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Development of a class-selective enzyme immunoassay for urinary phenolic glucuronides

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Abstract

Class-selective immunoassays for the measurement of glucuronides in human urine can aid evaluation of human exposure to complex mixtures of xenobiotics. Therefore, an enzyme immunoassay (EIA) for the group-selective detection of phenolic β-D-glucuronides was developed. The immunoconjugate was formed by coupling *p*-aminophenyl-β-D-glucuronide to the carrier protein thyroglobulin leaving an exposed glucuronic acid. Rabbits were injected with the immunogen in order to raise polyclonal antibodies. The resulting antibody #1339 (serum dilution: 1/5000) has been used in combination with a *p*-aminophenyl-1-thio-β-D-glucuronide-peroxidase-conjugate as an enzyme tracer (tracer dilution: 1/60 000). The resulting competitive assay is sensitive for phenyl-β-D-glucuronide (IC_{50} = 401 ng/ml), *p*-nitrophenyl-β-D-glucuronide (IC_{50} = 244 ng/ml), *p*-acetamidophenyl-β-D-glucuronide (IC_{50} = 239 ng/ml) and *p*-aminophenyl-β-D-glucuronide (IC_{50} = 434 ng/ml). Little or no cross-reactivities were observed for potential urinary cross-reactants. A combination of high-performance liquid chromatography (HPLC) and immunoassay was developed in an approach to apply the developed EIA for the determination of four different phenolic glucuronides in complex matrices such as human urine. The hyphenated technique HPLC–EIA may be used to monitor human exposure to a combination of related toxic agents including benzene, phenol and phenylamines which are all known to be excreted in urine as their phenolic glucuronic acid conjugates.

Keywords: Immunoassay; Phenolic compounds; Glucuronides; ELISA; Integrated HPLC–ELISA

1. Introduction

Routine biomonitoring of human exposure to xenobiotics requires rapid, inexpensive, and reliable techniques that can detect urinary metabolites at low levels. These demands can be met by immunochemical methods which are widely used not only in biochemistry, endocrinology or medical chemistry, but also increasingly in environmental and toxicological fields [1]. Thus, immunoassays have proved to be suitable for the rapid and inexpensive detection of urinary biomarkers such as mercapturates as indicators of exposure [2–6]. In addition to 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.

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Typically, people are not exposed to an individual toxic compound alone, but rather to a combination of several environmental pollutants. Simultaneous exposures to a mixture of potentially harmful chemicals may derive from water supplies, industrial occupational exposure, or through household items [11–14]. Phenol and phenolic compounds such as *p*-aminophenol, *N*-acetyl-*p*-aminophenol, *p*-nitrophenol, benzene or aniline are agents to which humans are exposed. Phenol is a major metabolite of benzene and a component of disinfectants and antiseptics. It is used in the manufacture of phenolic resins, bisphenol A, and alkyl phenols [14]. Both aniline and its major metabolite *p*-aminophenol are, for instance, important intermediates for the synthesis of sulfur and azo dyes [14,15]. The *N*-acetyl derivative of *p*-aminophenol (acetaminophen) is used not only as an analgesic and antipyretic, but also to manufacture azo dyes or photographic chemicals [14,15] and can be deacetylated in vivo to the nephrotoxic *p*-aminophenol [16,17]. *p*-Nitrophenol is a metabolite of the highly toxic insecticide methyl parathion and is used for the synthesis of pesticides and pharmaceuticals [15,18,19]. Benzene is a component of gasoline and a feedstock for synthetic organic chemical production. Humans are regularly exposed to benzene as a constituent of gasoline fumes, automobile exhaust, and tobacco smoke [20–22]. The exposure to these chemicals may cause severe negative health effects and results predominantly in the urinary excretion of their phenolic glucuronic acid conjugates [22–35].

