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Determination of ethyl glucuronide, a minor metabolite of ethanol, in human serum by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A rapid and sensitive determination procedure using liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) has been developed for the determination of ethyl glucuronide (EtG) in human serum. Samples were precipitated with methanol, centrifuged and the supernatant was evaporated to dryness followed by reconstitution with distilled water. As mobile phase 30 m*M* ammonium acetate–acetonitrile (30:70, v/v) was utilized. The base peak observed at m/z 221 was the $[M-H]^-$ ion of EtG, which was detectable in satisfactory sense. The detection limit was 0.03 μg/ml in the selected ion monitoring mode. A calibration graph constructed for EtG in serum gave good linearity over the range from 0.1 to 25 μg/ml. This paper also presents the application of this LC–ESI-MS procedure to the analysis of authentic serum samples.

Keywords: Ethanol; Ethyl glucuronide

1. Introduction

Driving under the influence of alcohol (DUI) is among the most serious of social problems throughout the world. Generally, blood alcohol testing serves as the most accurate method for the determination of DUI cases. However, a certain time-loss usually arises between the seizure of a drunk driver and the time of blood sampling, which often leads to a notable decrease in the alcohol concentration since ethanol has a high metabolism. In most countries, including Japan, a series of extensive legal procedures must be completed prior to involuntary drawing of the driver's blood. In the worst cases, only a trace amount of alcohol is detectable in blood, although a police officer had taken an obvious ethanol-positive result by preliminary breath testing.

In order to help forensic chemists take unequivocal proof of alcohol consumption, ethyl glucuronide (EtG), a minor metabolite of ethanol, is now gaining much attention [1,2]. EtG is formed from ethanol by conjugation with UDP-glucuronic acid and was first isolated in 1952 by Kamil et al. from rabbits' urine [3]. Since 1994 when EtG was first synthesized by

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Schmitt et al. [4], its determination has been carried out not only in human serum and urine samples [4], but also in hair strands [5,6]. Schmitt et al. also performed kinetic studies and reported that the EtG concentrations peaked 2 to 3.5 h after ethanol has reached its maximum, and EtG is still detectable up to 8 h after complete ethanol degradation [7]. The determination of EtG serves as a forensic tool to clarify the metabolic pathway of ethanol. Cases with contamination doubts (e.g., with a disinfectant) can be also proven [8]. Therefore, the development of rapid and reliable procedure for EtG becomes of great interest for forensic chemists, as well as for traffic investigation such as hit-and-run cases.

The determination of EtG has been performed by GC–MS after acetylation, which converts the highly polar glucuronide into a substance of higher volatility [4–8]. In analytical toxicology, LC–MS is increasingly employed as a quick, yet sensitive confirmatory method to detect polar substances without tedious pretreatment steps like derivatization [9]. So far, the authors have already successfully applied the LC–MS methodology for the detection of various illicit drugs including their glucuronides [10–13].

The present work aims to develop a quick and sensitive LC–MS procedure for the detection of EtG in human serum without any complex sample pretreatment steps. For this purpose, both chromatographic and instrumental parameters were optimized. In order to confirm the validity of the LC–ESI-MS procedure, EtG concentration was determined by this method for authentic serum samples of a volunteer drinker as well as suspected drunk drivers.

2. Experimental

2.1. Materials

EtG was synthesized from ethanol and triacetyl-α-D-6-bromoglucosiduronic acid methyl ester according to reference literature [4]. A standard stock solution of 1.0 mg/ml EtG was prepared in distilled water. Spiked serum samples were daily prepared by adding known amounts of the standard solution to blank human serum which had been sampled from healthy volunteers who had not consumed any alcoholic drinks or foods for 5 days. Acetonitrile and methanol were of HPLC grade, and all organic and inorganic reagents used were of analytical grade or better. Mobile phase was filtrated through a 0.45 μm membrane filter prior to the use.

2.2. Instruments

LC–ESI-MS was performed on a Platform (Micromass, UK) mass spectrometer equipped with PU-980 pumps (Jasco, Tokyo). Analyses were performed with electrospray ionization in the negative mode. The ion source temperature, capillary voltage, corn voltage were set at 80°C, −3.0 kV and −30 V, respectively. A TSKgel Amide-80 (250 mm×4.6 mm I.D., Tosoh, Tokyo) column was used with a TSK guardgel Amide-80 (15 mm×3.2 mm I.D.) guard column which was connected to the separation column. As mobile phase 30 m*M* ammonium acetate (NH_4OAc)–acetonitrile (30:70, v/v) was used at a flow rate of 0.8 ml/min. The eluted mobile phase was split, with 0.08 ml/min eluate being introduced into the mass spectrometer.

