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Journal

Analytical and Bioanalytical Chemistry, 407(24)

ISSN

1618-2642

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Publication Date

2015-09-01

DOI

10.1007/s00216-015-8918-5

Peer reviewed

HHS Public Access

Author manuscript

Anal Bioanal Chem. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:

Anal Bioanal Chem. 2015 September ; 407(24): 7263–7273. doi:10.1007/s00216-015-8918-5.

An immunoassay for the detection of triclosan-*O*-glucuronide, a primary human urinary metabolite of triclosan

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Abstract

Triclosan-*O*-glucuronide (TCSG) is one of the primary urinary metabolites of the antibacterial compound triclosan or TCS that is found in many personal care products and consumer goods. We have developed a competitive, indirect heterologous ELISA for the detection of the target TCSG in urine. Such an ELISA for TCSG could be developed as a useful tool to measure this important biomarker of human exposure to TCS. Immunogens were prepared by conjugating TCSG to thyroglobulin, via heterobifunctional cross-linkers AEDP or 3-[(2-aminoethyl)dithio] propionic acid•hydrochloride and TFCS or N-[ε-trifluoroacetylcaproyloxy]succinimide ester. The coating antigen was prepared by the direct conjugation of TCSG to bovine serum albumin. Antibodies raised in rabbits 2619, 2621 (immunogen TCSG-AEDP-Thy) and 2623 (immunogen TCSG-TFCS-Thy) and the coating antigen were screened and characterized to determine their optimal concentrations. The optimized ELISA, developed with antibody 2621, gave an IC_{50} value of 2.85 ng/mL, with the linear range (IC_{20} – IC_{80}) determined to be 2.6 – 24.8 ng/mL. Selectivity of the assay was assessed by measuring cross-reactivity of antibody 2621 to related congeners such as the aglycone TCS, triclosan-*O*-sulfate, triclocarban, a polybrominated diphenyl ether derivative and 3-phenoxybenzyl alcohol glucuronide. There was virtually no recognition by antibody 2621 to any of these cross-reactants.

Keywords

triclosan-*O*-glucuronide; urinary metabolite; biomarker; polyclonal antibodies; ELISA

Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol: TCS), a chlorinated phenol, is a lipid-soluble, broad-spectrum microbiocide [1] that is predominantly used as an active ingredient in a wide variety of soaps, deodorants, toothpastes, cosmetics, fabrics, plastics and other consumer products. TCS is typically used at concentrations of 0.1–0.3% by weight in personal care products. Use of personal care products is the most common form of direct human exposure. New and emerging evidence on the potential health issues surrounding the use of TCS include contact dermatitis [2],[3] endocrine disruption [4–9], antibiotic cross-resistance (in laboratory settings) [10] and the formation of carcinogenic by-products such

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as dioxins and chlorinated phenols [11–13]. James et al [6] demonstrated that TCS (0.09 nM) is an inhibitor of estrogen sulfonation in sheep placental tissues. TCS interacts with phase I enzymes, causing induction of P450 isoforms of the CYP1A or CYP2B subfamily that is linked to the toxicity and endocrine disrupting effects [14]. *In vitro* cell-based and nuclear-receptor-responsive bioassays have shown TCS (1.2 μM) to be a potent channel sensitizer in a RyR (ryanodine)-mediated bioassay. At higher TCS concentrations (10 μM), weak AhR (aryl hydrocarbon receptor) activity was exhibited. TCS was a potent antagonist in both ER (estrogen)- and AR (androgen)-mediated bioassays [4]. It was also a powerful disruptor of Ca^{2+} regulation. Further investigations showed that *in vitro*, TCS possessed intrinsic estrogenic and androgenic activity [15]. At higher concentrations of 10 μM, TCS displaced estradiol from estrogen receptors of MCF-7 human breast cancer cells and from recombinant human ERα/ERβ. TCS also inhibited testosterone from binding to the rat androgen receptor. Investigations with male rats [7] showed that exposure to TCS decreased serum levels of testosterone and the activity of vital steroidogenic enzymes. The binding of TCS to the ryanodine receptor in mice has resulted in cardiac fibrillation and arrest. TCS impaired the excitation-contraction coupling (ECC) of cardiac and skeletal muscles both *in vitro* and *in vivo* [16]. More recently, work from the Tukey laboratory [17] observed that TCS is a liver tumor promoter in mice. These observations caution that there may be effects on human and animal health because exposure (at environmentally relevant concentrations) can occur.

