Preliminary Immunochemical Test for the Determination of Ethyl Glucuronide in Serum and Urine: Comparison of Screening Method Results with Gas Chromatography–Mass Spectrometry

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Abstract
Ethyl glucuronide is a highly specific metabolite of ethanol that is formed by enzymatic conjugation of ethanol with glucuronic acid. Because of its suitability as a marker of excessive alcohol consumption in serum with low blood-alcohol concentration and as a consumption marker in serum and urine, especially after the breakdown of ethanol, demand exists for a simple and fast analytical procedure, which is rarely possible using mass spectrometric determination methods. For this reason, we developed an immunochemical screening procedure (ELISA) in which polyclonal antibodies are bound to the walls of microtiter plates. To test suitability, 335 authentic serum and 186 urine samples were examined using immunochemistry and gas chromatography–mass spectrometry (GC–MS). The serum (urine) samples with cutoff values of 0.31 mg/L (1.33 mg/L) yielded false-negative results in 9.5% (24.3%) and false positives in 8.4% (23.2%) of cases. Specificity was calculated at 91.6% (76.8%) and sensitivity at 90.5% (75.7%). Test efficiency was 90.8% (76.3%). The study shows that ethyl glucuronide and therefore alcohol consumption can be detected in immunochemical screening of serum in a similar manner as current drugs, but the method is of limited value for urine. A GC–MS confirmation continues to remain a necessity.

Introduction
Ethyl glucuronide (EtG) is a phase II metabolite of ethanol that is only formed as a consequence of ethanol consumption. Approximately 0.5–1.6% of the quantity of alcohol consumed is conjugated with activated glucuronic acid (1). The formation of EtG has been shown to depend on the serum-ethanol concentration. EtG peaks 2 to 3.5 h later in blood than ethanol, and subsequently its concentration decreases exponentially (2). The half-life of EtG ranges from 2 to 3 h (3). EtG was reported to be detectable in urine up to five days after heavy alcohol consumption. Therefore, EtG can be regarded as a marker of excessive alcohol consumption and relapse (4–8). Furthermore, EtG can be detected in human hair. A positive result is always associated with alcohol consumption (9).

Besides gas chromatography–mass spectrometry (GC–MS) (2,3) and liquid chromatography (LC–MS) (10) methods, demand exists for an easy-to-perform preliminary test with high sample throughput for rapid diagnosis analogous to immunochemical screening of drugs (11), for example, in clinical diagnosis of alcoholism relapse or evaluation of fitness to drive. In cooperation with BioGenes GmbH Berlin, we developed an ELISA in the form of a microtiter assay, which has already been reported after immunization of rabbits with EtG conjugate (12).

The objective of our research was a practical evaluation of the EtG enzyme immunoassay with a larger sample number. Of interest were the determination of diagnostic test parameters such as sensitivity, specificity, and test efficiency as well as the determination of cutoff value dependence on sample material and use.

Materials and Methods

Sample series
The samples were obtained in drinking tests and regional hospitals. In the study, only one sample of a matrix was determined for each test subject or patient. In all, 186 authentic urine and 335 serum samples were collected and analyzed. The study plan was presented to and approved by the Ethics Committee of the Faculty of Medicine of the University of Heidelberg (Application No.: 130/94).
Immunochemical examination using EtG ELISA

The antigen that was used for immunization was EtG directly linked to a carrier molecule. The carrier molecule was the enzyme peroxidase linked to the functional group of EtG (-COOH, carboxyl group at Cs). For the production of polyclonal antibodies, rabbits were immunized with this antigen. The antibody is directed towards the structure of EtG. The reproducibility of the antibodies between different batches is high because seven rabbits were immunized at the beginning, but only the pooled blood of one of the rabbits was used to coat the microtiter plates. Furthermore, the antibodies were purified by affinity chromatography.

One-hundred microliters of urine sample (1:20) and serum (1:5) diluted with sample dilution buffer were placed into the wells of the microtiter plates, and 50 μL of the enzyme conjugate working solution, consisting of enzyme conjugate solution and conjugate dilution buffer in a ratio of 1:150 for urine and 1:80 for serum, was added. After closing the wells with adhesive foil, the plate was shaken by the Vortemp 56 EVC (by National Labnet, Edison, NJ) for 30 min at 200 rpm/min and 20°C. Then the content of the wells was emptied with Nunc-Immuno™ Wash 8 (Rochester, NY) and rinsed four times with twice-distilled water. Subsequently, 150 μL dye solution (3,3',5,5'-tetramethylbenzidine) was added, and the wells were again closed with adhesive foil and incubated in the dark for 30 min. After the addition of 50 μL stopping reagent (1N hydrochloric acid), measurement of extinction was carried out at a wavelength of 450 nm (Dynex, MRX microplate recorder, St. Paul, MN). Extinction is inversely proportional to EtG concentration. Each sample was assayed in duplicate. Automated analysis using Revelation software from Dynex Technologies and analysis software written in-house was performed after data transmission from the photometer to the PC over an online link. Calibration was carried out with standard EtG solutions of 0, 0.01, 0.03, 0.1, 0.3, 1, and 100 mg/L urine or serum.

