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TECHNICAL NOTE

A novel and an effective analytical approach for the LC-MS determination of ethyl glucuronide and ethyl sulfate in urine

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Abstract An alternative liquid chromatography–mass spectrometry (LC-MS) method based on no discharge (ND) atmospheric pressure chemical ionization (APCI) was developed for the simultaneous determination of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine in negative ion conditions. Abundant $[M\text{-}H]^-$ species of EtG and EtS were obtained, allowing to reach limits of quantification (0.1 μg/ml for both analytes), accuracy, and precision comparable to those proposed in the literature. Additionally, the LC-ND–APCI-MS method proved to be reliable, requiring little maintenance even when high throughput analyses (i.e., 6,000 samples per year) were required.

Keywords Liquid chromatography · Mass sprectrometry · Ethyl glucuronide · Ethyl sulfate

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are nonvolatile, water-soluble ethanol metabolites that have been proposed as efficient markers of alcohol intake and abuse [1–3]. They can be quantified in body fluids for an extended time period after complete elimination of alcohol, being detectable in serum up to 8 h [4] and in urine up to 80 h after ethanol elimination [5].

A number of analytical methods have been developed for their determination in biological matrices such as gas chromatography–mass spectrometry with derivatization [4, 6], liquid chromatography–mass spectrometry (LC-MS) [7], liquid chromatography–multiple mass spectrometry (LC-MS/MS) [8, 9], liquid chromatography with pulsed electrochemical detection [10], capillary zone electrophoresis [11, 12], and immunochemical tests [13].

The use of electrospray ionization (ESI) and LC-MS/MS methods on the most recent, high-performance mass spectrometric instrumentation enabled the *mise au pointe* of methods fulfilling guideline requirements for forensic confirmation of analytes and exhibiting low limits of detection (LOD) and low limits of quantification (LOQ) after simple or null sample pretreatment [14]. However, a loss of sensitivity due to matrix effects (defined as the combined effect of all components of the sample other than the target analyte on the measured analyte quantity [15]) has already been described using ESI, when instruments with on-axis interfaces are employed and automated analyses of large sample batches are performed [15, 16]. Indeed, minute droplets originating from the ionization process are capable of inflowing the entrance capillary together with desolvated ions, thus creating a thin layer of neutral species on the entrance capillary inner surface. At high temperature, these neutrals may undergo thermal decomposition and activate the inner surface against EtG and EtS decreasing the intensity of the $[M\text{-}H]^-$ signal. In order to overcome these problems, an alternative LC-MS approach based on the use of no discharge (ND) atmospheric pressure chemical ionization was tested for the analysis of EtG and EtS in urine in a forensic setting where thousands of samples need to be processed in a year.

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Materials and methods

EtG and d_5-EtG were purchased from Medichem (Promochem, Milano, Italy); EtS and d_5-EtS were prepared by chemical esterification of sulphuric acid with ethanol and d_5-ethanol [17]. Water was obtained from a Milli-Q Academic system (Millipore, Bedford, MA, USA). Acetonitrile (Sigma, Milano, Italy), formic acid (Sigma, Milano, Italy), and methanol (Fluka, from Sigma, Milano, Italy) while Isolute-NH_2 solid phase extraction columns were obtained from Varian (Harbor City, CA, USA).

Individual stock solutions of EtG, d_5-EtG (internal standard, IS), EtS, and d_5-EtS (IS) were prepared in methanol at a concentration of 1 mg/ml and kept for 6 months at −20°C. Working solutions of EtG and EtS were prepared weekly at a concentration of 5 μg/ml in water. The IS working solutions were prepared weekly at 5 μg/ml in water. Calibration and quality control samples were prepared by adding suitable amounts of the standards to blank urine, obtaining concentrations in the working range of 0.1–5 μg/ml in urine. Urine samples (20 ml) were collected from subjects whose driving license had been revoked for alcohol-related problems and were following a regranting protocol. EtG and EtS-free urine samples were collected from healthy volunteers who had not consumed alcohol in the last 7 days. All specimens were stored in plastic containers at −20°C prior to analysis. To urine (125 μl), IS working solutions (200 μl), acetonitrile (2 ml), and 6 M HCl (50 μl) were added. After centrifugation at 3,500×g, the supernatant was transferred to an aminopropyl Isolute-NH_2 solid phase extraction column, previously activated by methanol (2 ml), water (2 ml), and acetonitrile containing 0.1% formic acid (2 ml). The column was washed with pentane (2 ml) and dried under vacuum for 10 min. Elution of analytes was performed with 2 ml of water, 0.1% formic acid, and 10 mmol/l ammonium formate (pH 3). EtG and EtS were recovered in the first 0.8 ml of eluate. The whole procedure corresponds to 1: 6.4 urine dilution; 20 μl of eluate was used for injection.