It is estimated that the annual production volume of TCS in the U.S. is close to a million pounds, one-hundredth of the annual production volume of its structurally similar congener, the flame retardant - polybrominated diphenyl ethers (PBDEs). Yet, TCS is found to have a higher environmental persistence than the PBDEs [18]. Thus, the environment serves as an indirect source of human exposure. The extensive use of TCS has been steadily increasing over the past twenty years resulting in its continuous release into the environment, especially contamination of aquatic ecosystems [19–21]. TCS has some tendency to bio-accumulate in biosolid-amended soils as well as sediments and is persistent during anaerobic digestion processes [22,23]. Even if TCS availability to aquatic biota is reduced by biodegradation, the transformation products – methyl-triclosan and chlorinated phenols are toxic and more resistant to degradation. Macherius et al. [24] investigated the metabolism of TCS, methyl triclosan and triclocarban after uptake into carrot cell cultures and in TCS contaminated soils. Eight new phase II metabolites of TCS were found in carrot cell cultures as well as in intact carrot plants grown in TCS-contaminated soil.

TCS is active against a wide variety of microorganisms. Therefore, it is interesting to speculate on the effect of TCS on beneficial bacteria in the environment as well as in the human body. Although to date, there are no known reports that TCS destroys the beneficial microbes in the soil, its effect on human gut microbiota, such as the bifidobacterial strains that have distinctive benefits to the human body, has been minimally explored [25]. TCS exposure has been linked to obesity patterns observed in the U.S.[26]. The authors theorize that the potential of TCS to possibly alter the gut microflora and endocrine function could be associated with increased body mass index.

Immunoassays

Although TCS may represent an important healthcare and sanitary tool when used in clinical settings, constant human and animal exposure to the chemical poses a health and environmental hazard. Considering the high magnitude of human exposure to TCS as well as its substantial environmental persistence, there are sufficient reasons to be concerned about its impact on human health and hence more efforts must be made to monitor TCS in humans. The current analytical methods available for TCS monitoring include traditional tools such as gas chromatography-mass spectrometry (GC-MS) [27–29]that are generally time-consuming.. Since with GC-MS, TCS rather than its glucuronide metabolite normally is monitored, a heavy atom labeled glucuronide internal standard or at least careful characterization of the hydrolysis step need to be included. In contrast to mass spectrometric detection methods, immunoassays are fast do not require a multi-step sample preparation process, and are adaptable to on-site detection. In spite of the merits, immunoassays cannot replace conventional methods like high-performance liquid chromatography and GC-MS altogether and are best used in conjunction with them. Nonetheless, use of a quantitative immunoassay can be a cost- and time-effective alternative.

TCSG

In humans, *in vivo*, TCS is readily metabolized to its oxygen-linked glucuronide (uridine-diphospho-glucuronosyl transferase or UGT-catalyzed) and sulfate conjugates (sulfotransferase or SULT-catalyzed). The glucuronide is the main metabolite eliminated primarily in the urine [30,31]. Developing an immunoassay for the main metabolite, TCS-*O*-glucuronide (TCSG), in order to assess human exposure, offers several key advantages over an assay for the parent TCS [32] – **1**. Being the major human urinary metabolite, TCSG is an excellent biomarker for evaluating human exposure. **2**. Urine samples can be directly analyzed as the need for hydrolysis of the conjugate to free TCS is eliminated. **3**. Immunoassays are run under aqueous conditions and the glucuronide is more polar and has a greater solubility compared to the parent TCS which is lipophilic. **4**. Being a larger molecule with additional hydrogen bonding sites than the parent TCS, an enhanced antibody recognition and binding to the glucuronide will result in higher affinity and selectivity.

Materials and Methods

Materials

The hapten coupling reagents, bovine serum albumin (BSA), thyroglobulin (Thy), Tween 20, and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich. Co. (St. Louis, MO). Heterobifunctional cross linkers, AEDP (3-[(2-aminoethyl)dithio]propionic acid hydrochloride) and TFCS (N-[ε-trifluoroacetylcaproyloxy]succinimide ester) were purchased from Thermo Fisher Scientific (Houston, Texas). Skimmed milk was purchased from BD Biosciences. Goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP) was purchased from Abcam (Cambridge, MA). ELISA was performed on 96-well polystyrene microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Sunnyvale, CA) in dual wavelength mode (450–650 nm).

