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Evaluation of a commercial LC-MS/MS assay for the quantification of ethyl glucuronide in urine for alcohol intake monitoring

Evaluierung eines kommerziellen LC-MS/MS Testkists zur Bestimmung von Ethylglucuronid im Urin zum Monitoring des Alkoholkonsums

DOI 10.1515/labmed-2015-0029
Received March 4, 2015; accepted March 30, 2015; previously published online April 28, 2015

Abstract

Background: A commercially available in vitro diagnostics (IVD)-approved mass spectrometric assay for the quantification of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine (RECIPE Chemicals+Instruments GmbH, Munich, Germany) was verified for monitoring of recent alcohol intake after transplantation.
Methods: For sample preparation, 50 µL of urine sample was mixed with an isotope-labeled internal standard solution. After centrifugation, 5 µL of the supernatant was analyzed by LC-MS/MS in a total run time of 3 min. An API 6500 tandem mass spectrometer (AB SCIEX, Toronto, Canada) combined with a Shimadzu UFLC system (Duisburg, Germany) was applied.
Results: The limits of quantification for the commercial assay were 0.07 mg/L for EtG and 0.03 mg/L for EtS in urine. The coefficient of variation for both analytes was lower than 7% (within-day) and 15% (between-days). Accuracy ranged between 101 and 144% for samples from an external quality assurance program. The comparison of the commercial test kit and an established LC-MS/MS method showed a very good agreement for EtG (r=0.96) and EtS (r=0.97) over a broad urine concentration range.
Conclusions: The commercial IVD-certified LC-MS/MS assay is suitable for the analysis of EtG and EtS in human urine[0] to assess recent alcohol intake in transplant monitoring.

Keywords: ethyl glucuronide; tandem mass spectrometry; urine.

Zusammenfassung

Hintergrund: Ein kommerzieller Testkit für die Analytik von Ethylglucuronid (EtG) und Ethylsulfat (EtS) (RECIPE Chemicals+Instruments GmbH, Munich, Germany) im Urin wurde für die Eignung zur Abschätzung eines kürzlich zurückliegenden Alkoholabusus im Rahmen des Transplantmonitorings untersucht.
Methoden: Für die Probenaufarbeitung wurden 50 µL Urin mit der internen Standard-Lösung versetzt. Für die Messung und Quantifizierung von EtG und EtS wurde ein API 6500 Tandem Massenspektrometer (AB Sciex, Toronto, Canada) und ein UFLC System der Fa. Shimadzu (Duisburg, Germany) eingesetzt. Isotopenmarkierte interne Standards dienten der Quantifizierung beider Analyte. Die analytischen Kenndaten der Methode wurden erhoben. Die Methode wurde für 50 Urinproben mit einer etablierten LC-MS/MS Methode verglichen.
Ergebnisse: Die Bestimmungsgrenzen des kommerziellen Testkits betrugen 0.07 mg/L für EtG und 0.03 mg/L für

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EtS im Urin. Für EtG und EtS wurden intra-assay sowie inter-assay Variationskoeffizienten von <7% und <15% ermittelt. Die Richtigkeit bei Ringversuchsproben lag zwischen 101–144%. Für Urinproben wurde eine Korrelation von r=0.96 (EtG) und r=0.97 (EtS) zwischen dem kommerziellen Testkit und einer etablierten LC-MS/MS Methode ermittelt.

Schlussfolgerungen: Der kommerzielle IVD-zertifizierte Testkit ist für die Quantifizierung von EtG und EtS zur Abschätzung des kurzfristigen Alkoholkonsums im Urin von Patienten im Rahmen des Transplantmonitorings geeignet.

Schlüsselwörter: Tandem Massenspektrometrie; Ethylglucuronid; Urin.

Introduction

For an assessment of a patient's history of alcohol consumption, information on recent but also about long-term alcohol intake and the amount of alcohol (heavy or low dose) is of high interest. Carbohydrate-deficient transferrin (CDT) has been widely used to screen alcohol consumption in patients prior to and after liver transplantation. However, CDT elevation is only highly specific of long-term heavy alcohol consumption (alcohol intake for more than 14 days and more than 60 g ethanol/day). False positive or negative results occur in patients with severe liver diseases or genetic variability in the serum transferrin distributions. Moreover, hyperbilirubinemia, which is often observed in patients with liver fibrosis, disturbs immunological and chromatographic assays.