Accordingly, a class-selective immunoassay can be applied for the screening of human exposure to a group of chemicals using their urinary phenolic glucuronides as biological biomarkers. The verification of EIA measurements by conventional analytical instruments such as GC or GC/MS [18,36–38] will be essential, but could be confined to samples with relevant concentrations of the target analytes. Further, class specific immunoassays are excellent post-column detector systems following separation by HPLC [39–41]. The prior separation of certain phenolic glucuronides by HPLC and the subsequent quantification by immunological methods can be a helpful tool in clinical and forensic toxicology or doping control, where drugs and poisons are usually screened in biological fluids. Thus, the development of a class-selective immunoassay and the potential of an integrated HPLC–EIA system for the direct analysis of phenolic β-D-glucuronides in human urine will be described.

2. Experimental

2.1. Chemicals and reagents

p-Aminophenyl-β-D-glucuronide (*p*-APG) as a hapten for the immunoconjugate was synthesized as described below (Section 2.3). Phenyl-β-D-thioglucuronide was obtained from Molecular Probes, Inc. (Eugene, OR) and α-naphthyl-β-D-glucuronide from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Ethyl-β-D-glucuronide was generously supplied by Sension GmbH (Augsburg, Germany). *p*-Aminophenyl-1-thio-β-D-glucuronide (ThioAPG) and *o*-aminophenyl-β-D-glucuronide (*o*-APG) for conjugation to peroxidase, the standards phenyl-β-D-glucuronide (PG), *p*-nitrophenyl-β-D-glucuronide (*p*-NPG), and *p*-acetamidophenyl-β-D-glucuronide (*p*-AAPG), bovine serum albumin (BSA), the carrier protein thyroglobulin (porcine), Tween-20, 3,3′,5,5′-tetramethylbenzidine (TMB) and chemicals examined as cross-reactants were obtained from Sigma Chemical Co. (St. Louis, MO). Horse-radish peroxidase (HRP) was purchased from Boehringer (Mannheim, Germany). 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 in syntheses, conjugations, for buffers, and for HPLC were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA).

2.2. Instruments

An ^{1}H-NMR spectrum at 300.1 MHz was measured for *p*-APG on a General Electric QE-300 spectrometer (Bruker NMR, Billercia, MA) in Me_2SO-d_6 solution at ambient temperatures. ^{1}H-chemical shifts are expressed in parts per million (ppm) downfield from internal tetramethylsilane (TMS). Further, *p*-APG was analyzed by direct flow injection using a Quattro-BQ mass spectrometer (VG Biotech, Altrincham, UK) and ACN/H_2O (1:1 v/v) as mobile phase. An Isco μLC-500 syringe pump delivered the mobile phase at 5 μl/min. Injection volume was 10 μl. The analyte

was dissolved in ACN/H_2O (1:1 v/v). Large quantities (approximate amount injected: 100 nmol) were analyzed so that minor reaction products could be observed. The cone lens was set to 50 V, the source temperature 70°C and the scan range 100–1000 *m/z*. The capillary voltage was set at −3.5 kV. The mass spectrometer was operated in negative ion mode.

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 mm × 4.6 mm i.d., 5 μm was employed for separation (Western, Analytical Products, Inc., Murrieta, CA). The injection volume was 10 μl, the flow-rate 0.5 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 [42]).

Absorbances (*A*) in 96-well plates (F 96 Maxisorb Nunc-Immunoplate, Roskilde, Denmark) 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.

2.3. Hapten synthesis

p-Aminophenyl-β-D-glucuronide was prepared as follows (modified from [43]): a solution of *p*-nitrophenyl-β-D-glucuronide (80 mg, 0.254 mmol) and a catalytic amount of palladium (10 wt.% on activated carbon) in MeOH (15 ml) was stirred under hydrogen for 30 min at room temperature. The resulting suspension was filtered. The retentate was dissolved in water and filtered to separate the reaction product from the catalyst. The filtrate was dried under nitrogen and subsequently washed with MeOH to obtain the hapten as a brown powder in 70% yield (Fig. 1). The structure was confirmed by ^{1}H-NMR and electrospray ionization mass spectrometry (ESI). Purity was supported by HPLC measurements at different wavelengths. HPLC: R_t = 5.1–5.2 min, ^{1}H-NMR (Me_2SO-d_6): 6.72 (d, J = 8.8 Hz, 2H); 6.49 (d, J = 8.8 Hz, 2H); 5.32 (d, J = 4.9 Hz, 1H); 5.14 (d, J = 4.7 Hz, 1H); 4.72 (d, J = 7.5 Hz, 1H); 3.72 (d, J = 9.6 Hz, 1H); 3.39–3.17 (m, 4H); 3.16 (d, J = 3.2 Hz, 1H); 2.5 (s, 2H). ESI mass spectra (*m/z*): $[M - H]^-$: 284 (100%); $[2M - H]^-$: 569 (0.2%); $[2M - 2H + Na]^-$: 591 (0.1%), [M − Gluc − H] (fragment ion formed by the loss of glucuronide moiety): 108 (30%).