Buffers—All buffers were prepared with ultrapure deionized water. Phosphate buffered saline was 10 mM phosphate pH 7.4, 1X PBS. Wash buffer (PBST) was 1X PBS containing 0.05% Tween 20. Coating buffer (pH 9.6) was 15 mM Na_2CO_3, 34.88 mM $NaHCO_3$. Blocking agent was 1% skimmed milk in PBS. Substrate buffer (pH 5.5) was 0.1 M sodium acetate/citrate buffer. For the substrate solution, 0.4 mL of 0.6% TMB (in dimethylsulfoxide, w/v) and 0.1 mL of 1% H_2O_2 were added to 25 mL sodium acetate-citrate buffer. Stop solution was 2 N H_2SO_4. For purification of antisera by magneto-precipitation, sodium phosphate buffer was prepared by mixing 0.03 g of $NaH_2PO_4.H_2O$ and 0.11 g of Na_2HPO_4 (anhydrous) in water to a volume of 10 mL after adjusting the pH to 7.4 with 1 N NaOH solution.

Methods

Synthesis of 6-[5–chloro-2-(2,4-dichlorophenoxy)-phenoxyl]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid (riclosan-*O*-glucuronide or *TCSG*)—Fig. 1 illustrates the synthetic pathway for triclosan-*O*-glucuronide (TCSG). TCSG was prepared starting with the methylation of a glucuronolactone, *1*, to obtain the corresponding glucopyranuronate, *2*. Compound *2* was then treated with 30% hydrobromic acid in acetic acid to yield the bromomethyl ester, *3*. Compound *3* was treated with triclosan in a Koenigs-Knorr reaction to obtain the per-gluc ester, *4*, which was then hydrolyzed to obtain TCSG.

Methyl 1,2,3,4-tetra-O-acetyl-D-glucopyranouronate, 2—Sodium hydroxide was dissolved in 500 mL of methanol, followed by the addition of 60 g (0.34 mole) of glucuronolactone, *1*. The mixture was stirred for an hour at 25 °C and methanol was removed under reduced pressure. The resulting syrup was treated by dropwise addition of acetic anhydride (150 mL) in pyridine (100 mL) on an ice bath, maintaining the temperature of the mixture below 40 °C. The reaction mixture was stored at 4 °C overnight, which aided in the recrystallization of methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranouronate, *2* (62.6 g, 73% yield; melting point 176.8 – 178 °C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.03 (1H, d, *J* = Hz), 5.57 (1H, t, *J* = Hz), 5.02 (2H, m, *J* = Hz), 4.73 (1H, d, *J* = Hz), 3.62 (3H, s, *J* = Hz), 2.0 (12H, m, *J* = Hz)

Methyl (2,3,4-tri-O-acetyl-R-D-glucopyranosyl bromide)uronate, 3—To a cooled solution of methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranouronate, *2* (5 g, 0.013 mole) dissolved in dichloromethane (10 mL), was added 16 mL (6 equiv, 0.08 mole) of 30% hydrobromic acid in acetic acid. Initially, the solution turned brown and after ~ 4.5 h, became light yellow in color. The resulting mixture was left to stir overnight over an ice bath. For the isolation of the bromo ester, *3*, the reaction mixture was slowly added to ~ 100 mL of chloroform. This solution was added in a slow, drop-wise fashion to a cooled solution of saturated sodium bicarbonate (~ 600 mL). The contents were transferred into a separatory funnel. The chloroform layer was washed twice with distilled water, followed by a saturated sodium chloride solution. The chloroform was dried over anhydrous sodium sulfate, filtered and evaporated to obtain a yellow syrup. Diethyl ether (20 mL) was added to the syrup, warmed to dissolve all the material and the compound was recrystallized at −20 °C. White crystals of bromo-glucuronic acid methyl ester, *3* separated out overnight, which were then dried (3.9 g, 73% yield, melting point 106 – 107 °C) and used for further analysis. ^{1}H NMR

(300 MHz, $CDCl_3$): δ 6.63 (1H, dt, *J* = 4.1, 0.6 Hz), 5.60 (1H, t, *J* = 9.8 Hz), 5.22 (1H, dd, *J* = 10.3, 9.5 Hz), 4.84 (1H, dd, *J* = 10.0, 4.0 Hz), 4.57 (1H, dd, *J* = 10.3, 0.7 Hz), 3.75 (3H, s), 2.34 – 1.87 (13H, m).