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are direct and specific phase II metabolites of ethyl alcohol. <0.1% of the ingested ethanol amount [1–3] is metabolized by glucuronidation. EtG is recommended to uncover a recent alcohol intake in different clinical and forensic settings [4]. With a detection time up to 24 h after intake of 0.25 g/kg and up to 48 h after intake of 0.50 g/kg, EtG in urine aims to extend the detection time window for recent alcohol consumption [5]. The detection time in blood is considerably shorter [6, 7]. The recommended EtG threshold differs between 0.1 and 0.5 mg/L depending on the forensic or clinical question. To avoid the detection of unintentional intake of ethanol (ethanol vapors, cosmetics, chocolate, juices), a cutoff of 0.5 mg/mL is recommended [8, 9] for clinical purposes, such as liver transplantation. Due to a limited stability of EtG, the parallel analysis of EtS enables the assessment of storage influences and therefore lowers the risk of generating clinical false positive [10] (e.g., due to *Escherichia coli* contamination) and false negative [5, 11] (bacterial degradation) results. This is of interest if samples have to be shipped to the laboratory.

In the present study, the first IVD-certified commercially available test kit for the tandem mass spectrometric analysis of EtG and EtS in urine was verified and compared to an established and fully validated LC-MS/MS method for application in clinical setups.

Materials and methods

The commercially available ClinMass® complete kit (RECIPE Chemicals+Instruments GmbH, Munich, Germany) for EtG and EtS quantification in urine samples was established on an API 6500 triple quadrupole tandem mass spectrometer (AB Sciex, Toronto, Canada) connected with an isocratic pump (LC-20AD, Shimadzu, Duisburg, Germany) and an autosampler (SIL-20AC, Duisburg, Germany). Mobile phase and HPLC column were included in the ClinMass® test kit (RECIPE Chemicals+Instruments GmbH, Munich, Germany). Pump parameters were slightly modified. A flow rate of 0.5 mL/min, instead of 0.2 mL/min, was used for isocratic elution which resulted in a total analysis time of 3 min. Sample preparation was performed according to manufacturer's instructions: 50 µL of spontaneous urine sample and 1000 µL of internal standard solution were mixed, vortexed and centrifuged. It is recommended to store urine samples at temperatures of 2–8 °C or lower with stabilities of at least 7 days. At room temperature, abacterial urine can be stored for 4 days. The supernatant was quantitatively transferred into autosampler vials, and 5 µL was injected into the HPLC system. Ionization was achieved in the negative ion mode with an ionization voltage of −4500 V. Further ion source parameters were set as follows: temperature=450 °C, gas 1 (nebulizer gas)=70 psi and gas 2 (heater gas)=50 psi. The following quantifier and qualifier transitions were monitored for EtG and EtS and their corresponding isotope-labeled internal standards: EtG *m/z* 221.0/75.2 and 221.0/84.8, EtG-$^2H_5$ *m/z* 226.0/75.2 and 226.0/84.8, EtS *m/z* 124.8/96.8 and 124.8/79.8, EtS-$^2H_5$ *m/z* 129.9/97.8 and 129.9/79.8, respectively. The second quadrupole (collision cell) was used with collision energies between 22 and 40 V and collision cell exit potentials between −5 and −11 V. For urinary EtG and EtS quantification, a six-point calibration curve (calibration range: EtG 0.1–9.9 mg/L, EtS 0.02–1.9 mg/L; ClinCal® Calibrators Set, RECIPE Chemicals+Instruments GmbH, Munich, Germany, MS8713) was applied.

Evaluation experiments included the determination of the limits of detection (signal/noise ratio=3) and lower limits of quantification (signal/noise ratio=6). Within- and between-days imprecision as well as accuracy was based on results from commercial quality control materials (ClinChek® Controls, three urine levels, RECIPE Chemicals+Instruments GmbH, Munich, Germany) analyzed 10 times during 1 day and on five consecutive working days. Furthermore, four external proficiency testing samples (Society for Toxicologic and Forensic Chemistry (GTFCh), Heidelberg, Germany) were analyzed to assess accuracy of the commercial assay. Method comparison was performed with residuals of 50 urine patient samples (ethical

approval Leipzig 082-10-190-42010) which had been externally analyzed with a DIN/EN/ISO 17025 accredited LC-MS/MS method [12]. Differences between urinary EtG and EtS concentrations determined by the commercial test kit and the established LC-MS/MS assay were identified via Bland-Altman Plots and Bablok Passing regression.