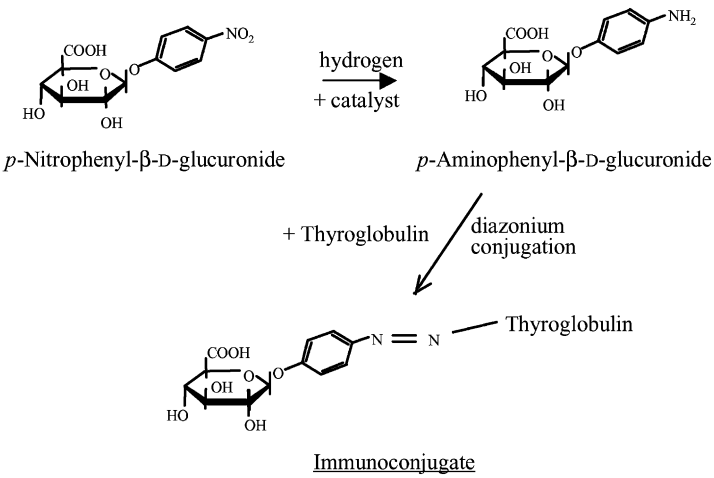

Fig. 1. Synthesis of hapten and immunoconjugate.

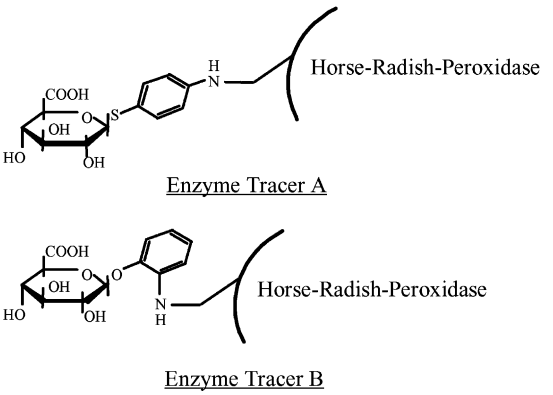

Fig. 2. Hapten–enzyme conjugates. Two haptens were attached via their amino groups to the aldehyde groups of oxidized horse-radish-peroxidase (periodate procedure).

2.4. Preparation of the immunogen conjugate

The diazonium procedure was applied for the hapten-carrier conjugation (Fig. 1). Details have been described by Goebel [43] and Kreissig et al. [44].

2.5. Preparation of enzyme tracers

Two different enzyme tracers (A and B, Fig. 2) were synthesized by coupling ThioAPG or *o*-APG to peroxidase (POD) from horse-radish. The activation of POD by periodate oxidation for reductive amination coupling to amine containing haptens has been described in detail by Kreissig et al. [44].

2.6. Immunization

Control sera were collected from three New Zealand white rabbits prior to the initial immunizations. After the first immunization with 1 mg of the immunogen in PBS, emulsified in Freund's complete adjuvant (1:1 v/v), additional immunizations using Freund's incomplete adjuvant were given. Booster injections were carried out every 4 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.