Establishing EtG/ELISA calibration curves

Raw data. Optical density was plotted against concentration as calibration points for EtG in serum and urine.

Software. The calibration curve was described with a logistic regression using software written in-house.

Determination of EtG with the internal standard

tert-butyl glucuronide using GC–MS

The former GC–MS method was performed without internal standard. We describe the synthesis of tert-butyl glucuronide and the modified GC–MS method using the internal standard.

Synthesis of tert-butyl glucuronide

Triacetyl-α-D-6-bromogluicosiduronic acid methyl ester (BGAME) was used as the base for the synthesis of tert-butyl glucuronide. tert-Butyl glucuronide was produced by deacetylation of tert-butyl-β-D-6-triacetyl-glucosiduronic acid methyl ester (t-BuGAME). This synthesis was generally equivalent to that of EtG (2). The conversion of the two synthesis steps is represented in Figure 1.

t-BuGAME

In a two-necked flask with reflux condenser and magnetic stirring, 1 g (2.5 mmol) BGAME was dissolved in 20 mL absolute benzene under exclusion of light. Five milliliters of 52.8 mM tert-butanol was added to this mixture. Subsequently, the reaction mixture was heated to boiling, and within 3 h 1.5 g silver carbonate was added and the mixture stirred overnight at room temperature. The next morning, the liquid was centrifuged off the deposited bromide precipitate. Evaporating the solvent in a water jet pump vacuum resulted in a faintly yellow, pasty residue from which colorless crystals were obtained after recrystallization with ethanol.

The yield was 0.34 g product (35%). The product is freely soluble in methanol and chloroform and obviously insoluble in ethanol.


t-BuG

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late in absolute methanol was added. The reaction mixture turned a faint yellow color and was stirred overnight. Evaporating the solvent in a water jet pump vacuum provided a yellow residue that was suspended in a barium hydroxide solution (0.03%, 5 mL). After 1 h the precipitate was removed by centrifugation. The pH value of the faintly yellow alkaline centrifugate was adjusted to 6 by slowly dripping in an oxalic acid solution (9%). After centrifugation, the solution was evaporated to dryness under vacuum. The residue was dissolved in a few drops of methanol and shaken without heating. Subsequently, diethyl ether was added in drops with a Pasteur pipette, whereupon a pure white product precipitated. The white residue was broken up with a spatula and allowed to settle. After decanting and filtering, the residue was completely dried in an ultrahigh vacuum at room temperature.

The yield was 0.2 g (92%) colorless powder with a melting point of 159°C (decomposition) that was readily soluble in water and methanol and obviously insoluble in chloroform, diethyl ether, and ethanol.

Characterization and determination of purity

To secure the identity of the product a microanalysis was conducted. In addition, nuclear magnetic resonance (NMR) spectra and an infrared (IR) spectrum of the solid (KBr pressed disk) were recorded.

Elementary analysis

The elementary analysis showed a purity of ≥ 98.9%.

NMR spectroscopy

NMR spectroscopy details were as follows: 1H NMR (BRUKER: DRX 500 MHz, CD3OD, HDO suppression, TMS as internal standard): δ ppmI = 4.45 (d, J1,2 = 8 Hz, 1H), 3.66 (m, J5,4 = 7 Hz, 1H), 3.56 (d, J4,3 = 9 Hz, 1H), 3.42 (d, J3,4 = 9 Hz, 1H), 3.16 (t, J2,1 = 8 Hz, 1H), 1.27 (s, 9H).

13C NMR (BRUKER: DRX 500 MHz, CD3OD, TMS as internal standard): δ ppmI = 98.8 (C-1), 77.9 (C-5), 77.0 (C-3), 76.6 (C-CH3), 75.0 (C-2), 73.9 (C-4), 29.0 (3 CH3).

IR spectrum

IR details were as follows: IR (KBr pressed disk, IR spectrometer IPS 66; Bruker, Forchheim; wave number range: 400–4400 cm⁻¹ measured with 32 scans): wave number [cm⁻¹] = 3600–3200: (O-H) valence vibrations; 2980, 2920: (C-H) valence vibrations of the C-H and CH3 groups; 1730: (C=O) valence vibrations; 1400–1470: (C-H) deformation vibrations of the C-H and CH3 groups; 1370: (O-H) deformation vibration of the hydroxy groups; 1270, 1190: (C-O) valence vibration of the hexose tert-butyl ether grouping.