Results

The chromatographic separation of EtG and EtS in a patient's urine sample with a total run time of 3 min is presented in Figure 1. Retention time of EtG and EtS are 0.8 min and 2.4 min, respectively. Limit of detection (LOD) was found to be 0.03 mg/L for EtG and 0.01 mg/L for EtS in urine. The lower limit of quantification (LLOQ) of the commercial assay was found to be 0.07 mg/L for EtG and 0.03 mg/L for EtS. Calibration curves for EtG in urine ranged from 0.1 to 9.9 mg/L and for EtS from 0.02 to 1.91 mg/L (r>0.99 for both analytes). Method imprecision was investigated for each analyte. The within- and between-days imprecision are presented in Table 1.

Accuracy of the commercial assay in urine ranged between 96 and 133% for EtG and 97 and 121% for EtS for the commercial controls. In Table 2, results from external quality proficiency testing samples (GTFCh, Heidelberg, Germany) are presented. Generally, accuracy was found to be between 101 and 144% for both analytes in urine.

The commercial assay was compared to an established and validated LC-MS/MS assay [12] by measuring 50 residual urine samples for each analyte. Pearson coefficients of correlation were 0.96 for EtG (concentration range 0.19–8.34 mg/L) and 0.97 for EtS (concentration range 0.02–4.06 mg/L). In Figure 2, absolute method differences are presented by Bland-Altman Plots. The absolute and relative differences between the commercial and the validated LC-MS/MS method were −0.7 mg/L (−23%) for EtG and −0.3 ng/mL (−33%) for EtS. Bablok Passing regression between results from patient samples obtained by the commercial and the established LC-MS/MS assay showed the following slopes (95% confidence interval) and intercepts (95% confidence interval): 1.29 (1.17–1.45)

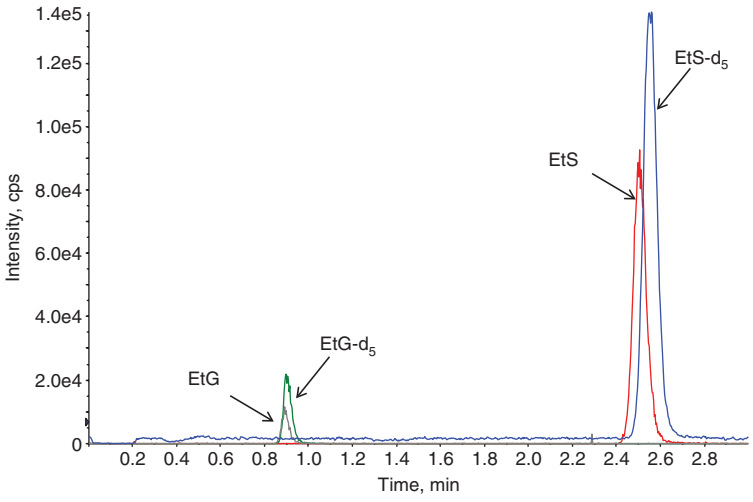

Figure 1: LC-MS/MS chromatogram of EtG and EtS in urine.

Table 1: Within- and between-day variability and accuracy in urine quality control samples.

Analyte	Within-day CV (n=10)		Between-day CV (n=5)		Accuracy, %
	Mean, mg/L	CV, %	Mean, mg/L	CV, %	Mean (range)
EtG	0.11	7.2	0.12	15.4	117 (86–133)
	1.98	5.4	2.11	4.3	105 (96–106)
EtS	0.05	6.0	0.05	8.4	113 (97–121)
	0.77	4.8	0.81	4.8	103 (95–108)

CV, coefficient of variation.

Table 2: Results from external quality proficiency testing (GTFCh, Heidelberg, Germany).

Sample	EtG				EtS			
	Measured conc., mg/L	Mean conc. (LC-MS/MS), mg/L	Target conc., mg/L	Accuracy, %	Measured conc., mg/L	Mean conc. (LC-MS/MS), mg/L	Target conc., mg/L	Accuracy, %
1	0.75	0.68	0.60	125.0	1.59	1.26	1.10	144.5
2	1.50	1.38	1.35	111.1	0.73	0.70	0.72	101.4

Conc., concentration. Target concentration=mean of submitted concentrations received from all participants applying either LC-MS, LC-MS/MS, GC-MS or immunoassays (without outliers).