2.7. Enzyme immunoassay procedure

The optimized assay was conducted using three well replicates for each standard concentration or sample. Coating: microtiter plates were coated overnight at 4°C with 300 μl per well of the diluted antiserum (1:5000) in carbonate buffer (pH 9.6, consisting of Na_2CO_3, 1.7 g/l and $NaHCO_3$, 2.86 g/l). Washing: three 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 $NaH_2PO_4{\cdot}H_2O$ (1.38 g/l) and Na_2HPO_4 (9.94 g/l). Washing: two times as before. Immunoreaction: standard or sample (200 μl per well) was added plus 50 μl enzyme tracer A diluted in PB (1:60 000) and incubated for 90 min at room temperature on a horizontal shaker. Washing: three times as before. Enzyme reaction: 200 μl substrate buffer ($NaH_2PO_4{\cdot}H_2O$, 18.2 g/l; Na_2HPO_4, 0.99 g/l; urea hydrogen peroxide, 282 mg/l) and 100 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 15–20 min with 50 μl 2N H_2SO_4 and the absorption measured with the microplate reader.

2.8. Cross-reactivity

The selectivity of the optimized assay was characterized by testing 11 different glucuronides. In addition, two structurally related sugar derivatives (*p*-nitrophenyl-β-D-galacturonide and *p*-nitrophenyl-β-D-glucopyranoside), glucose, free glucuronic acid, several unconjugated phenols and the main constituents of urine (urea, hippuric acid and creatinine) were tested for cross-reactivities. *p*-AAPG had the lowest IC_{50} (Fig. 3) and therefore, was used as the reference analyte for the calculation of cross-reactivities (% CR) [(concentration of *p*-AAPG at 50% (B/B_0)/concentration of the cross-reactant at 50% $(B/B_0) \times 100$)].

2.9. Matrix effects

p-NPG was selected as a representative glucuronide standard for the evaluation of matrix effects.

2.9.1. Solvent

Standard curves of *p*-NPG were prepared in PB-buffer (pH 7.6) containing 0, 10, and 20% (v/v) methanol to determine the effects of solvent. The pH

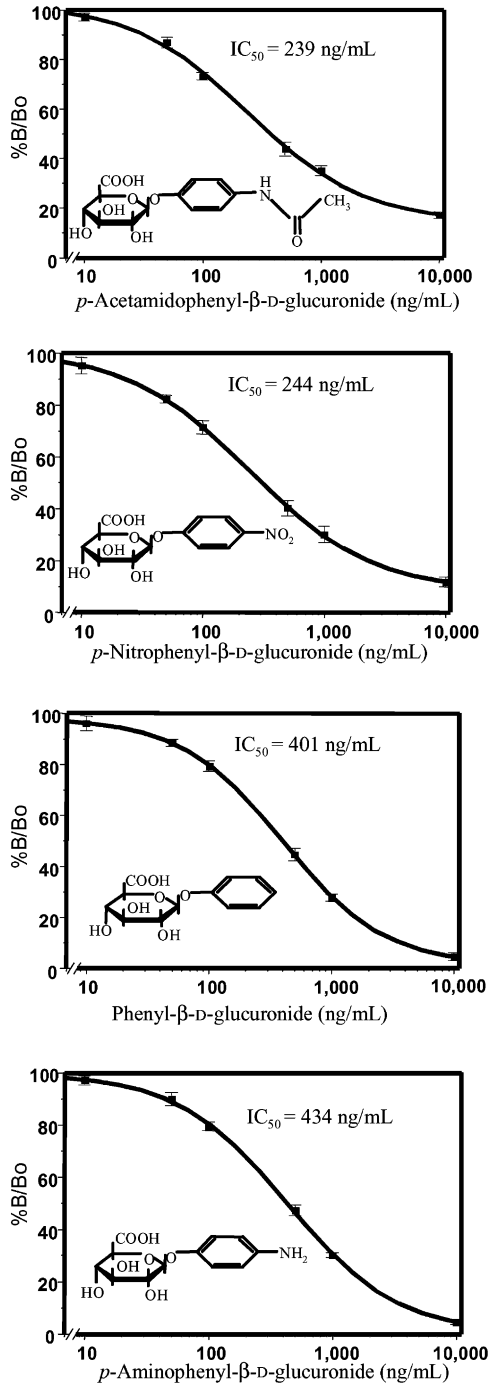

Fig. 3. EIA calibration curves of four different phenolic glucuronides using antisera #1339 (dilution: 1:5000) and enzyme tracer A (dilution: 1:60 000). All inhibition curves represent the average of five curves with standard deviation. The absorptions are converted to $\%(B/B_0)$-values. The coefficients of regression were >0.99 in each case.

values were adjusted to pH 7.6 with 1 M NaOH if necessary.

