Detection of Ethyl Glucuronide Using Pulsed Electrochemical Detection following Reversed-Phase High Performance Liquid Chromatography

CAN 101

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Introduction

Ethyl glucuronide (EtG) is a non-volatile, hydrophilic metabolite of ethanol, and its determination in biological samples, especially urine, has gained popularity as a biomarker of alcohol consumption. The ability to determine EtG in ante- and post-mortem samples has become especially important and provides applications in both clinical toxicology and forensic cases. EtG is a specific and sensitive biomarker of alcohol consumption because it may be detected when low levels of alcohol are consumed, and may be detected for up to 80 h after the elimination of alcohol from the body.[1]

Reported methods include use of reversed-phase liquid chromatography (LC) with pulsed electrochemical detection (PED), which provides a simple, sensitive, and direct detection of EtG in post-mortem urine samples.[2,3] This method isocratically separates EtG from methyl glucuronide (MetG), which is used as the internal standard. A C18 bonded-phase column is used in the separation, with a mobile phase consisting of 1% acetic acid/acetonitrile (98/2; v/v). Post-column addition of NaOH provides PED with a detection environment for all glucuronides. PED employs a gold working electrode for the detection of glucuronides. The reported limit of detection for this method is 0.03 µg/mL and the repeatability at the limit of quantitation is 1.7% relative standard deviation (RSD). The method requires a solid-phase extraction (SPE) step to remove any interferents from urine samples before analysis. The SPE provides a recovery of approximately 50 ± 2 %. Validation of this method is observed from the results of 29 post-mortem urine samples that agreed with the certified determinations. Advantages of this method include the direct and sensitive detection of glucuronides using a less expensive method of detection.[2]

This application note discusses improvements made to the previous method.[3] One change involves mobile phase optimization, in which alternative organic modifiers are studied. Another change is the addition of a wash step, which prevents carryover of the sample matrix between runs. Adjustments made to the SPE provide a much higher sample recovery. The internal standard is also changed to ProG in order to prevent elution with matrix interferents.

Equipment

Chromatography

Dionex DX-500 Series chromatography system consisting of:

- GP40 Gradient Pump (including an on-line degasser module)
- ED40 Electrochemical Detector
- IP20 Isocratic Pump
- AS3000 Autosampler (Spectra-Physics)
- LC30 Column Oven
- PeakNet® Software, version 5.21

Other Equipment

Electrochemical instrumentation* consisting of:

- Bipotentiostat, Model AFRDE4 (Pine Instrument Company)
- ASYST Scientific Software (Analyst Software Technologies)
- DAS-20 AD/DA Expansion Board (Keithley Data Acquisition)

Water purification system consisting of:

- Elix® 3 electrodeionization station
- Milli-Q® A-10 water purification system (Millipore)

Vacuum filtration apparatus (Fisher Scientific)

SPE cartridge:

- Sep-Pak® amino propyl cartridges (Waters)

Vacuum manifold apparatus:

- Spe-ed Mate 10 (Applied Separations)

Centrivap™ (Labconco)

Other Consumables

0.2 µm PTFE membrane filter (Alltech Associates Inc.)

Autosampler vials (0.5 mL, Alltech P/N 98048)

*Equipment used to help develop the application, but not needed to execute the application.

Reagents and Standards

Sodium hydroxide, 50% (w/w) (VWR Scientific Products Corp.)
Acetic Acid, HPLC grade (Fisher Scientific)
t-Butanol (Fisher Scientific)
Acetonitrile (Fisher Scientific)
Deionized water (used for preparation of all standards and eluents)
Ethyl glucuronide (EtG) (Aurora Analytics LLC)
Propyl glucuronide (ProG) (Aurora Analytics LLC)
Surine™ (Dyna-Tek Inc.)
Purified water with a resistivity of 18 MΩ

Conditions

Column:	Acclaim® PolarAdvantage, 5 µm, 120Å, 250 × 4.6 mm
Guard Column:	Vydac® C18 column, 5 µm 7.5 × 4.6 mm (W. R. Grace)
Operating Pressure:	2000 psi
Temperature:	30 °C
Degas:	Always on
Injection Volume:	50 µL
Injection Loop:	50 µL
Detection:	Pulsed Electrochemical, gold working electrode (1.0 mm diameter), Ag/AgCl reference electrode (Model 46333; Dionex)
Collection Rate:	2 Hz
Needle Height:	2 mm
Flush Volume:	400 µL
Run Time:	30 min

Eluent/Reagent Preparation

Mobile Phase Consisting of 1% Acetic Acid, 0.5% t-Butanol

Prepare mobile phase in a 2 L volumetric flask. First add 1 L of DI water to the flask. Then deliver 20 mL of acetic acid (HPLC grade) to the flask, using a volumetric pipet. *t*-Butanol solidifies at room temperature, so gentle heating in a flask of water is necessary before use. Use a volumetric pipet to add 10 mL of the warm *t*-butanol to the flask. Bring to volume with DI water and invert the flask several times to ensure proper mixing of the solution. After preparation, install on the system and store under nitrogen.

