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Fresenius J Anal Chem (2001) 369 : 273–279 © Springer-Verlag 2001

SPECIAL ISSUE PAPER

Norbert Staimer · Shirley J. Gee · Bruce D. Hammock

Development of a sensitive enzyme immunoassay for the detection of phenyl-β-D-thioglucuronide in human urine

Received: 22 August 2000 / Revised: 7 November 2000 / Accepted: 8 November 2000

Abstract Immunoassays for the measurement of glucuronides in human urine can be a helpful tool for the assessment of human exposure to toxic chemicals. Therefore an enzyme immunoassay (EIA) for the specific detection of phenyl-β-D-thioglucuronide was developed. The immunoconjugate was formed by coupling *p*-aminophenyl-β-D-thioglucuronide to the carrier protein thyroglobulin leaving an exposed glucuronic acid. The hapten-protein conjugate was adsorbed to gold colloids in order to enhance the immunogenic effect. Rabbits were injected with the immunogold conjugates to raise polyclonal antibodies. The resulting competitive assay showed an inhibition by phenyl-β-D-thioglucuronide at sample concentrations of 23.0 ± 1.3 ng/mL (50% B/B_0) and a high cross-reactivity to *p*-aminophenyl-β-D-thioglucuronide (120%). Little cross-reactivities (< 2%) were observed for potential urinary cross reactants. In addition human urine samples were incubated with β-glucuronidase in order to investigate the EIA for specific matrix effects. An integration of high-performance liquid chromatography (HPLC) and EIA was developed in an attempt to decrease the matrix effects and increase the sensitivity of the overall method. The hyphenated technique HPLC-EIA may be used to monitor human exposure to toxic thiophenol which is excreted by mammals as urinary phenyl thioglucuronide.

Introduction

To protect the environment and public health, monitoring, evaluation and enforcement are areas that require strong analytical support. Thus rapid, inexpensive, and reliable techniques are essential for the routine assessment of human and environmental exposure to hazardous chemicals. These demands can be fulfilled by immunochemical methods which are increasingly used in environmental and toxicological fields [1]. Immunoassays have proved to be suitable for the rapid and inexpensive detection of urinary biomarkers such as mercapturates as indicators of exposure [2–6]. Besides the formation of mercapturates, glucuronidation is a major phase II detoxication pathway. A superfamily of UDP-glucuronyltransferases (UGTs) catalyzes the biotransformation of numerous endobiotic and xenobiotic compounds, including drugs, pesticides or carcinogens [7–10]. The enzymatically synthesized metabolites are water soluble, excreted as glucuronic acid conjugates in human fluids and hence, suitable as urinary biomarkers of exposure.

Thiophenol is a high production volume (HPV) U.S. chemical manufactured in or imported into the United States in amounts over 500,000 kg per year . It is on the regulatory list of extremely hazardous substances and on the HPV challenge program chemical list [11, 12]. The exposure to thiophenol may cause severe negative health effects and is expected to be excreted in urine as its thiophenolic glucuronic acid conjugate [13–16]. It was identified as a principal contributer to flavor-tainting found in fish and causes conversion of oxyhemoglobin to methemoglobin in human red blood cells [17, 18]. Human exposure to thiophenol has the potential to occur at sites of its manufacture as a pesticide and pharmaceutical intermediate by dermal contact or inhalation. It has been found in effluents from petroleum refineries and pulp mills [19]. Further, thiophenol is permitted for direct addition to food as a flavoring agent [20].

Conventional analytical methods for glucuronides in urine rely on time-consuming multi-step sample clean-up procedures, including hydrolysis and extraction. Some methods require analyte derivation for subsequent analysis by GC or GC/MS [21–23]. Therefore, a sensitive immunoassay for phenyl-β-D-thioglucuronide can be applied for the monitoring of human exposure to thiophenol using the urinary phenolic conjugate as a biomarker.

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Further, immunoassays are excellent post column detector systems following separation by HPLC. The combined technique improves selectivity, sensitivity and confidence as well in the identification of the relevant compounds [24–26]. The prior separation of certain phenolic glucuronides by HPLC and the subsequent quantification by immunological methods can be useful in clinical and forensic toxicology where drugs and poisons are usually screened in biological fluids. Thus the development of an immunoassay and the potential of an integrated HPLC-EIA system for the direct analysis of phenyl-β-D-thioglucuronide in human urine will be described.

Experimental

Chemicals and reagents. Phenyl-β-D-thioglucuronide (ThioPG) was obtained from Molecular Probes, Inc. (Eugene, OR). *p*-Aminophenyl-β-D-thioglucuronide (ThioAPG) as hapten for the immunoconjugate and *o*-aminophenyl-β-D-glucuronide (o-APG) for the covalent coupling to the surface of amine binding 96-well plates, bovine serum albumin (BSA), the carrier protein thyroglobulin (porcine), Tween 20, 3,3′, 5,5′-tetramethylbenzidine (TMB), horse-radish peroxidase conjugated goat anti-rabbit immunoglobulin (GAR-HRP) and chemicals examined as cross-reactants were obtained from Sigma Chemical Co. (St. Louis, MO). *p*-Aminophenyl-β-D-glucuronide was synthesized as outlined by Staimer et al. [27]. Enzymatic hydrolysis was carried out using β-glucuronidase from *Helix pomatia*, Type H-2 (Sigma Chemical Co., St. Louis, MO). Other chemicals and organic solvents used for the synthesis of the immunoconjugate, for buffers, and for HPLC were purchased from Aldrich Chemical, Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA).