3,4,5-Triacetoxy-6-[5–chloro-2-(2,4-dichlorophenoxy)-phenoxy]-tetrahydropyran-2-carboxylic acid methyl ester, 4—To a solution of bromo-glucuronic acid ester, *3* (3 g, 0.01 mole) in dry dichloromethane (100 mL), were added triclosan (TCS) and silver carbonate (2.1 g, 0.01 mole). The mixture was stirred under argon at 25 °C for 12 h. The reaction mixture was filtered to remove the silver salt and washed with aqueous 2N potassium hydroxide and water. The resulting solution was evaporated and applied to flash chromatography (elution with 80/20 followed by 70/30 hexanes/ethyl acetate) to afford TCS-glucuronic acid methyl ester (1.1 g, 25% yield). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.74 (1H, d, *J* = 2.7 Hz), 7.42 (1H, d, *J* = 2.4 Hz), 7.05 (1H, d, *J* = 8.4 Hz), 6.83 (1H, d, *J* = 9.0 Hz), 5.94 (1H, d, *J* = 4.8 Hz), 5.02 (2H, m), 4.37 (1H, t, *J* = 3.9 Hz), 4.25 (1H, d, *J* = 8.1 Hz), 3.66 (3H, s), 2.03 (12H, m)

6-[5–Chloro-2-(2,4-dichlorophenoxy)-phenoxyl]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid (TCSG)—TCS-glucuronic acid ester, *4* (0.3 g, 0.004 mole) was dissolved in methanol (5 mL), and 1 M sodium hydroxide-methanol solution (2 mL) was added. This mixture was stirred on an ice bath for 24 h. After neutralization with 1 M hydrochloric acid-methanol solution, the mixture was evaporated in vacuo. The residue was washed with diethyl ether and ethyl acetate to remove any unreacted impurity. Purification was done by silica gel column chromatography (stepwise elution with ethyl acetate/methanol: 10, 20 and 30% methanol). The resulting eluate was evaporated in vacuo and washed with diethyl ether to obtain a pale yellow powder of TCSG. (8% yield). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.32 (1H, s), 7.64 (1H, d, *J* = 3.7 Hz), 7.32 (1H, d, *J* = 2.4 Hz), 7.27 (1H, dd), 7.23 (1H, dd), 7.15 (1H, d, *J* = 7.4 Hz), 6.86 (1H, d, *J* = 8.3 Hz), 5.88 (1H, d, *J* = 4.8 Hz), 4.55 (2H, m), 3.91 (1H, t, *J* = 3.9 Hz), 3.73 (1H, d, *J* = 8.1 Hz).

Preparation of immunizing antigens—TCSG was coupled to thyroglobulin to yield the immunogen via two heterobifunctional cross-linkers, AEDP (3-[(2-aminoethyl)dithio]propionic acid•hydrochloride) and TFCS (N-[ε-trifluoroacetylcaproyloxy]succinimide ester)TCSG (Fig. 2).

Cross-linking of TCSG to thyroglobulin by AEDP—*N*-hydroxysuccinimide, NHS (0.032 mmol, 0.0037 g) and dicyclohexyl carbodiimide, DCC (0.01 g, 0.03 mmol) were added to TCSG (0.01 g, 0.02 mmol) dissolved in 0.1 mL of dry *N*,*N*'-dimethylformamide (DMF). The mixture was stirred overnight at 4 °C under a nitrogen atmosphere. The dicyclohexyl urea (whitish) precipitate was removed by centrifugation at 13,000 rpm for 10 min. This resulted in an activated ester form. AEDP (0.01 g, 0.02 mmol) dissolved in triethylamine (0.002 g, 0.02 mmol, 3 μL) was added to the activate ester solution (~ 0.1 mL) and stirred overnight at 4 °C. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC (0.01 g, 0.04 mmol) dissolved in 0.3 mL DMF was added to the mixture and allowed to react at 4 °C overnight. The following day, the reaction mixture was

added dropwise (15 μL every 10 minutes) to a thyroglobulin solution (25 mg thyroglobulin in 5 mL of 0.05 M borate buffer at pH 8) with vigorous stirring at 4 °C for 24 h. Unreacted small molecules were removed by dialysis. The reaction mixtures containing the immunogen were dialyzed (10,000 MWCO) under stirring against PBS (0.01 M, pH 7.4), for three days, with frequent changes of the PBS solution to remove the unconjugated free hapten.