Chromatographic separation was optimized by injection of pure standard solutions in a Hypercarb (Thermo, San José, CA, USA) column (150×2.1 mm; 5 μm) that allows the separation of highly polar compounds with closely related structures. A Spectra System P4000 pump (ThermoFinnigan, San José, CA, USA) was used for gradient elution at a constant flow rate of 0.2 ml/min. The mobile phase compositions were: (a) water, 0.1% formic acid, 10 mmol/l ammonium formate, and pH 3 and (b) acetonitrile, 0.1% formic acid, and 10 mmol/l ammonium formate. The elution was programmed as follows: initial conditions 0% B for 30 s, linear gradient to 80% B in 3 min, 80% B hold from 3.5 to 7 min, and linear gradient to 0% B in 2 min. Re-equilibration time was 6 min with a total run time of 15 min. The injection volume was 20 μl. Analyte signal intensities were enhanced by postcolumn addition of acetonitrile (0.4 ml/min). All mass spectrometric measurements were obtained using an LCQ-Duo (Finnigan, San José, CA, USA) ion trap mass spectrometer equipped with an APCI source operating in negative ion mode. Optimization of the ion source and MS parameters was performed by direct infusion of a 2.5 μg/ml solution of EtG and/or EtS with a syringe pump at 40 μl/min, coupled with a T-union to the outlets of two LC pumps, one delivering the mobile phase (water, 0.1% formic acid, 10 mmol/l ammonium formate, and pH 3) at 0.2 ml/min and the other delivering acetonitrile at 0.4 ml/min.

The optimized no discharge APCI parameters were: source vaporizer temperature 500 C and 600°C for EtG and EtS, respectively, entrance capillary voltage 10 V, sheath gas (nitrogen) 40 arbitrary units (a.u.), and entrance capillary temperature 160°C. The corona discharge voltage was switched off.

For resonant collision-induced dissociation experiments, a supplementary radio frequency (rf) voltage ("tickle voltage") in the range of 0–5 V was applied to the end caps of the ion trap. Helium was used as target gas, at a pressure of 1.46×10^{-3} Pa. During LC-MS runs, acquisition was split into two segments; in the first (3–5.5 min), MS/MS was performed on precursor ions at m/z 221 and m/z 226 for EtG and d_5-EtG (acquiring product ions in the range m/z 50–230), respectively, and in the second (5.5–7 min), the mass spectrometer was operated in single ion monitoring mode for ions at m/z 125 (EtS) and m/z 130 (d_5-EtS). A divert valve was used to switch the LC flow to waste from 0 to 3 min and from 7 min to the end of the run.

Results and discussion

The aim of the study was to develop a LC-MS method for EtG and EtS using an alternative ionization method that avoids deposition of neutral species on the entrance capillary inner surface encountered using an on-axis ESI source. This occurrence, particularly relevant when multiple LC injections of urine extracts have to be performed daily, causes a dramatic decrease in the instrument sensitivity that can be restored only by extensive cleaning of the entrance capillary or its substitution.