Instruments. The HPLC separations were carried out using a Hewlett Packard pump system (HP 1100 Series) connected to a diode-array detector (Hewlett Packard, HP 1050 Series). The data were collected and integrated by a HP Chem Station for LC, A.06 Revision (Hewlett Packard). An Aquasil C_{18} column, 250 × 4.6 mm I.D., 5 µm was employed for separation (Western, Analytical Products, Inc., Murrieta, CA). The injection volume was 20 µL, the flowrate 0.7 mL/min and detection was effected at 254 nm. The mobile phase consisted of MeOH/H_2O (30 : 70 v/v), 0.5% triethylamine, 2% glacial acetic acid, pH 4.0 (modified from [28]).

Absorbances (A) in amine binding surface 96-well plates (Corning Costar, Cambridge, MA) were measured with a UV_{max} microplate reader (Molecular Devices, Inc., Sunnyvale, CA) in dual wavelength mode (450–650 nm) and in part converted to $\%B/B_0$ values according to the formula: $\%B/B_0 = (A–A_{excess})/(A_{control}–A_{excess}) \times 100$. Inhibition curves were processed with the Soft*max* software package (Molecular Devices, Inc., Sunnyvale, CA) using a four-parameter curve fitting algorithm.

Preparation of the hapten-protein conjugate. Ten mg of ThioAPG were dissolved in 0.5 mL *N*,*N*–dimethylformamide (DMF) and 0.5 mL H_2O. The pH was adjusted to 2.0. The mixture was stirred at 4 °C after the addition of 400 mg sodium nitrite (dissolved in 0.4 mL H_2O). Thirty mg of ammonium sulfamate in 0.3 mL H_2O were added and the mixture was stirred for 20 min (mixture A). Thyroglobulin (100 mg) was dissolved in 10 mL borate buffer (200 mmol boric acid, pH 10.0) (mixture B). Mixture A was added dropwise under stirring and pH control to mixture B and the red colored product was stored for 30 min at 4 °C. The hapten-protein conjugate (Fig. 1) was dialyzed for three days against 10 L of distilled H_2O (with three changes of water) and kept in the freezer at –20 °C (modified from [29, 30]).

Preparation of the gold immunogen. Twenty mL of 60 nm gold colloid solution (Goldmark Biologicals, Phillipsburg, NJ) were diluted with 20 mL H_2O giving a gold particle concentration of circa 10^{10}/mL. The pH value of the gold colloid solution was carefully adjusted to 8.0 with 0.1 M NaOH using a gel filled electrode. ThioAPG-thyroglobulin conjugate (1 mg) and 20 µg of an adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, Sigma Chemical) were diluted in H_2O (1 mL). The mixture of the hapten-protein conjugate and the adjuvant peptide (1 mL) was added rapidly to 20 mL of the gold colloid solution. After 2 min NaCl (0.200 g) was added. The solution with the immunogold conjugate (Fig. 1) was divided in half, each portion (10.5 mL) added to 15 mL Corex glass tubes (Corning Costar) and centrifuged (20 min, 10,000 g) in order to reduce the volume. The supernatants were removed and the pellets resuspended in 1 mL of NaCl solution (0.09%) (modified from [31]).

Immunization. Control sera were collected from two New Zealand white rabbits prior to the initial immunizations. The first intravenous (i.v.) immunization with 1 mL of the gold immunogen solution (= 0.5 mg protein) in 0.09% NaCl was accompanied by a subcutaneous (s.c.) injection with 0.5 mg of the hapten-protein conjugate in 0.5 mL PBS emulsified in Freund's complete adjuvant (1:1 v/v). Additional i.v. and s.c. immunizations using Freund's incomplete adjuvant for the s.c. injections were given. Booster injections were carried out every 3 weeks. Test bleeds were taken 10 days after each injection in order to screen the antisera for their specific antibody titer. The sera were isolated after blood coagulation by centrifugation for 10 min at 4 °C. Final bleeds were used for EIA development.

Enzyme immunoassay procedure. Assays were conducted using two well replicates for each standard concentration or sample. *Coupling:* o-APG was covalently coupled to the surface of amine binding 96-well plates (Corning Costar) precoated with a layer of

