right heart (*n*=13) | 0.31 | 0.16−1.15 |

chromatograms obtained from an autopsy blood sample. The chromatograms provided good resolution and separation. The quantified concentrations of ethanol in blood are shown in Table 2. Ethanol was detected in all samples, and the median concentrations were 0.72 mg/mL for femoral vein blood and 0.31 mg/mL for right heart blood. These results indicated that ethanol could be quantified in whole blood samples collected at autopsies by NeedlEx®-GC/MS. Additionally, this method will be a useful alternative for laboratories that perform forensic toxicological investigations.

One of the current disadvantages of this method is the limitation in the number of target compounds, such as 1-propanol, which can be produced during decomposition and putrefaction processes[1,2]. Therefore, further studies are needed to establish a method that can simultaneously quantify ethanol, 1-propanol, and other VOCs in whole blood.

Additionally, preliminary experiments showed that quantification of ethanol using the present method was possible at a lower level of 2.5 μg/mL. These results suggested that the present method may be applicable to clinical and forensic toxicology.

## Conclusions

In this study, we developed and validated a method involving NeedlEx®-GC/MS for the quantification of ethanol in whole blood. The applicability of this method was demonstrated by measuring the concentrations of ethanol in whole blood samples from autopsies. This method was found to be suitable for forensic and clinical toxicology analyses. We are currently trying this technique for the detection of other VOCs in human body fluids.

## Conflict of Interest

The authors have no conflicts of interest directly relevant to the content of this article.

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