As an alternative to ESI conditions, we tested the use of an APCI source without any corona discharge as recently proposed by Cristoni et al. [18]. Using ND-APCI, a clear increase of the abundance of protonated (or deprotonated) molecules and a concomitant lowering of chemical background and clustering reactions have been observed for analytes of different chemical classes [19]. Looking at these data and considering the ionic nature of EtG and EtS in

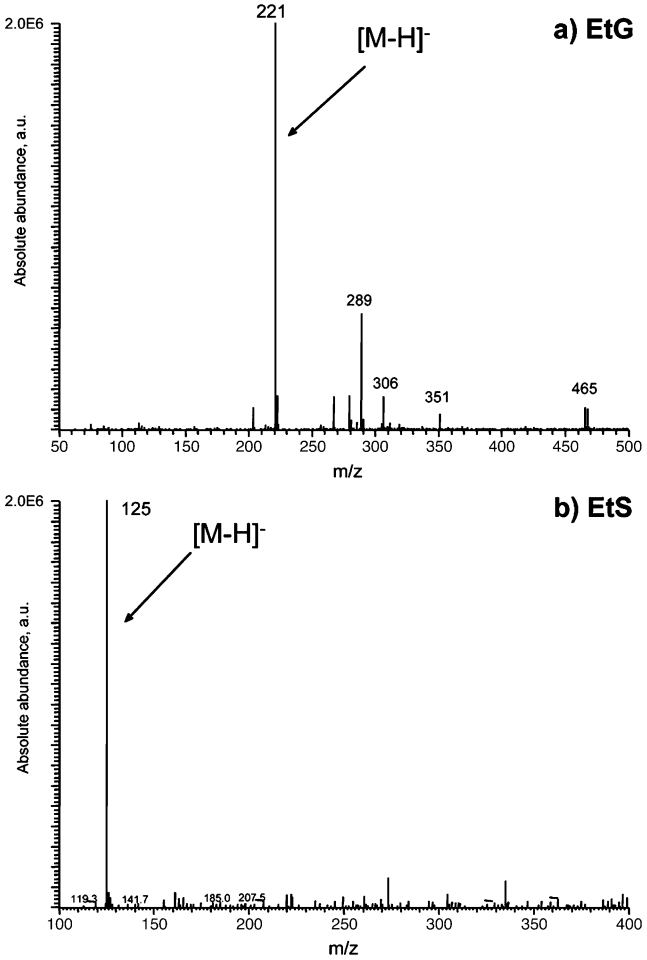

Fig. 1 ND-APCI spectra of **a** EtG and **b** EtS

aqueous solution, ND-APCI was considered a promising substitute for ESI.

The mass spectra obtained by infusing 2.5 μg/ml standard solutions of EtG and EtS are shown in Fig. 1. In both cases, the deprotonated molecules led to the most intense signals; worth noting is the low level of chemical background, typical for that obtained by the ND-APCI approach. Once the optimized ionization conditions for the target analytes was reached, a LC-ND–APCI-MS method was developed for the quantitative simultaneous determination of EtG and EtS in urine after solid phase extraction.

For the preliminary validation of the method, specificity was checked by running 20 different blank urine samples. In every case, signal-to-noise (*S*/*N*) values were lower than three for selected ion traces. The percentage deviations of relative abundances of the diagnostic ions acquired in the scan function were determined by duplicate analysis of six different urine samples spiked at 0.5 and 2 μg/ml of EtG and EtS, performed on two separate days. RSDs were <20% for urine at 0.5 μg/ml and <14% for urine at 2 μg/ml for both EtG and EtS. Calibration curves were linear in the range 0.1–5 μg/ml in urine. The correlation coefficients of all individual curves exceeded 0.991. When concentrations in real samples were presumed to be higher, sample dilution was undertaken in order to fall into the linearity range.

The LOQ was established at 0.1 μg/ml for both analytes on the basis of the accuracy of five independent determinations at this concentration (deviation from nominal value within 20%). LODs, defined as analyte concentrations giving rise to an *S*/*N* ratio=3, were found to be 0.05 μg/ml for both EtG