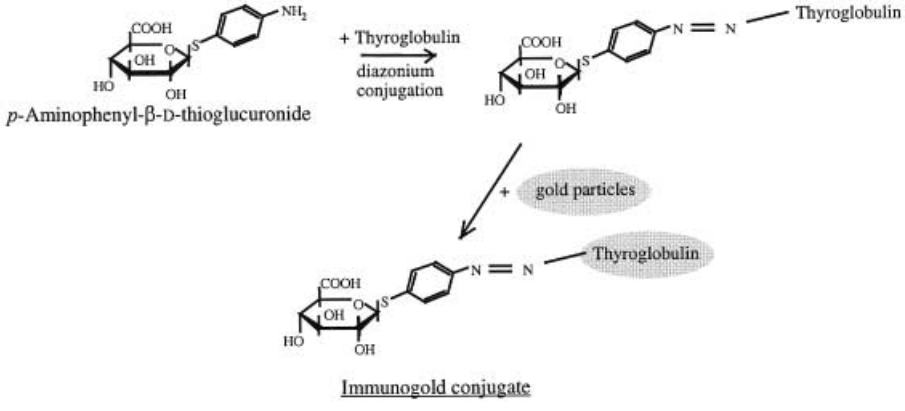

Fig. 1 Synthesis of the immunogold conjugate

reactive N-oxysuccinimide esters. For that purpose o-APG was diluted to a concentration of 0.04 mg/mL carbonate buffer (pH 9.6, consisting of Na_2CO_3, 1.7 g/L and $NaHCO_3$, 2.86 g/L). The o-APG solution (100 µL per well) was added and incubated 1 h at room temperature on a horizontal shaker. *Washing:* 3 times with 300 µL per well of a Tween 20 solution (0.05% in H_2O). *Blocking:* 30 min at room temperature with 300 µL per well of a 2.0% BSA solution in phosphate buffer (PB), pH 7.6, consisting of $NaH_2PO_4 \times H_2O$ (1.38 g/L) and Na_2HPO_4 (9.94 g/L). *Washing:* 2 times as before. *Immunoreaction I:* standard or sample (50 µL per well) was added plus 50 µL of antiserum diluted in PB (1:1,000) and incubated for 60 min at room temperature. *Washing:* 4 times as before. *Immunoreaction II:* 100 µL of GAR-HRP per well in PB (1:5000) was added and incubated for 60 min at room temperature. *Washing:* 4 times as before. *Enzyme reaction:* 100 µL substrate buffer ($NaH_2PO_4 \times H_2O$, 18.2 g/L; Na_2HPO_4, 0.99 g/L; urea hydrogen peroxide, 282 mg/L) and 50 µL chromogen (500 µL phosphoric acid, 98%, in 1 L distilled water plus 288 mg TMB, dissolved in 100 mL dimethylsulfoxide, and 12 mg/L penicillin G) were added. The POD reaction was stopped after 10–15 min with 50 µL 2 N H_2SO_4 and the absorption measured with the microplate reader.

Assay optimization. Five different sugar derivatives (*p*-aminophenyl-β-D-thioglucuronide, *o*- and *p*-aminophenyl-β-D-glucuronide, *p*-aminophenyl-β-D-thiogalactopyranoside, and *p*-aminophenyl-β-D-thioglucopyranoside) were tested with two different antisera using a two dimensional titration. Inhibition was measured for those antibody and coupling antigen combinations giving a reasonable absorbance (0.7 – 1.0). The combination giving the highest sensitivity for phenyl-β-D-thioglucuronide (ThioPG) was selected for further assay development.

Cross-reactivity. The selectivity of the optimized assay was characterized by testing six different glucuronides. In addition two structurally related sugar derivatives (*p*-aminophenyl-β-D-thiogalactopyranoside and *p*-aminophenyl-β-D-thioglucopyranoside), glucose, free glucuronic acid, several unconjugated phenols and the main constituents of urine (urea, hippuric acid and creatinine) were tested for cross-reactivities. ThioPG was used as the reference analyte for the calculation of cross-reactivities (% CR) [(concentration of ThioPG at 50% B/B_0 / concentration of the cross-reactant at 50% B/B_0) × 100].

Matrix effects. Solvent: Standard curves of ThioPG were prepared in PB-buffer (pH 7.6) containing 0, 10, and 20% (v/v) methanol to determine the effects of solvent. pH values were adjusted to pH 7.6 with 1 M NaOH if necessary.

Ionic strength: The effects of ionic strength on the quantitation of ThioPG were evaluated by preparing analyte solutions in 80 mM PB, 80 mM PB + 75 mM NaCl, and 80 mM PB + 150 mM NaCl at pH 7.6.

pH values: The tolerance of the assay for different pH values (pH 6.5, 7.6, and 8.5) was tested using ThioPG as target analyte.

Urine: The effects of the urine matrix were evaluated by preparing various concentrations of three different human urine samples (A, B, C) in PB (0.001, 0.01, 0.1, 1, 2 and 5%) and measured by the developed EIA. The urine samples (A, B, C) were collected in the morning from the same person, but on different days. To investigate the EIA for specific matrix effects, diluted urine (1% in PB) was incubated 4 h at 37 °C with β-glucuronidase solution (final concentration: 1000 Fishman units/mL). As a control, diluted urine (1% in PB) was kept 4 h at 37 °C. PB was added to the control sample instead of β-glucuronidase solution. In addition, a mixture of *p*-nitrophenyl-β-D-glucuronide (p-NPG; 100 µg/mL PB) and ThioPG (100 µg/mL PB) was treated with β-glucuronidase (final concentration: 1000 Fishman units/mL) and measured by HPLC in order to test the enzyme for its effectiveness (500 µL aliquots were diluted with 500 µL MeOH and filtered through a 0.45 µm syringe filter. Then, 20 µL of the filtrate was injected into the HPLC). As a control, phosphate buffer (PB) was spiked with a mixture of p-NPG and ThioPG standards (100 µg/L each) and kept 4 h at 37 °C. PB was added to the control sample instead of β-glucuronidase solution.