Sample preparation for EtG determination in serum and urine using GC-MS

Serum or urine (200 µL) was mixed with 50 µL of a 0.1 mg tert-butyl glucuronide per milliliter of methanol solution and 950 µL methanol in a 1.6-ml centrifuge vessel, shaken, and kept overnight at 4°C. Precipitated protein was centrifuged off at 14,000 rpm/min in 20 min, and 900 µL of the supernatant was evaporated under a gentle stream of nitrogen at 40°C. The dry residue was derivatized with 100 µL acetic anhydride and 25 µL pyridine for 30 min at 60°C. After evaporation to dryness in a nitrogen stream at 40°C, the residue was dissolved in 250 µL chloroform, shaken for 10 min, and transferred through a membrane filter (Spartan 13/30, Schleicher & Schuell, Dassel, Germany) into a GC vial microinsert. One microliter of the clear solution was examined by GC–MS.

Analysis instrument and parameters

A Hewlett-Packard GC–MSD (Hewlett Packard) model 6890 was used. The injector temperature was 250°C; transfer line temperature was 290°C.

The quartz capillary column was a 12.5-m × 0.25-mm i.d. CP-SIL 5 (Chrompack, Müllheim, Germany). The flow rate was 1.2 mL/min

The temperature program was 60°C held for 1 min, increased to 320°C at 20°C/min, and held 1 min.

The ions used for identification and quantitation (underlined) of EtG and tert-butyl glucuronide (internal standard) in a calibration curve for serum. Calibrators in milligrams EtG per liter serum against optical density.

![Figure 2. Calibration curve for serum. Calibrators in milligrams EtG per liter serum against optical density.](image)

![Figure 3. Calibration curve for urine. Calibrators in milligrams EtG per liter urine against optical density.](image)

| Table I. Intra-assay Variation Coefficients in ELISA for Serum and Urine |
|-------------------|-------------------|-------------------|
| EtG (mg/L) | EtG in serum (%) | EtG in urine (%) |
| 0.3 | 10.9 | 11.5 |
| 1 | 12.8 | 16.8 |
| 3 | 15.2 | 19.3 |
Initially, the immunoassay was validated with respect to the intra- and inter-assay variation coefficients and recovery. To determine the intra-assay variation coefficients, three concentrations were tested in a 10-fold batch using ELISA. The results are given in Table I. To determine the interassay variation coefficient for EtG in serum and urine, a concentration of 0.5 mg/L was determined five times in an eightfold batch using ELISA. Interassay variation coefficients of 10.5% for serum and of 11.9% for urine were obtained.

The calibration curve was linear in the range from 0.25 to 50 mg EtG/L serum and from 0.25 to 500 mg EtG/L urine. Recovery was 70%. The detection limit (probability of a false-positive result: α-error = 1%) was 0.1 mg/L in serum and urine (13). The quantitation limit (probability of a false-positive result: α-error = 1%) was 0.25 mg/L in serum and urine (13). The intra-assay variation coefficient was 8.5% (concentration: 3 mg/L), and the interassay variation coefficient was 9.5% (concentration: 3 mg/L).

Results

To examine recovery, spiked serum and urine samples were prepared in an at least threefold batch and examined at least twice with ELISA. The added concentrations lay between 0.5 and 1.3 mg/L for serum and in the range from 0.5 to 41.5 mg/L for urine. Recovery rates of 89–100% for serum and 40.8–100% for urine were achieved. The calibration curves for serum and urine are represented in Figures 2 and 3. An analytical description of the course of the curve succeeded with a logistic regression model (R = 0.99).

The cutoff values of 0.31 mg/L for serum and 1.33 mg/L for urine employed in this study resulted from ROC plots (Figures 4 and 5). The areas under the ROC function were 0.96 for serum and 0.85 for urine. With specificities of 91.6% (serum) and 76.8% (urine), sensitivities of 90.5% (serum) and 75.7% (urine), and test efficiencies of 90.8% (serum) and 76.3% (urine), false-negative results were obtained in 9.5% of serum cases and 24.3% of urine cases (Tables II and III).

For comparison of ELISA to GC–MS, the positive and negative serum and urine results are presented in decision tables (Tables II and III).

The correlation coefficients calculated between the GC–MS and ELISA procedures resulted in values of R = 0.93 for serum (n = 335) and R = 0.74 for urine (n = 186).