2.3. Sample pretreatment

Whole blood samples were centrifuged at 2200 *g* for 5 min, and clear supernatants were separated. To 0.2 ml of the supernatants were added 1.0 ml methanol for deproteinization, followed by centrifugation at 11 000 *g* for 3 min. The clear supernatants separated were then evaporated to dryness under a gentle stream of nitrogen. The residue was finally reconstituted in 100 μl distilled water, with 5 μl aliquots being submitted to LC-MS analysis.

3. Results and discussion

3.1. Optimization of chromatographic conditions

3.1.1. Separation column

In our previous study [10], we demonstrated the successful determination of morphine glucuronides by LC–MS using a hydrophobic ODS-type separation column. However, for EtG, which is an

extremely polar compound, the use of a hydrophilic column was thought to be preferable. The authors concluded to employ an acrylamido-type column, although such type of columns have not been frequently used in LC–MS analysis.

Antiquated ESI interfaces did not allow a large flow rate of mobile phase. Currently, it has become possible to spray a relatively large flow rate, even 1 ml/min, of mobile phase. However, in our previous experiment [14], a flow rate of 50–100 μl/min resulted in the highest sensitivity. Therefore, the following methods were compared in this study: (1) separation with a conventional column (4.6 mm I.D.), followed by split of the eluate, and (2) the use of a semi-micro column (the same column packing, 2.0 mm I.D.) without split of the eluate. Although the latter gave a ten times higher detection sensitivity, chromatographic stability was sometimes interfered even by pump pulsation. Moreover, injection of large amounts of biological extracts resulted in a shorter column life, and required frequent column cleaning. On the contrary, the conventional column did not suffer from such problems yet gave satisfactory sensitivity. Thus, an acrylamido-type conventional column was employed at a mobile phase flow rate of 0.8 ml/min, followed by splitting of the eluate at a ratio of 1:10.

3.1.2. Mobile phase

3.1.2.1. Effect of ammonium acetate on retention time. We employed a rather simple sample pretreatment, which included deproteinization of the serum samples with methanol, followed by evaporation of the separated supernatant. As this pretreatment can not remove inorganic salt components in blood, interference with such salts was anticipated to influence chromatographic efficiency. Variable retention time was observed for EtG in urine in our separate LC–MS study [15]. This phenomenon also occurs in serum samples, though it was much slighter than that in urine. Therefore, the effect of inorganic salts was investigated using a series of 10 μg/ml EtG aqueous solutions containing 0%, 0.05% and 0.1% each of sodium chloride. Generally, LC–MS analysis does not allow the use of non-volatile buffers such as phosphate salt buffer. However, NH_4OAc buffer, which can be vaporized in the interface, worked effectively for improving chromatographic efficiency [14]. The effect of NH_4OAc concentration in mobile phase was examined using above-mentioned model samples. The use of water–acetonitrile (30:70, v/v) markedly postponed the retention time (t_R) at a higher NaCl concentration; 3.1 min (0% NaCl), 4.7 min (0.05% NaCl) and 5.4 min (0.1% NaCl). The addition of NH_4OAc shifted the t_R as follows: 6.13 min (0%), 6.46 min (0.05%) and 6.53 min (0.1%), where the mobile phase was 10 m*M* NH_4OAc–acetonitrile (30:70, v/v). Finally, less variable t_R was given at higher NH_4Ac concentration; 9.17 min (0%), 9.23 min (0.05%) and 9.26 min (0.1%) with 30 m*M* NH_4OAc–acetonitrile (30:70, v/v). These results indicate that substantial ion strength is effective in minimizing interference with inorganic salts in biological matrix. This was probably because the silanol moiety left intact on the column packing was masked with NH_4OAc, which was very effective in reducing the interference.