Post-Column Sodium Hydroxide

In order to prepare 2 L of 600 mM sodium hydroxide, add 63 mL of 50% (w/w) NaOH via a graduated cylinder to 1 L of water in a 2 L volumetric flask. Bring to volume, gently stir the solution, and store under nitrogen to prevent exposure to carbon dioxide.

Wash Step Consisting of Acetonitrile/Water (50:50 v/v)

Prepare solution by adding 1 L of acetonitrile to 1 L of water. Stir the solution for several minutes to ensure proper mixing.

Standard Preparation

No purification of the MetG, EtG, and ProG standards is necessary. Prepare glucuronide solutions at desired concentrations and store at 4 °C.

Sample Preparation

Surine is a synthetic urine matrix without many of the disadvantages of human urine, such as odor, foam, and biohazard disposal requirements. Solid phase extraction (SPE) is performed on both the Surine and urine samples to remove the glucuronide from each sample. Pretreat all samples (0.2 mL) with 100 µL of 1 M HCl and 0.4 mL of acetonitrile. Condition the aminopropyl cartridges by adding and removing 3 mL of methanol, 3 mL of distilled water, and 3 mL of acetonitrile consecutively. Load each sample onto the SPE cartridge consisting of a 500 mg sorbent bed of aminopropyl stationary phase, with a rate of 0.5 mL/min. Use a vacuum apparatus to pull the sample through the device. Wash the cartridge with 3 mL of acetonitrile, and use the vacuum for 10 min to remove any moisture. Elute the glucuronides by washing the bed with 2 mL aliquots of 2% ammonia in methanol. Collect the eluent in 12 × 75 mm culture tubes and dry in a Centrivap concentrator. Reconstitute the glucuronides with 1.00 mL of water.

Results and Discussion

Pulsed Voltammetry

Pulsed Voltammetry (PV) has been used to characterize analyte response and to select the time parameters for a PED waveform.[4] For this application, PV was used to optimize the mobile phase composition. Previously, acetonitrile (CH_3CN) was used as an organic modifier in the mobile phase.[2] Figures 1A and 1B show the effects of the mobile phase on the signal for MetG. Clearly, Figure 1A shows that as the concentration of CH_3CN in the mobile phase increases, the signal from MetG decreases significantly. The cyano group on the CH_3CN adsorbs to the electrode surface which in turn blocks adsorption sites for the MetG and decreases its response. The need for an organic modifier, which has functional groups that adsorb equally or less than the hydroxyl groups of the glucuronides, was apparent. Tertiary alcohols were found to provide the necessary properties of an acceptable organic modifier. A similar study was done with *t*-butanol (*t*-BuOH); Figure 1B shows that as the concentration increases, the signal of MetG is relatively unaffected. This alternative modifier provides much better results without complications. Only 0.5% *t*-butanol was required, because it has a higher solvent strength than the CH_3CN.[5] The PV studies also show a maximum signal-to-noise ratio at a detection level of +100 mV and an increase in sensitivity and long-term stability.[6] Please note that as glucuronides all have a similar response, the separation of these compounds must occur prior to detection.