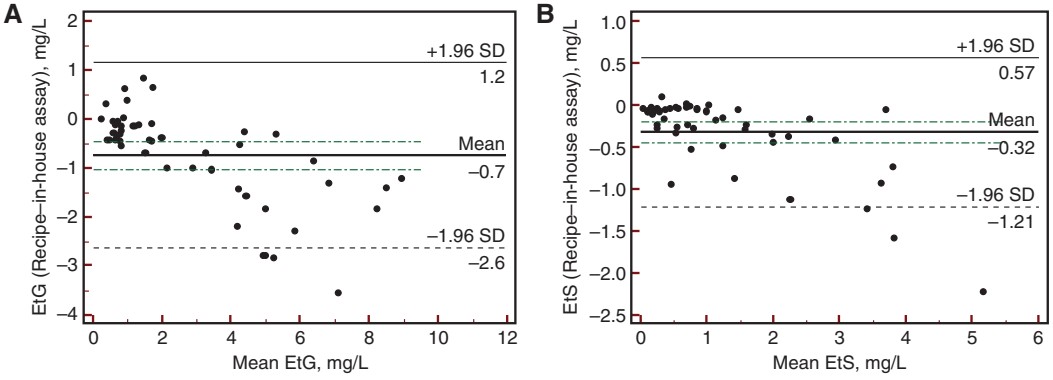

Figure 2: Bland-Altman-Plots for urinary concentrations of EtG and EtS analyzed by the commercial assay and the validated LC-MS/MS method.

and −0.06 (−0.20 to 0.19) for EtG and 1.19 (1.09–1.30) and 0.02 (0.02–0.08) for EtS.

Discussion

In this study, we used a commercially available tandem mass spectrometric assay for the quantification of EtG and EtS in urine. The manual sample pretreatment procedure was easy to handle and is comparable to the established LC-MS/MS method. An enhancement of flow rate by optimization of source temperature of the API 6500 QTrap resulted in a reduction of run time from 5 min to 3 min, which significantly improves the throughput of the analytical platform.

The determined LODs and LLOQs of 0.03 mg/L and 0.07 mg/L for EtG and 0.01 mg/L and 0.03 mg/L for EtS, respectively, of the commercial assay were comparable to those stated in the manual. The within- and between-days imprecision of the commercial assay were in the acceptable range of 5–15% for EtG and 5–8% for EtS. Mean inaccuracy of commercial controls was found to be below 17%. However, the analysis of two urine samples from an external proficiency testing program showed accuracies between +1 and +44% for EtG and EtS concentrations. The target concentration values represent the mean of submitted concentrations received from all participants applying either LC-MS, LC-MS/MS, GC-MS or immunoassays. The comparison of the submitted analyte concentration determined with this assay compared to results retrieved by LC-MS/MS methods revealed a slightly better accuracy, which lay between –11% and +26%, which indicates that with the application of LC-MS/MS methodology, higher concentrations of EtG and EtS are determined. The established LC-MS/MS method even quantifies higher EtG and EtS concentrations compared to the commercial assay. However, the Bland-Altman plot shows a very good agreement in the concentration range until 2 mg/L EtG or EtS. Method differences in higher concentration ranges may be caused by different calibrator sets. Nevertheless, method comparison revealed a very good correlation between both LC-MS/MS methods for EtG and EtS quantification.

Besides quantification of EtG, the normalization of urinary EtG concentrations to creatinine levels to compensate for urine dilution, either intentional or unintentional, will be elucidated in further studies.

Conclusions

The commercial LC-MS/MS testkit is a valuable certified assay for analysis of EtG in urine samples to assess recent alcohol consumption.

Acknowledgments: We acknowledge RECIPE Chemicals+Instruments GmbH (Munich, Germany) for granting the assay reagents and control material.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: This publication is supported by LIFE – Leipzig Research Center for Civilization Diseases, Universität Leipzig. LIFE is funded by means of the European Union, by the European Regional Development Fund (ERDF) and by means of the Free State of Saxony within the framework of the excellence initiative.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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