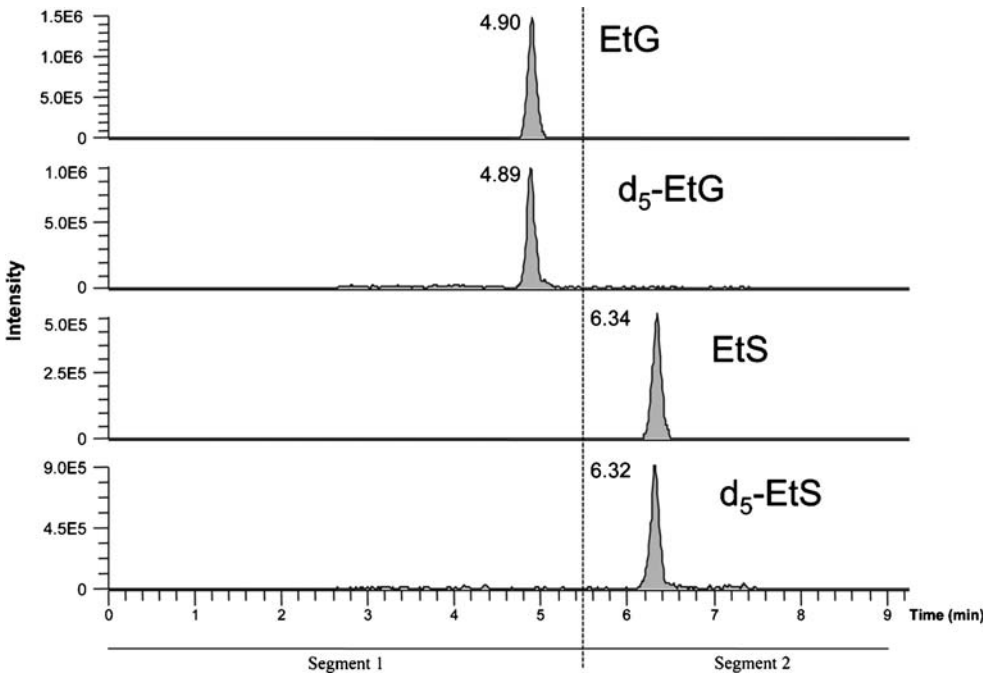

Fig. 2 Chromatograms of a positive urine sample (EtG 1.7 μg/ml and EtS 0.8 μg/ml). *Segment 1*, MS/MS of m/z 221 and 226 (product ion scan). *Segment 2*, single ion monitoring of ions at m/z 125 and 130

and EtS. These values were also obtained by five independent determinations. Intra- and interassay precision, as percent relative standard deviation, assessed by extracting and analyzing four replicates of spiked samples (at 0.5, 1, and 2 μg/ml) on three consecutive days (n=12) was satisfactory being <20% in urine for both analytes. Accuracy, in the absence of a certified reference material, was evaluated as percent relative error (i.e., the percentage deviation of the mean result for all analyses from the nominal value of a spiked sample) on the basis of the total data set (n=12) at the reported three concentrations and averaged within±18% of the target values with a RSD of ≤15% for both analytes.

The method described was primarily developed to detect EtG and EtS in urine samples collected from subjects whose driving license was suspended for alcohol related problems. As an example, the ion chromatogram related to EtG, d_5-EtG, EtS, d_5-EtS obtained for a real sample under ND-APCI conditions is reported in Fig. 2. What is most interesting is that the signal intensity of both $[M-H]^-$ and the ISs were constant after repeated injections of urine extracts in LC.

The license regranting protocol includes the unannounced collection of three to five urine samples over a period of 2–4 weeks. Data obtained from EtG and EtS analysis are used to evaluate the reliability of the alcohol intake declared by the subjects during medical examination and together with other alcohol biomarkers to outline their drinking pattern. A cutoff of 0.3 μg/ml is applied to distinguish accidental alcohol intake (e.g., from medication or food) from conscious alcohol intakes. On the whole, more than 6,000 urine samples from 1,500 subjects need to be processed each year. The LC-ND–APCI-MS method showed to be really robust reducing routine source maintenance and allowing 24 h automated analysis of large batches of samples.