Integrated HPLC-EIA. Aliquots of human urine (500 µL) were spiked with 5 µg ThioPG standard. MeOH (500 µL) was added, mixed on a vortex, centrifuged at 10,000 g for 10 min and the supernatant filtered through a 0.45 µm syringe filter. Then, 20 µL of the filtrate (0.1 µg standard) was injected into the HPLC system (flow rate: 0.7 mL/min). Fractions (120 µL) were collected every ten seconds in 96 well polystyrene microtitre plates (Dynatech Lab. Inc., Chantilly, Virginia). A "pH- adjustment solution" (120 µL; 31.5 mL PB plus 8. 5 mL 1 M NaOH) was added to each fraction. Aliquots (50 µL) were transferred to the amine binding microtitre plates and measured by the developed EIA as described before. It follows that from the injection onto the HPLC to the EIA detection a dilution of about 24 times was used.

Results and discussion

Hapten design and gold immunogen preparation

Harris et al. recently reported a specific immunoassay for *p*-nitrophenyl-β-D-glucuronide by coupling the glucuronic acid moiety of the compound to the carrier protein[1]. In this paper we used a different strategy in order to ensure the antibody selectivity for the *S*-glucuronic acid conjugate of thiophenol (Fig. 1): ThioAPG was conjugated to thyroglobulin *via* diazo-coupling of the aminophenyl moiety leaving the *S*-glucuronic acid part exposed. Thyroglobulin was chosen as the carrier protein because of its good solubility characteristics in aqueous buffer solutions and its excellent capability of imparting immunogenicity to covalently coupled haptens [32]. The diazonium conjugation used for the synthesis of hapten-carrier immunogen has the advantage of producing red colored products indicating a successful coupling reaction. However, the estimation of the degree of coupling (hapten-density) from the absorption spectrum of the resulting azoproteins was not possible because of the variety of side chains that undergo reaction. The absorbancy at several wavelengths depends on the ratio of each type of side chain that has undergone reaction and can only be determined with suitable model compounds [33].

Gold conjugates are able to improve the immunogenic effect of endogenous compounds such as neurotransmitter [31]. Therefore, glucuronide–thyroglobulin conjugates were adsorbed to gold colloids in order to guarantee a strong antigenic response to the glucuronic acid moiety of the hapten-protein complex.

Screening of the sera and assay optimization

The antisera of two rabbits (#508 and #509) were tested against several aminophenolic carbohydrates covalently coupled *via* the aminogroup to the surface of 96 well mi-

[1] Harris AS, Goodrow MH, Jones AD, Gee SJ, Hammock BD (2000) Chem Res Toxicol (submitted)

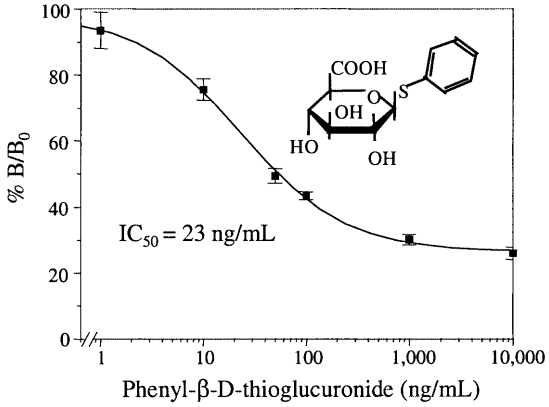

Fig. 2 EIA calibration curve for phenyl-β-D-thioglucuronide using antiserum #508 (dilution: 1:1,000) and *o*-aminophenyl-β-D-glucuronide as coupling antigen (concentration: 0.04 mg/mL). The inhibition curve represents the average of 3 curves with standard deviation (n = 3). Assays were conducted in triplicate for each concentration on separate plates using 2 well replicates per plate. The absorptions are converted to % B/B_0-values. The coefficients of regression were > 0.99 in each case

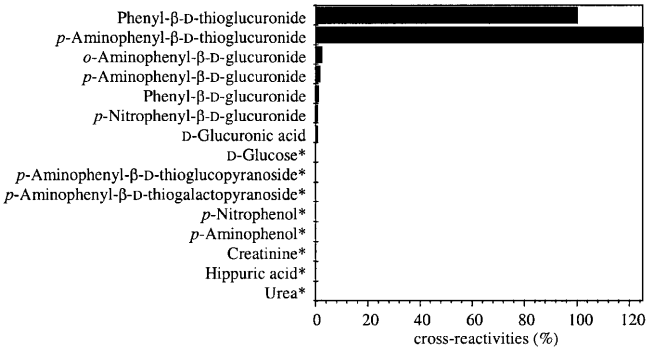

Fig. 3 Cross-reactivity of polyclonal antiserum #508. Cross-reactivities were calculated for each compound as (IC_{50} of phenyl-β-D-thioglucuronide/IC_{50} of cross-reactant) × 100. * no inhibition at 100 µg/mL

crotitre plates as described before. Both antisera exhibited no titers for *p*-aminophenyl-β-D-thiogalactopyranoside and *p*-aminophenyl-β-D-thioglucopyranoside (absorbance < 0.4 at a coupling antigen concentration of 10 mg/mL and antibody dilution of 1:2,000 in each case). The antisera #508 and #509 showed high titers for all immobilized glucuronides (absorbance > 1.0) at an antibody dilution of 1:2000 and coupling antigen concentration of 0.1 mg/mL. Inhibition experiments using a combination of antiserum #508 and o-APG as coupling antigen showed the best sensitivity for the target analyte ThioPG. Therefore, antiserum #508 was used for further assay development. The optimized assay with antiserum #508 (dilution 1:1,000) and immobilized o-APG (concentration 0.04 mg/mL) is highly sensitive for ThioPG (Fig. 2).