3.1.2.2. Effect of ammonium acetate on sensitivity. Our previous basic experiments [14] shows that the concentration of NH_4OAc in the mobile phase strongly affected to the mass fragmentation and the spectrometric sensitivity. As mentioned earlier, higher NH_4OAc concentrations successfully minimized inorganic salt interference. However, as shown in Fig. 1, higher NH_4OAc concentration resulted in a remarkable decrease of the intensity of the base peak at m/z 221. Thus, the mobile phase 30 m*M*

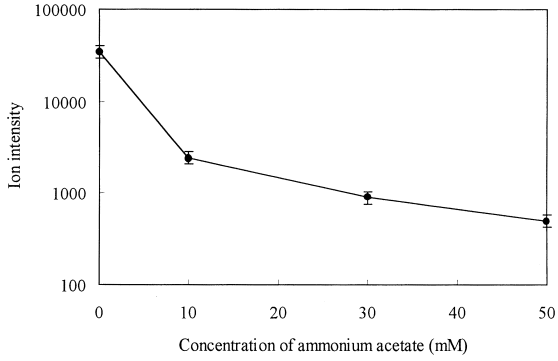

Fig. 1. Effect of ammonium acetate on the peak intensity.

NH_4OAc–acetonitrile (30:70, v/v) was employed in all further experiments below.

3.2. Optimization of interface parameters

3.2.1. Capillary voltage

In LC–ESI-MS, the capillary voltage remarkably affects to the ionization efficiency of the interface. The voltage was optimized in the negative mode, using a 10 μg/ml aqueous solution of the analyte standard. A capillary voltage of −3.0 kV gave the maximum ion intensity for the base peak at m/z 221.

3.2.2. Corn voltage

The optimization of corn voltage is also indispensable for the determination of polar analytes like EtG [14]. A corn voltage of −30 V led to the highest base peak response. Three μl aliquots of 10 μg/ml aqueous solution of analyte standard were injected. Fig. 2 depicts the LC–ESI-MS chromatograms and the mass spectrum of EtG obtained from a spiked serum sample at 10 μg/ml under the optimized conditions in the full-scan mode.

3.3. Quantitative analysis

In order to ensure the reliability of the present procedure, a calibration graph was constructed for EtG using a series of spiked serum samples (0.1, 0.5, 2.5, 10 and 25 μg/ml) employing absolute calibration method. The abundance of the base peak was monitored in the selected ion monitoring mode. The calibration graph gave good linearity over the range from 0.1 to 25 μg/ml ($y=-52+1280x$, $r=0.998$). The detection limit was 0.03 μg/ml (S/N=3). The recovery of EtG at 2.0 μg/ml in serum was 78.1%, and the relative standard deviations were 5.6% (within-day, $n=5$) and 11.5% (between-day, $n=3$). Although LC–ESI-MS usually gave simple mass spectra, its quantitative efficiency was slightly better than the conventional GC–MS determination [4].

3.4. Application to authentic blood samples

The optimized LC–ESI-MS procedure was applied to the analysis of serum samples of a volunteer drinker. The volunteer (36-year-old male, 60 kg) consumed 0.95 l of wine (about 60 g of ethanol) within 1 h. Informed consent was obtained from the subject. Blood samples were drawn up to 8 h after drinking. Fig. 3 shows the serum EtG concentrations

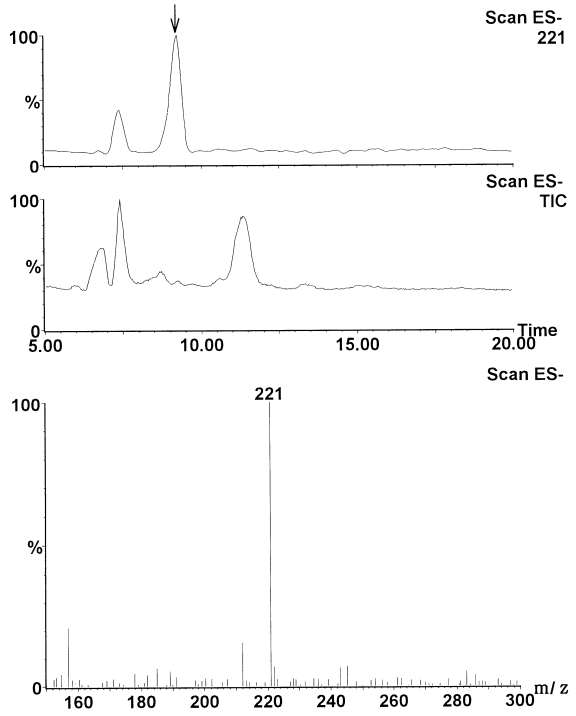

Fig. 2. Ion chromatogram at m/z 221 (top), total-ion chromatogram (middle), and the mass spectrum of ethyl glucuronide (bottom) obtained from a spiked serum sample at 10 μg/ml.