2.9.2. Ionic strength

The effects of ionic strength on the quantitation of *p*-NPG were evaluated by preparing analyte solutions in 80 mM PB, 80 mM PB + 150 mM NaCl, and 80 mM PB + 300 mM NaCl at pH 7.6.

2.9.3. pH values

The tolerance of the assay for different pH values (pH 6.5, 7.6, and 8.5) was tested using *p*-NPG as analyte.

2.9.4. Urine

The effects of the urine matrix were evaluated by preparing various concentrations of two different human urine samples (I and II) in PB (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 10%) and measured by the developed EIA. The urine samples (I and II) were collected in the morning from the same person, but on different days.

2.10. Integrated HPLC–EIA

Aliquots of human urine (500 μl) were spiked with 25 μg (=50 ppm) of four different standards (PHG, *p*-APG, *p*-AAPG, *p*-NPG). 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 10 μl of the filtrate (0.25 μg standard) was injected into the HPLC system. The eluate was collected in the form of 30 s fractions (250 μl). Phosphate buffer (250 μl) and 1 M NaOH solution (75 μl) was added to each fraction in order to adjust the pH-value to 7.6 for subsequent measurement by the developed EIA. It follows that from the injection onto the HPLC to the EIA detection a dilution of about 100 times was determined.

3. Results and discussion

3.1. Hapten design and immunogen conjugate preparation

Harris et al. [3,45] recently reported a specific immunoassay for *p*-NPG by coupling the glucuronic

acid moiety of the compound to the carrier protein and leaving the nitrophenyl-group exposed [45]. In this paper, we used a different strategy in order to broaden the antibody selectivity for phenolic glucuronides (Fig. 1): the commercially available *p*-NPG was reduced to *p*-APG and conjugated to thyroglobulin via diazo-coupling of the aminophenyl moiety as described before. Thus, the specific group of the glucuronide was used for conjugation with the general glucuronic acid part distal from the carrier protein. The synthesis of the hapten was done without protection of the glucuronic acid moiety resulting in a good yield (70%) and high purity (>98%). 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 [46]. The diazotization method used for the synthesis of hapten-carrier immunogen conjugate 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 [47].

3.2. Enzyme tracer synthesis

All immunochemical reactions follow the Law of Mass Action, a reversible antibody-analyte equilibrium competition with an antibody–hapten–enzyme (or protein) conjugate. The level of detection of an assay often can be improved by using heterologous enzyme-labeled haptens with an affinity to the antibody smaller than that of the analyte [48]. Therefore, *p*-ThioAPG and *o*-APG were chosen as haptens for the synthesis of two different enzyme tracers in order to develop a heterologous immunoassay. In addition, the affinity of the antibody to the tracer can be slightly decreased by using different coupling procedures for the immunogen and the hapten–enzyme conjugate: *p*-ThioAPG or *o*-APG were coupled via the amino-group to HRP by a reductive-amination-mediated hapten-carrier conjugation and held together by secondary amine bonds (Fig. 2). *p*-APG, however, was cross-linked via the amino-group to thyroglobulin by creating a diazo linkage (Fig. 1).

3.3. Assay optimization

Two different enzyme tracers (A and B, Fig. 2) were tested with three different antisera using a two-dimensional titration to determine the best dilution of the enzyme tracer and antiserum. Because enzyme tracer B was hardly recognized by all antisera inhibition was measured for those antibody and enzyme tracer A combinations giving a reasonable absorbance (0.7–1.0). The combination giving the highest sensitivity for *p*-NPG was selected for further assay development. The optimized assay with enzyme tracer A (1:60 000) and antibody #1339 (1:5000) resulted in a strong signal and low background. Fig. 3 shows the calibration curves for four different glucuronide standards with 50% (B/B_0)-values (IC_{50}) between 239 and 434 ng/ml in assay buffer.