Cross-reactivity

The optimized immunoassay does not cross-react with urea, hippuric acid, creatinine, unconjugated phenols, and glucose (no inhibition at 100 µg/mL) and showed only a weak inhibition by the free glucuronic acid. No cross-reactivities were observed for *p*-aminophenyl-β-D-thiogalactopyranoside and *p*-aminophenyl-β-D-thioglucopyranoside indicating that both the carboxy-group and the position of the hydroxy group of the glucuronic acid are crucial for the recognition of ThioPG by the developed immunoassay (Fig. 3). These results are in accordance with the pioneering work of Landsteiner and Goebel. Landsteiner has pointed out the important role played by acid groups in determining the specifity of certain azoproteins [34]. Chemo-immunological studies on conjugated carbohydrate-proteins reported by Goebel have emphasized the fact that the spatial relationship of the less polar hydroxyl groups suffices to determine the serological specificity of carbohydrates [29]. Further, the sulfur atom to which the glucuronyl moiety is attached is important as well, since the tested *O*-glucuronides were hardly recognized by the antibodies generated in this study (Fig. 3). A recently developed EIA for phenolic *O*-glucuronides showed almost no cross-reactivities for both methyl- and ethyl *O*-glucuronides [27]. Because an analogous immunoconjugate design was used for the EIA described in this paper, cross-reactivities of the antiserum #508 with alkyl-thioglucuronides are unlikely. This might be of importance, since there are indications for an endogenous thioglucuronide (methyl-β-D-thioglucuronide) in the urine of normal humans [35]. The high cross-reactivity to *p*-aminophenyl-β-D-thioglucuronide exhibited by antiserum #508 was expected and might also be true for glucuronic acid conjugates of several aromatic sulfur compounds such as *o*-aminothiophenol, *o*- or *p*-chlorothiophenol, *p*-fluorothiophenol, and *p-tert*-butylthiophenol which are all used as intermediates for pharmaceuticals and pesticides. These possible cross-reactivities exclude an exact quantification of ThioPG in urine samples containing unknown glucuronic acid conjugates of thiophenol derivatives, but allow the application of the EIA for the screening of samples for such metabolites in low concentrations. However, prior separation of thiophenolic glucuronides by HPLC and subsequent quantification by the developed EIA can be achieved by analogy with a recently described method for the detection of urinary phenolic *O*-glucuronides [27].

Matrix effects

The evaluation of matrix effects is an important step in immunoassay development, especially if the assay will be applied to crude urine samples typically associated with considerable variations in salt concentration and pH value. In addition the effect of different MeOH concentrations was tested as well, because the developed assay was coupled to HPLC using a methanol containing mobile phase: Figure 4 shows that the assay was tolerant to high MeOH concentrations. However, the assay was sensitive

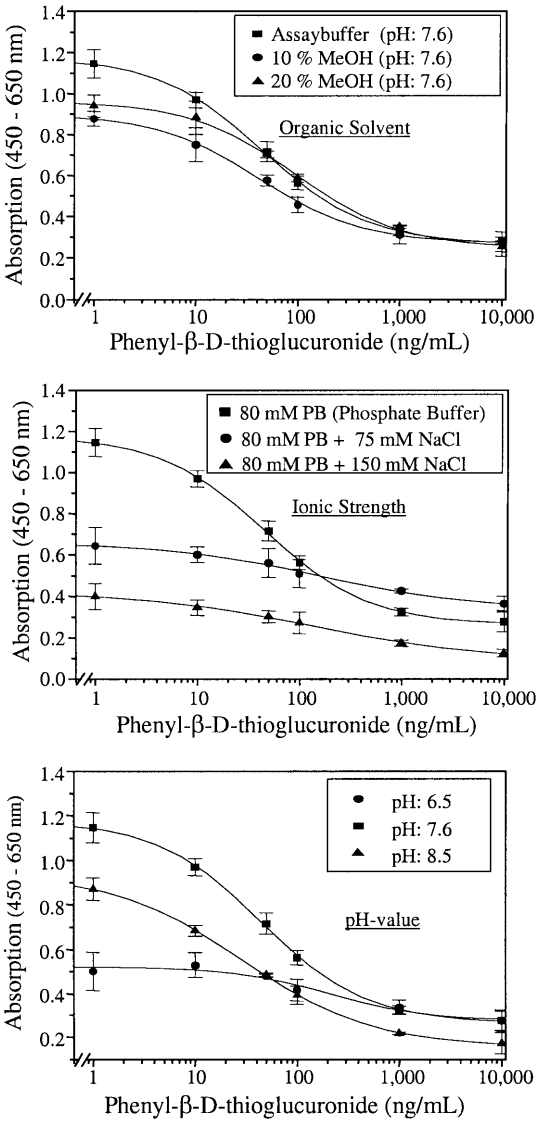

Fig. 4 EIA competition curves of phenyl-β-D-thioglucuronide prepared with various % methanol, ionic strengths, and pH values. All inhibition curves represent the average of 3 curves with standard deviation (n = 3) using antiserum #508 (dilution: 1:1,000) and *o*-aminophenyl-β-D-glucuronide as coupling antigen (concentration: 0.04 mg/mL). Assays were conducted in triplicate for each concentration on separate plates using 2 well replicates per plate. The coefficients of regression were > 0.99 in each case