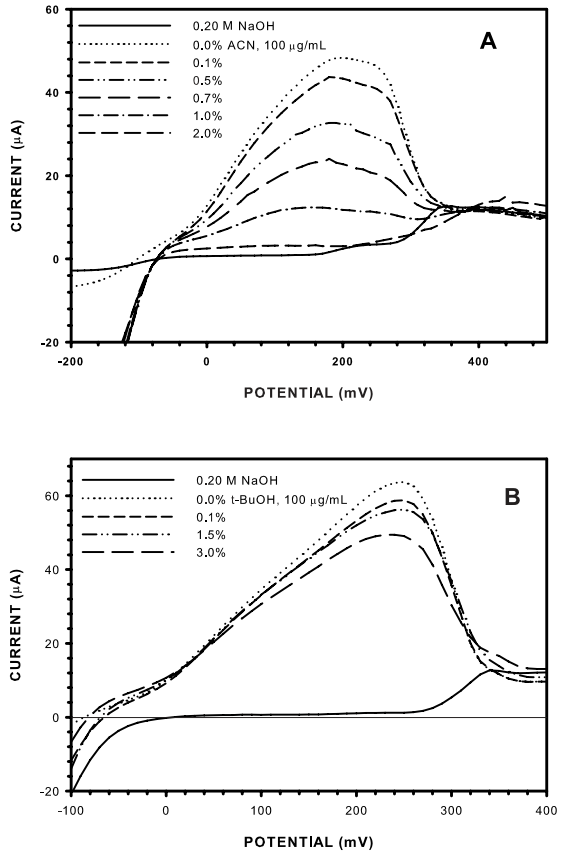

Figure 1. PV responses as a function of detection potential for MetG, 100 µg/mL, at Au RDE in 0.17 M NaOH and increasing increments of (A) CH_3CN from 0.1% to 2% and (B) t-BuOH from 0.1% to 3%. Condition: 900 rpm rotation speed.

Solid-Phase Extraction (SPE)

A few adjustments were made to the previously reported SPE procedure.[2] First, the use of MetG as an internal standard had a flaw: MetG elutes among matrix interferents in the chromatogram. Using n-proply glucuronide (ProG) as an alternative internal standard prevents any overlap with the interferents, because ProG elutes after and behaves similarly to EtG. In the previous SPE procedure, the 3 M HCl caused a white film to form during the drying step that was difficult to reconstitute with water. A lower concentration of 1 M HCl does not form this residue and therefore eases the reconstitution. Alteration in sample pretreatment also yielded improvements in recovery. Previously, 1.0 mL of sample was pretreated with 3 mL of CH_3CN, but SPE recovery was further optimized by using only 0.2 mL of urine with 0.4 mL of CH_3CN. Overall, the optimization improved recovery to 84 ± 3% (previously reported recovery was 50%[2]), and decreased the required sample volume.

Chromatography

In general, all of the glucuronides were easily separated by reversed-phase chromatography with a mobile phase consisting of 1% acetic acid/ 0.5% *t*-butanol (by volume). Preparation for the reversed-phase separation required the addition of 1% acetic acid to the mobile phase, to ensure the glucuronides were neutral. We selected this mobile phase by testing for conditions in which the EtG and ProG peaks were baseline resolved and exhibited the best column efficiency and peak shape. As shown in Figure 2, retention times for EtG and ProG were 4.94 ± 0.01 min. and 8.83 ± 0.01 min., respectively. Previously, the results showed a signal-to-noise (S/N) of 50 for EtG with CH_3CN; optimization of the method drastically increased the S/N to 230 with *t*-butanol. Implementation of a wash step with high solvent strength prevented the carry-over of the sample matrix between runs. The wash step consisted of the addition of CH_3CN/water (50/50, v/v) for 10 min followed by a reequilibration to the separation conditions for 10 min. A guard column was placed before the analytical column to protect it from potentially harmful impurities. A postcolumn addition of 600 mM NaOH, delivered by an isocratic pump, is essential to PED. The optimized chromatographic run is 30 min.

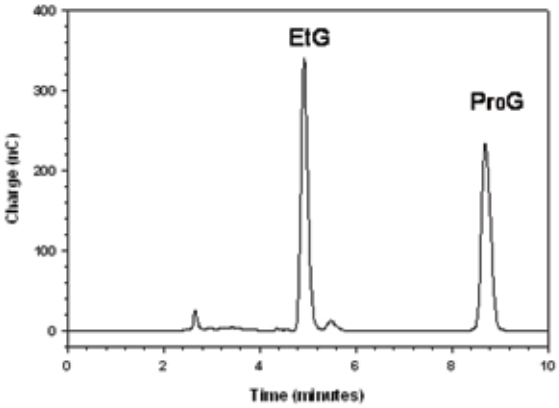

Figure 2. A representative chromatogram of EtG and ProG at a concentration of 10 µg/mL each. Conditions: columns: Acclaim PolarAdvantage 120 Å, 5 µm, 250 × 4.6 mm, (Dionex), and Vydac C18, 5 µm, 7.5 × 4.6 mm guard; mobile phase: 1% acetic acid/0.5% t-butanol (by volume); flow-rate: 1.0 mL/min; postcolumn reagent: 600 mM NaOH at 0.4 mL/min.