Discussion

The results of the EtG immunoassays were compared to those of an independent analysis method (GC–MS).

In the case of the sigmoid calibration curve (Figures 2 and 3), the coefficients of variation increase with increasing concentration. There are five calibration levels between 0 mg/L and 1 mg/L, but no calibrant between 1 mg/L and 100 mg/L. With our calibration samples, there are only small errors near the cutoff limits, where the yes/no decision is made. The semi-

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<th>Table II. Comparison of ELISA to GC–MS Results for Serum*</th>
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* n = 335; cutoff = 0.31 mg/L.

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<th>Table III. Comparison of ELISA to GC–MS Results for Urine*</th>
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* n = 186; cutoff = 1.33 mg/L.
quantitative result is not so important because every positive result is confirmed by GC–MS.

The reason why the recovery rates of the urine samples ranged from 40.8 to 100% is not yet known. Different substances may interfere in urine than in serum. We have different matrix effects, other pH values, and other enzymes present, so the situation is quite different.

The ROC plots, the cutoff values, and the area under the ROC function indicate that the assay is suitable for serum but not for urine.

Cross-reactions occurring in native samples led to false-positive results in 6 serum and 26 urine cases, which is equivalent to a share of 8.5% and 23.2%, respectively, with respect to negative GC–MS cases. Interference from cross-reacting substances had a particularly negative effect in urine. In an earlier study (12), the following cross-reactivities were identified: methyl glucuronide (6%), propyl glucuronide (2.6%), butyl glucuronide (0.4%), glucuronic acid (0.02%), and 2-glucose (0.0006%).

Interference from the glucuronides of isobutanol, 2-methyl, and 3-methyl butanol-1 may be classified as minor because of decreasing cross-reactivity in the homologous series (< 1%) (11). Effects due to free glucuronic acid are not anticipated because it is almost exclusively present as UDP glucuronic acid (14). Interference from pathological glucose concentrations in serum and urine could not be detected in the false-positive cases.

Despite much higher urine sample dilution (1:20 instead of 1:5) in comparison with serum, cross-reacting substances preferentially interfere with this matrix because higher concentrations of the body's and possibly endogenous glucuronides, whose effect could not be examined so far, were present. Appropriate steps must still be examined in this case with an immunochromatographic search for cross-reacting agents but they will probably prevent a simple performance of the test without sample preparation.

False-negative results were found in 25 serum and 18 urine cases, which is equivalent to a share of 9.5% and 24.3%, respectively, relative to all positive GC–MS cases. The cause for this was a 3- to 13-fold lower detection limit of the GC–MS method for serum and urine compared with the immunochromatographic cutoff. We expect that in these cases EtG was not bound to immobilized antibodies but to other structures in the biological sample and therefore escaped detection (11). This portion is obviously not always detected in the GC–MS method and explains a recovery rate of 70% after protein precipitation.

Diagnostic test parameters such as sensitivity, specificity, and test efficiency are dependent on the selected cutoff value. For purposes of optimization, diagnostic parameters for various cutoff values were calculated. The optimum was located at the point at which the tangent touches the ROC curve. This was used as a criterion for determining the cutoff values (Figures 4 and 5).

Compared with the pilot study, a lower cutoff value for serum and a higher value for urine resulted (12).

All diagnostic parameters are dependent on the examined sample material. When only blanks are examined, a better specificity results for the same decision limit. By contrast, better sensitivity results with the same cutoff value if samples with a high analyte content are measured.

The occurrence of this tendency becomes visible in a comparison of the results of the pilot study (12) to those of a larger sample number; using a higher percentage of blanks than in the pilot study led to a larger number of false-positive results with a similar cutoff value. Therefore, it was important to measure as many samples as possible with concentrations near the decision limit. Our stock of samples consisted of blanks and samples with low and high EtG content. A multitude of samples was found near the cutoff value.

Comparison of ELISA measurements to GC–MS shows that the immunoassay is a suitable preliminary test for the EtG determination in serum. In practical application, sensitivity may be further raised by lowering the cutoff value. When using the value of 1 mg/L urine suggested in the pilot study, sensitivity rises to 91% and specificity drops to 68%.

Because more cross-reactions to structurally similar substances may be expected in polyclonal tests, the development of a test with monoclonal antibodies should be advantageous (15). Production of monoclonal antibodies is in progress (16).

Conclusions

Currently, the immunoassay provides semiquantitative results and is suitable as a preliminary test for the preselection of sera. ELISA is only suitable to a limited extent for urine examination, and higher test specificity must be achieved to enable employing it in a more reliable manner.

Positive results must be checked with the confirmation procedure of choice, which is gas chromatography–mass spectrometry.

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References


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