to NaCl and pH values below 7.6. It is evident that glucuronic acid conjugates of organic compounds exist largely in ionized form because of the low pK_{COOH} of phenolic glucuronides (3.0–3.4) in biological fluids [36]. Thus antibodies generated by a biological process after injecting rabbits with glucuronic acid containing protein conjugates will be targeted predominantly on deprotonated glucuronides.

Internal checks by sample dilution are indispensable to reveal and eliminate matrix effects. Therefore different concentrations of three different (non-spiked) urine samples (A, B and C) were prepared and each dilution was

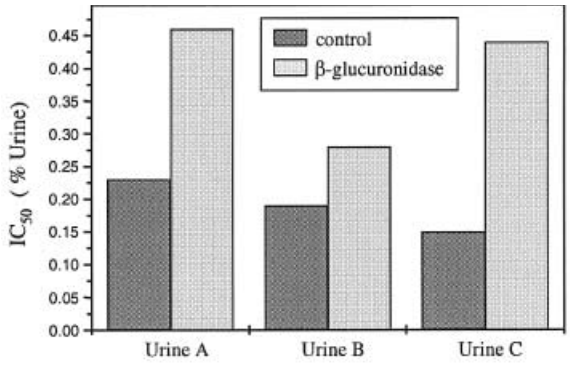

Fig. 5 Effect of urine matrix. Various concentrations of three different human urine samples (A, B, and C) in phosphate buffer were prepared and measured before (control) and after incubation with β-glucuronidase by the developed EIA. The resulting IC_{50} values were calculated by a four-parameter curve fitting algorithm (coefficients of regression: > 0.99). Sample preparation and EIA conditions are described in text

run by the developed immunoassay. The processed inhibition curves with IC_{50} values of 0.23% (sample A), 0.19% (sample B), and 0.15% showed that a substantial dilution of urine (>1:1,000) is required to reduce noticeably matrix effects (Fig. 5). This may be due to cross reacting substances at high concentrations with weak affinities for the applied antibodies or to the presence of competitive inhibitors possessing similar antibody affinities as the target analytes [37]. Most likely the former is the case because the typical normal human level of glucuronides in urine is very high and amounts to 300–450 mg per 24 h [36]. Urine samples A, B, and C were incubated with β-glucuronidase to verify the hypothesis that the revealed matrix effects are caused by cross-reacting glucuronides. First assay buffer (PB) was spiked with a mixture of *p*-nitrophenyl-β-D-glucuronide (p-NPG) and phenyl-β-D-thio-

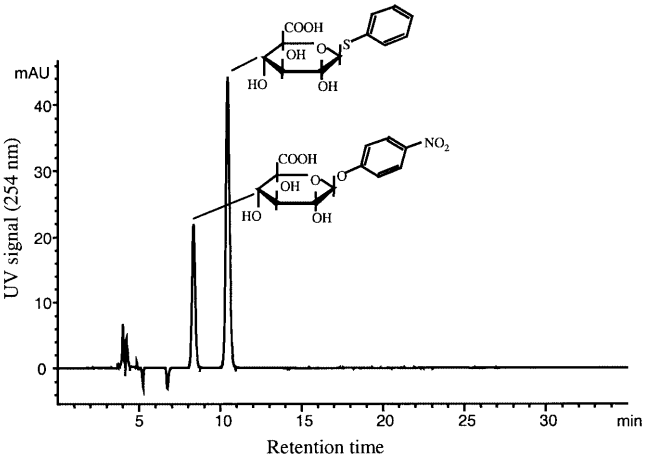

Fig. 6 HPLC chromatogram of glucuronic acid conjugates: *p*-nitrophenyl-β-D-glucuronide and phenyl-β-D-thioglucuronide standards were prepared in MeOH/phosphate buffer (1:1 v/v; 50 µg/mL), kept 4 h at 37 °C and injected into the HPLC. Chromatographic conditions are described in text