Analytical Figures of Merit

Quantitation was based on the peak height measurement of the analytical signal. Table 1 shows the analytical figures of merit for the PED method using a 4-potential waveform. Regression analysis demonstrates a linear response of PED over a range from 0.01 µg/mL to 10 µg/mL for both EtG and ProG. The limit of detection (LOD) in water was found to be 0.01 µg/mL for both analytes. Data for Surine analyses show a linear response for EtG, ranging from 0.08 µg/mL to 10 µg/mL. Table 1 also includes a comparison of %RSD for each compound, all at a S/N ratio of 10. This demonstrates the repeatablility of the analysis which included six repetitive injections at the limit of quantitation (LOQ). The table below also shows the reproducibility of EtG, 3.2% RSD, after the extraction, which was determined by six consecutive extractions of EtG at 5 µg/mL from the Surine matrix.

A blind study was performed in which samples were prepared by spiking water and Surine with EtG. The concentrations of the blind study samples covered a range which was predetermined given concentrations of standards. These measurements were performed in triplicate using the LC-PED method. The Surine matrix was prepared by applying the SPE to each sample, in triplicate. Table 2 shows the quantitation of EtG in each of the sample matrices. The data was determined using the ratio of the internal standard to EtG in the sample. Please note, % recovery values of ≥100 represent the amount of EtG recovered after using the internal standard to correct for loss by the extraction step. There was no significant difference between the amount added and recovered at a 95% confidence level. This supports the previous claim that the method is quantitative in both water and Surine samples. Table 2 also shows results from a study of human urine. Samples of human urine were spiked with EtG and diluted within the linear range of the assay for quantitation. The samples were analyzed in triplicate using SPE with the LC-PED method. It was found that there is no significant difference between the added and recovered amounts of EtG after SPE at a 95% confidence level. These results agree with those reported in previous work with the same experiment.[2]

Table 1. Quantitative Analysis of Glucuronides by PED

Compound	LOD[a] (µg/mL, pmol)	Linear Range nC = a (µg/mL) + b			LOQ[b] (µg/mL, pmol)	%RSD (n=6) at S/N=10
		a	b	r^2 (n = 8)		
EtG	0.01, 2	49.9	5.9	0.99967	0.02, 5	1.5
ProG	0.01, 2	24.8	2.4	0.99987	0.04, 9	2.0
EtG (Surine)	0.08, 20	20.8	1.6	0.99923	0.3, 70	3.2

[a]Limits of detection were determined at three times S/N from injections of concentrations that were within 10 times the calculated LOD.

[b]LOQ determined at S/N=10.

Table 2. Summary of Blind Study Results

Matrix	Sample	Amount EtG added (µg/mL)	Amount EtG found (µg/mL)	% Recovery (n=3)
Water	1	1.6	1.6 ± 0.02	100 ± 1
	2	2.9	3.0 ± 0.02	103 ± 1
	3	4.8	4.8 ± 0.04	100 ± 1
Surine	1	6.0	6.5 ± 0.5	108 ± 8
	2	6.3	6.6 ± 1.0	105 ± 15
	3	9.0	9.2 ± 0.3	102 ± 3
Urine	1	48.5	47.0 ± 2.0	97 ± 4
	2	95	94 ± 1.0	99 ± 1
	3	160	162 ± 2.0	101 ± 1

Conclusion

This application note provides the details of an improved method for the separation and detection of EtG in synthetic and human urine samples. *t*-Butanol as an organic modifier allows for the separation of analytes without suppression of the sample signal. With PED, *t*-BuOH provides higher sensitivity and a lower detection limit in comparison to acetonitrile. This discovery is generally applicable with PED methods of weakly-adsorbing analytes. Propyl glucuronide is a better internal standard than methyl glucuronide, as it avoids matrix interferents. Optimization of the SPE provides an increase in sample recovery to 84 ± 3%, as well as high reproducibility and a decrease in required sample volume. Implementation of a wash step with CH_3CN prevents carryover of the sample matrix into the following runs.

This application note has been kindly provided by a Dionex customer, and in the opinion of Dionex represents an innovative application of Dionex products. This application has not been tested in a Dionex applications lab and therefore its performance is not guaranteed.

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LPN 2113 PDF 09/08