References

1. Lands WE (1998) A review of alcohol clearance in humans. Alcohol 15:147–160
2. Wurst FM, Wiesbeck GA, Metzger JW, Weinmann W (2004) On sensitivity, specificity, and the influence of various parameters on ethyl glucuronide levels in urine—results from the WHO/ISBRA study. Alcohol Clin Exp Res 28:1220–1228
3. Wurst FM, Dresen S, Allen JP, Wiesbeck G, Graf M, Weinmann W (2006) Ethyl sulphate: a direct ethanol metabolite reflecting recent alcohol consumption. Addiction 101:204–211
4. Schmitt G, Droenner P, Skopp G, Aderjan R (1997) Ethyl glucuronide concentration in serum of human volunteers, teetotalers, and suspected drinking drivers. J Forensic Sci 42:1099–1102
5. Wurst FM, Kempter C, Seidl S, Alt A (1999) Ethyl glucuronide–a marker of alcohol consumption and a relapse marker with clinical and forensic implications. Alcohol Alcohol 34:71–77
6. Schmitt G, Aderjan R, Keller T, Wu M (1995) Ethyl glucuronide: an unusual ethanol metabolite in humans. Synthesis, analytical data, and determination in serum and urine. J Anal Toxicol 19:91–94
7. Nishikawa M, Tsuchihashi H, Miki A et al (1999) Determination of ethyl glucuronide, a minor metabolite of ethanol, in human serum by liquid chromatography–electrospray ionization mass spectrometry. J Chromatogr B Biomed Sci Appl 726:105–110
8. Bicker W, Lammerhofer M, Keller T, Schuhmacher R, Krska R, Lindner W (2006) Validated method for the determination of the ethanol consumption markers ethyl glucuronide, ethyl phosphate, and ethyl sulfate in human urine by reversed-phase/weak anion exchange liquid chromatography–tandem mass spectrometry. Anal Chem 78:5884–5892
9. Politi L, Morini L, Groppi A, Poloni V, Pozzi F, Polettini A (2005) Direct determination of the ethanol metabolites ethyl glucuronide and ethyl sulfate in urine by liquid chromatography/electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom 19:1321–1331
10. Shah R, Lacourse WR (2006) An improved method to detect ethyl glucuronide in urine using reversed-phase liquid chromatography and pulsed electrochemical detection. Anal Chim Acta 576:239–245
11. Krivankova L, Caslavska J, Malaskova H, Gebauer P, Thormann W (2005) Analysis of ethyl glucuronide in human serum by capillary electrophoresis with sample self-stacking and indirect detection. J Chromatogr A 1081:2–8
12. Novakova M, Krivankova L (2008) Determination of ethyl glucuronide in human serum by hyphenation of capillary isotachophoresis and zone electrophoresis. Electrophoresis 29:1694–1700
13. Zimmer H, Schmitt G, Aderjan R (2002) Preliminary immunochemical test for the determination of ethyl glucuronide in serum and urine: comparison of screening method results with gas chromatography–mass spectrometry. J Anal Toxicol 26:11–16
14. Weinmann W, Schaefer P, Thierauf A, Schreiber A, Wurst FM (2004) Confirmatory analysis of ethylglucuronide in urine by liquid chromatography/electrospray ionization/tandem mass spectrometry according to forensic guidelines. J Am Soc Mass Spectrom 15:188–193
15. Tang L, Kebarle P (1993) Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. Anal Chem 65:3654–3668
16. Enke CG (1997) A predictive model for matrix and analyte effects in electrospray ionization of singly charged ionic analytes. Anal Chem 69:4885–4893
17. Nalesso A, Frison G, Favretto D, Maietti S, Ferrara SD (2005) Synthesis and characterization of ethyl sulfate and D5-ethyl sulfate as reference substances for applications in clinical and forensic toxicology. Rapid Commun Mass Spectrom 19:3612–3614
18. Cristoni S, Bernardi LR, Biunno I, Tubaro M, Guidugli F (2003) Surface-activated no discharge atmospheric pressure chemical ionization. Rapid Commun Mass Spectrom 17:1973–1981
19. Turnipseed SB, Andersen WC, Karbiwnyk CM, Roybal JE, Miller KE (2006) No discharge atmospheric pressure chemical ionization: evaluation and application to the analysis of animal drug residues in complex matrices. Rapid Commun Mass Spectrom 20:1231–1239