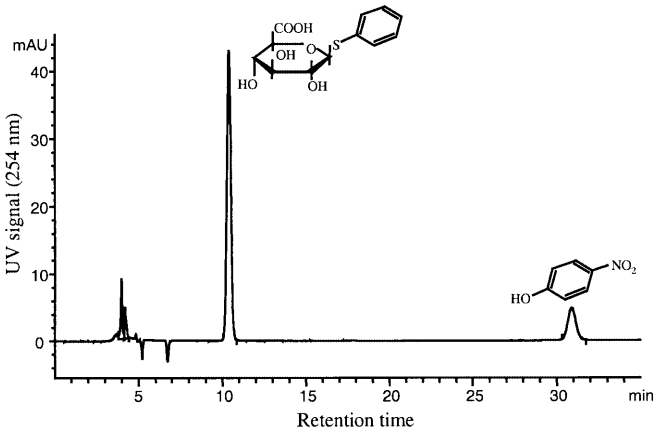

Fig. 7 Enzymatic hydrolysis: *p*-nitrophenyl-β-D-glucuronide and phenyl-β-D-thioglucuronide standards were prepared in MeOH/phosphate buffer (1:1 v/v; 50 μg/mL), incubated 4 h at 37 °C with β-glucuronidase solution (final concentration: 1000 Fishman units/mL) and injected into the HPLC. Chromatographic conditions are described in text

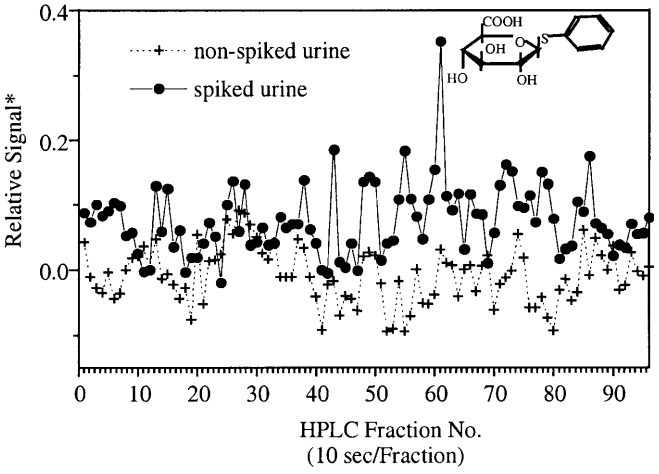

Fig. 8 Immunogram of human urine: non-spiked and spiked with phenyl-β-D-thioglucuronide. Sample preparation and chromatographic conditions as in text. *EIA signal was converted according to: A_{max}–A (A_{max} = maximum absorption, no analyte; A = absorption of the collected fraction)

glucuronide (ThioPG) standards, treated with β-glucuronidase and measured by HPLC in order to evaluate the effectiveness of the enzyme. The HPLC spectra show that the *O*-glucuronide was totally hydrolyzed, whereas the *S*-glucuronide is resistant to β-glucuronidase (Figs. 6, 7). Treatment of non-spiked urine with β-glucuronidase was able to remove the matrix interferences to a certain degree indicating that urinary glucuronides were partly responsible for the observed matrix effects (Fig. 5). In this context it is important to mention that Toennes and Maurer recently developed an improved enzymatic hydrolysis procedure for cleavage of urinary *O*-glucuronides using column-packed β-glucuronidase [38].

Integrated HPLC-EIA

The combined technique HPLC-EIA offers many advantages: The specificity of immunoassays enables the detection of compounds when ultraviolet or other detector systems would fail to recognize a sample peak because of a large background. The immunoassay described in this paper has the advantage that unconjugated phenolic compounds, which also absorb in the UV detector and may overlap chromatographically with their glucuronic acid conjugates in some cases, do not cross-react with the antiserum. Therefore, the EIA increases the selectivity of the overall method. In addition, HPLC can concentrate urine samples as well as remove materials interfering with the immunoassay. A prior HPLC step can improve both sensitivity and confidence in the identification of the resulting compound since it is unlikely that non-specific interferences in an immunoassay would have the same retention time as the target analyte.

Figure 8 shows an immunogram of human urine spiked ThioPG. The immunogram demonstrates that the hyphenated HPLC-EIA method can detect urinary ThioPG after injection of amounts as low as 0.1 μg. It follows that the integrated HPLC-EIA method can quantitate ThioPG at circa 10 μg/mL in whole urine even though the sample is diluted 24 times between injection and detection. The sensitivity of the overall method (compared to the high sensitivity of the developed EIA for ThioPG in assay buffer) is reduced. However, a 10 μg/mL quantification level for thiophenolic acid conjugates is in the range of several biological exposure indices (BEIs) recommended by the American Conference of Governmental Industrial Hygienists for phenolic compounds. The proposed BEI for total phenol (free and conjugated) in urine after benzene exposure is 50 mg/g creatinine which is equivalent to about 50 μg/mL [39].

Conclusion

The immunoassay reported here, used alone or in combination with HPLC, could provide a rapid, cost efficient, and reliable method for the biological monitoring of human exposure to the toxic chemical thiophenol by direct analysis of its urinary glucuronic acid conjugate.

Acknowledgements This project was funded by the National Institute of Environmental Health Sciences (NIEHS) Superfund Research Program (P42 ES04699), the National Institute of Environmental Health Sciences Center (P30 ES05707), and the Environmental Protection Agency Center for Ecological Health Research (CR 819658).

References

1. Hammock BD, Gee SJ (1995) In: Ragsdale NN, Kearney PC, Plimmer JR (eds) Eighth International Congress of Pesticide Chemistry. Options 2000, American Chemical Society, Washington, D.C.