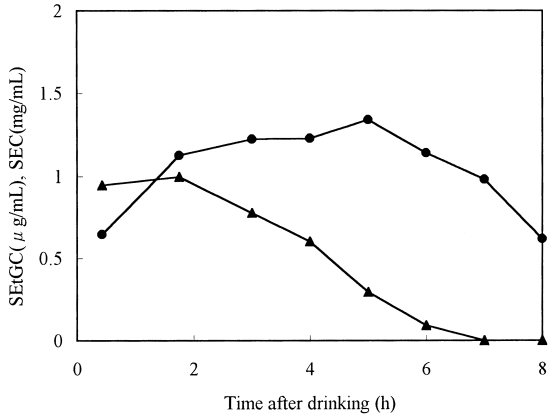

Fig. 3. Serum ethyl glucuronide concentrations (SEtGC, ●), and serum ethanol concentrations (SEC, ▲) of a volunteer drinker after intake of 0.95 liters of wine within 1 h.

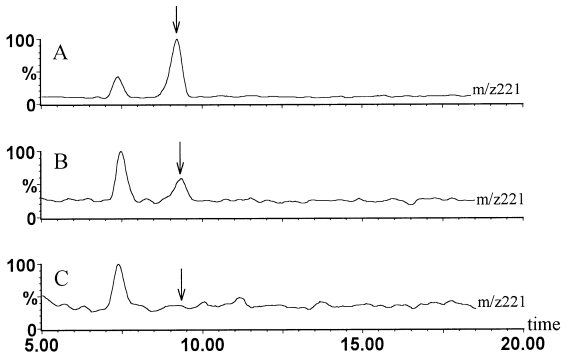

Fig. 4. Ion chromatograms at m/z 221 obtained from (A) spiked serum at 10 μg/ml, (B) the volunteer drinker's serum sample found to contain 1.34 μg/ml of EtG, and (C) blank serum.

(SEtGC) determined by the present LC–MS method, together with the serum ethanol concentrations (SEC) by headspace GC (*tert.*-butanol was used as I.S.). The EtG concentration peaked 5 h after the end of drinking, which was 3 h after that of ethanol. EtG was still detectable 8 h after the end of drinking, whereas ethanol disappeared 7 h after. Fig. 4 shows typical LC–ESI-MS results recorded in the full-scan mode for EtG in the serum sampled 5 h after the drinking. The EtG concentration was calculated to be 1.34 μg/ml. The characteristic peak at m/z 221 was clearly detected at a retention time of 9.3 min, which was identical to that of the analyte standard spiked to blank serum. The ion chromatograms at m/z 221 obtained from a blank serum and the spiked serum at 10 μg/ml are also presented in Fig. 4.

Furthermore, the serum EtG concentrations of five potential drunk drivers were determined by this method. Table 1 lists the results together with blood alcohol levels.

Table 1
Concentrations of ethyl glucuronide and ethanol in potential drunk driver's serum samples

Suspect no.	Concentration	
	ethylglucuronide (μg/ml)	ethanol (mg/ml)
1	7.69	0.25
2	2.57	1.20
3	1.24	0.34
4	2.43	1.34
5	4.70	1.40

As the relationship between the concentrations of ethanol and EtG is beyond the purpose of this study, this is described in a separate paper [7]. Even so, the determination of EtG could become necessary in certain cases, such as hit-and-run, in order to obtain a sufficient reason for the proof of drunk driving when the ethanol has already/almost disappeared at the time of an arrest due to its high metabolism. This present LC–MS procedure for EtG in serum, which does not need time-consuming sample pretreatment, would be useful for such purposes. Furthermore, EtG is more stable without derivatization than after acetylation or silylation. This might also contribute to the accuracy of the present LC–MS procedure.

4. Conclusion

Difficulties in developing a LC–MS procedure include limitations in mobile phase selection. Complications also exist in the selection of interface and the optimization of its operation conditions. It is, however, a promising technique, which allows determination of highly polar compounds like ethyl glucuronide without tedious derivatization. In spite of a simplistic sample pretreatment, we were able to minimize the interference with inorganic salts in serum matrix by addition of an appropriate concentration of ammonium acetate to the mobile phase. This simple and accurate LC–ESI-MS procedure for ethyl glucuronide in serum would therefore be a useful alternative in forensic toxicology.

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