3.4. Cross-reactivity

The optimized immunoassay does not cross-react with urea, hippuric acid, creatinine, unconjugated phenols, glucose and the free glucuronic acid (no inhibition upto 20 ppm). Almost no cross-reactivities were observed for *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-galacturonide indicating that both the carboxyl-group and the position of the hydroxyl-group of the glucuronic acid are crucial for the recognition of glucuronides by the developed immunoassay. 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 specificity of certain azoproteins [49]. 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 [43]. Further, the phenyl-group is important as well, since ethyl-, methyl- and even the naphthyl-*O*-glucuronides were hardly recognized by the antibodies generated in this study. Interestingly, the immunoassay showed only a weak inhibition by *ortho*-substituted nitro- and aminophenyl-glucuronides. However, phenyl-β-D-glucuronide, and *para*-substituted acetamido-phenyl-,

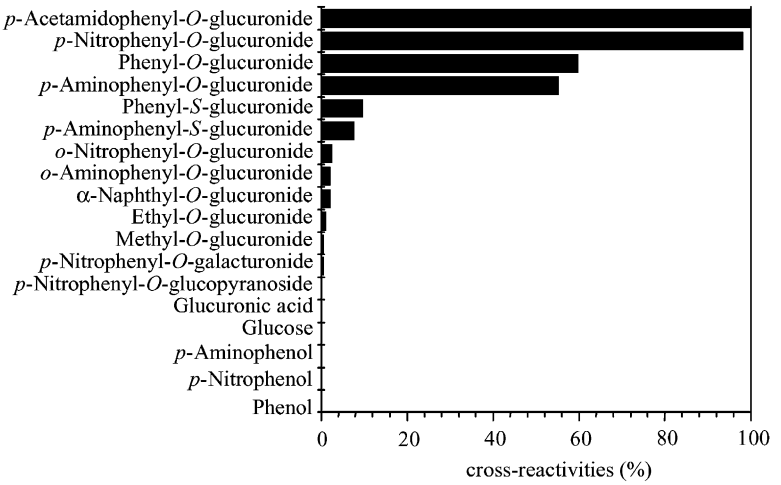

Fig. 4. Cross-reactivity of polyclonal antiserum #1339. Cross-reactivities were calculated for each compound as (IC_{50} of *p*-acetamidophenyl-β-D-glucuonide/IC_{50} of cross-reactant) × 100.

nitrophenyl-, and aminophenyl-β-D-glucuronides can be detected in the lower ppb (ng/ml) range (Figs. 3 and 4).

3.5. 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: Fig. 5 shows that the assay was tolerant to both higher NaCl and MeOH concentrations. However, the assay was sensitive to 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 [50]. 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 two different (non-spiked) urine samples (I and II) were prepared and each dilution was run by the developed immunoassay. The processed inhibition curves with an IC_{50} of 0.53% (sample I) and 0.14% (sample II) showed that a substantial dilution of urine (upto 1:1000) is required to reduce noticeably matrix effects (Fig. 6). The slopes of the inhibition curves in Fig. 6 are similar to the analyte slopes (Fig. 3) indicating that the interferences caused by highly diluted urine samples are actually specific. This may be due to cross-reacting substances at high concentrations and weak affinities for the applied antibodies or to the presence of competitive inhibitors possessing similar antibody affinities as the target analytes [51]. 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 [50]. The variation between the observed inhibition of the EIA by urine sample I ($IC_{50} = 0.53\%$) and sample II ($IC_{50} = 0.14\%$) may be due to different diets.