2. Gee SJ, Lucas AD, Hammock BD (1995) In: Curry PB, Iyengar S, Maloney PA, Maroni M (eds) Methods of Pesticide Exposure Assessment. Plenum Press, New York
3. Harris AS, Lucas AD, Kraemer PM, Marco M-P, Gee SJ, Hammock BD (1995) In: Kurtz DA, Skerritt JH, Stanker L (eds) New Frontiers in Agrochemical Immunoassay. AOAC International, Arlington, CA
4. Marco M-P, Hammock BD, Kurth MJ (1993) J Org Chem 58: 7548
5. Marco M-P, Nasiri M, Kurth MJ, Hammock BD (1993) Chem Res Toxicol 6: 284
6. Striley CAF, Biagini RE, Mastin JP, MacKenzie BA, Robertson SK (1999) Anal Chim Acta 399: 109
7. Burchell B, Brierley CH, Rance D (1995) Life Sciences 57: 1819
8. Burchell B, McGurk K, Brierley CH, Clarke DJ (1997) In: Sipes IG, Queen CAM, AJ Gandolfi (eds) Comprehensive Toxicology, Elsevier Science, New York
9. GreenMD, Tephly TR (1996) Drug Metab Dispos 24: 356
10. Oddy EA, Manchee GR, Coughtrie MWH (1997) Xenobiotica 27: 369
11. List of Extremely Hazardous Substances (last updated: February 4, 1999): access *via* the U.S. EPA website:http://www.epa.gov/swercepp/ehs/ehscas.html
12. HPV Challenge Program Chemical List (last updated June 6, 2000): access *via* the U.S. EPA website: http://www.epa.gov/opptintr/chemrtk/hpvchmlt.htm
13. National Institute for Occupational Safety and Health (1978) Publication No. (NIOSH) 78–213
14. Parke DV (1952) Detoxication Mechanism 2nd ed., Thesis Univ. London: quoted by Williams RT, Chapman and Hall, London, 1959
15. Parke DV (1968) The Biochemistry of Foreign Compounds. Volume 5. Pergamon Press, Oxford New York Toronto Sydney
16. Smith JE, Ross D, Graham AB, Skellern GG (1992) J Pharm Biomed Anal 10: 461
17. Heil TP, Lindsay RC (1988) J Env Science and Health. Part B: Pesticides, Food Contaminants, and Agricultural Wastes 23: 489
18. Amrolia P, Sullivan SG, Stern A, Munday R (1989) J Appl Toxicol 9: 113
19. Heil TP, Lindsay RC (1990) J Environ Sci Health. Part B: Pesticides, Food Contaminants, and Agricultural Wastes 25: 527
20. 21 CFR 172.515 (last updated: April 1, 1999). Food and Drugs. Database access *via* the Government Printing Office website: http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi
21. Hill RH, Shealy DB, Head SL, Williams CC, Bailey SL, Gregg M, Baker SE, Needham LL (1995) J Anal Toxicol 19: 323
22. Keski-Hynnila H, Andersin R, Luukkanen L, Taskinen J, Kostiainen R (1998) J Chromatogr A 794: 75
23. Pierce WM, Nerland DE (1988) J Anal Toxicol 12: 344
24. Hammock BD, Gee SJ (1995) In: Nelson JO, Karu AE, Wong RB (eds) Immunoanalysis. Emerging Technologies, American Chemical Society, Washington, D.C.
25. Kraemer PM, Lucas AD, Harris AS, Hammock BD (1995) In: Hock B, Niessner R (eds) Immunochemical Detection of Pesticides and their Metabolites in the Water Cycle. Deutsche Forschungsgemeinschaft. Research Report., VCH, Weinheim
26. Terplan G, Usleber E, Maertlbauer E, Schneider E, Dietrich R (1995) In: Hock B, Niessner R (eds) Immunochemical Detection of Pesticides and their Metabolites in the Water Cycle. Deutsche Forschungsgemeinschaft. Research Report., VCH, Weinheim
27. Staimer N, Gee SJ, Hammock BD (2001) Anal Chim Acta (in press)
28. Thompson M.J, Ballinger LN, Cross SE, Roberts MS (1996) J Chromatogr B 677: 117
29. Goebel WF (1936) J Exptl Med 64: 29
30. Kreissig S, Hock B (1991) Anal Lett 24: 1729
31. Pow DV, Crook DK (1993) J Neurosci Methods 48: 51
32. Hermanson GT (1996) Bioconjugate Techniques. Academic Press, San Diego
33. Williams CA, Chase MW (1967) Methods in Immunology and Immunochemistry. Academic Press, New York
34. Landsteiner K (1933) Die Spezifität der serologischen Reak tionen. Julius Springer, Berlin
35. Tangerman A (1984) In: Matern S, Bock KW, Gerok W (eds) Advances in Glucuronide Conjugation. Falk Symposium 40. MTP Press Limited, Lancaster Boston The Haque Dordrecht
36. Smith RL, Williams RT (1966) In: Dutton GJ (ed) Glucuronic Acid. Free and Combined. Chemistry, Biochemistry, Pharmacology, and Medicine. Academic Press, New York
37. Pengelly WL (1986) In: Bopp M (ed) Plant Growth Substances. Springer, Berlin
38. Toennes SW, Maurer HH (1999) Clin Chem 45: 2173
39. American Conference of Governmental Industrial Hygienists (1999): TLVs and BEIs, ACGIH, Cincinnati