3.6. 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

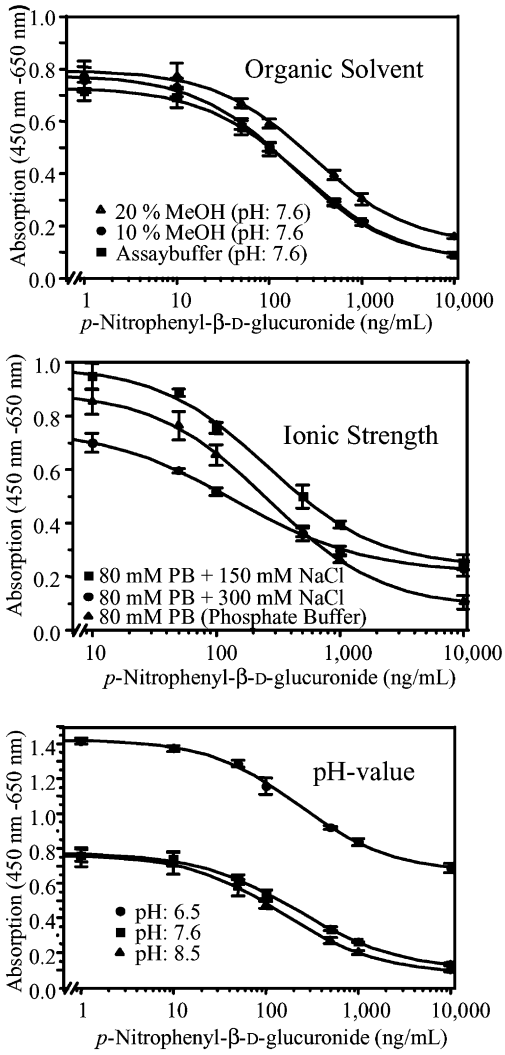

Fig. 5. EIA competition curves of *p*-nitrophenyl-β-D-glucuronide prepared with various %methanol, ionic strengths, and pH values. All inhibition curves represent the average of three curves with standard deviation using antisera #1339 (dilution: 1:5000) and enzyme tracer A (dilution: 1:60 000). The coefficients of regression were >0.99 in each case.

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. Alternatively, the same approach presented here could yield reagents for affinity chromatography of glucuronides. The resulting

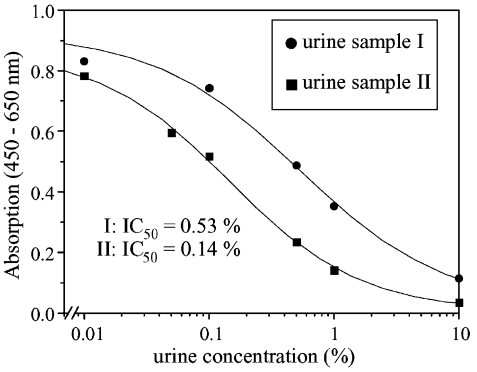

Fig. 6. Effect of urine matrix. Various concentrations of two different human urine samples (I and II) in phosphate buffer were prepared and measured by the developed EIA. The resulting inhibition curves were processed using a four-parameter curve fitting algorithm (coefficients of regression: >0.99). The absorptions represent the average of three replicates per microtiter plate (coefficients of variation: <10%). The EIA conditions are described in text.

glucuronide mixture could be speciated by a variety of HPLC, MS, or HPLC–MS procedures.

An HPLC method for the detection of single glucuronides [42] was modified in such a way that it is now possible to separate six different phenolic glucuronic acid conjugates within 20 min using an isocratic mobile phase (Fig. 7). In a preliminary study, human urine was spiked with four different standard glucuronides. The immunogram (Fig. 8) shows that the hyphenated HPLC–EIA method detects urinary *p*-aminophenyl-, *p*-acetamidophenyl-, phenyl- and *p*-nitrophenyl-β-D-glucuronides after injection of amounts as low as 0.25 μg. It follows that the integrated HPLC–EIA method can determine four structurally related glucuronides at 50 ppm in whole urine even though the sample is diluted more than 100 times between injection and detection (Section 2.10). The sensitivity of the hyphenated technique may be increased simply by injection of a larger volume and/or less diluted urine samples.

Nevertheless, a 50 ppm quantification level for phenolic glucuronic acid conjugates in urine already meets several biological exposure indices (BEIs) recommended by the American Conference of Governmental Industrial Hygienist for phenolic compounds. BEIs are reference values intended as guidelines for the evaluation of potential health hazards in the practice of industrial hygiene. The proposed BEIs for total

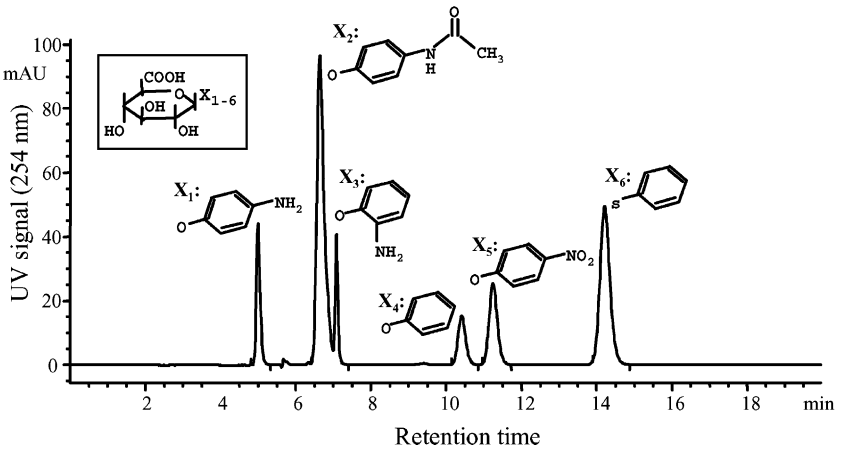

Fig. 7. HPLC chromatogram of glucuronic acid conjugates. Six different standards were prepared in MeOH/H_2O (1:1 v/v) and injected in different amounts: 1.5 μg (*p*-aminophenyl-β-D-glucuronide, phenyl-β-D-glucuronide, *o*-aminophenyl-β-D-glucuronide), 0.5 μg (*p*-nitrophenyl-β-D-glucuronide, phenyl-1-thio-β-D-glucuronide), and 0.25 μg (*p*-acetamido-phenyl-β-D-glucuronide). Chromatographic conditions are described in text.

p-aminophenol in urine after exposure to aniline and for total phenol (free and conjugated) after benzene exposure are 50 mg/g creatinine in each case which is equivalent to about 50 ppm. The BEI for total phenol in urine after exposure to phenol is 250 mg/g creatinine [52].

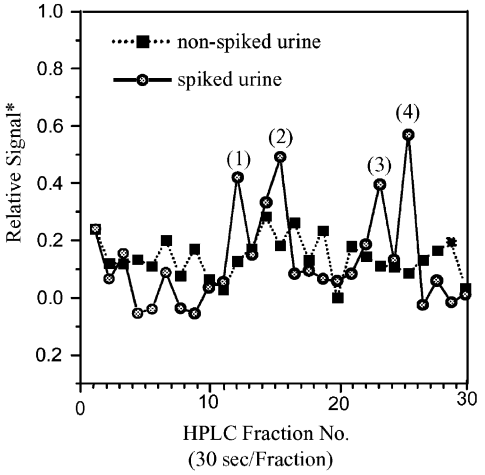

Fig. 8. Immunogram of human urine: non-spiked and spiked with (1) *p*-aminophenyl-β-D-glucuronide, (2) *p*-acetamido-phenyl-β-D-glucuronide, (3) phenyl-β-D-glucuronide, and (4) *p*-nitrophenyl-β-D-glucuronide. Sample preparation and chromatographic conditions as in text (*EIA signal was converted according to: $A_{max} - A$ (A_{max} = maximum absorption, no analyte; A = absorption of the collected fraction)).

In conclusion, the hyphenated technique HPLC–EIA reported here could provide a rapid, cost efficient, and reliable method for the biological monitoring of human exposure to a number of phenolic compounds by direct analysis of their urinary glucuronic acid conjugates.

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