Cross-linking of TCSG to thyroglobulin by TFCS—TFCS (0.01 g, 0.02 mmole) dissolved in 0.2 mL DMF was added to a thyroglobulin solution (25 mg in 5 mL of 100 mM sodium phosphate, 0.15 M NaCl, pH 7.4). After stirring the mixture at 4 °C for 2.5 h, the pH was adjusted between 7.8 – 8.1 with diluted 1 N NaOH and incubated for 2 h to remove the trifluroacetyl protecting group of the cross-linker. Meanwhile, N-hydroxysuccinimide, NHS (0.003 g, 0.02 mmol) and dicyclohexyl carbodiimide, DCC (0.004g, 0.02 mmol) were added to the hapten, TCSG (0.01 g, 0.02 mmol, 0.007 g) dissolved in ~ 0.1 mL of dry DMF. The mixture was stirred overnight at 4 °C under a nitrogen atmosphere. The NHS-activated ester of the TCSG hapten (0.02 mmol), was then added in a dropwise manner to the TFCS-linker attached thyroglobulin solution. Concentrations of the immunizing antigens, TCSG-AEDP-Thy and TCSG-TFCS-Thy were determined by the BCA (bicinchoninic acid) test to be 5 mg/mL.

Preparation of coating antigen, TCSG-BSA—*N*-Hydroxysuccinimide, NHS (0.004 g, 0.03 mmol) and dicyclohexyl carbodiimide, DCC (0.006 g, 0.03 mmol) were added to TCSG (0.01g, 0.02 mmol) dissolved in 0.1 mL of dry DMF. The mixture was stirred overnight at 4 °C under a nitrogen atmosphere. The activated ester of TCSG was added dropwise to a BSA solution (0.03 g of BSA in 5 mL of 0.05 M borate buffer at pH 8) as described above. The reaction mixture was dialyzed (10,000 MWCO) at 0 °C under stirring against PBS (0.01 M, pH 7.4), for three days, with frequent changes of the PBS solution to remove the unconjugated free hapten. Concentration of TCSG-BSA determined by the BCA test was 5 mg/mL.

Other haptens conjugated with BSA to test as coating antigens are shown in Fig. 3. They include (*E*)-3-(3-chloro-4-(2,4-dichlorophenoxy)phenyl)-2-methylacrylic acid, *5*, 3-(3-chloro-4-(4-chloro-2-hydroxyphenoxy)phenyl)propanoic acid, *6*, 2-(4-amino-2-chlorophenoxy)-5-chlorophenol, *7* and 6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexanoic acid, *8*.

Immunization and Antiserum Preparation—The immunization procedure followed a previously published protocol [33,34]. Four female New Zealand white rabbits 2619, 2621, 2623 and 2624 were each immunized with 500 μg of immunogen. Pre-immune bleeds were collected from each rabbit prior to immunization. In the initial immunization, rabbits 2619 and 2621 were immunized with TCSG-AEDP-Thy conjugate and rabbits 2623 and 2624 were immunized with TCSG-TFCS-Thy conjugate. Following this, each rabbit received 300 μg of immunogen in seven subsequent boosts. A test bleed was collected a month after the first immunization. Two weeks after the first test bleed collection, two boosts were given, separated by a two-week interval (second and third test bleeds). Following which, in 3 weeks, the fourth and fifth test bleeds were collected, each separated by a 3-week interval.

During this period of test bleed collection, rabbit 2624 died, which restricted the assay development to three rabbits (2619, 2621 and 2623). The fifth and sixth test bleeds were collected from boosts separated by a 4-week interval. Final bleeds were collected 4 months following the first immunization and all the seven boosts. Antiserum was obtained by centrifugation, stored at −20 C, and used without further purification.

All three antisera 2619, 2621 and 2623 were tested for recognition of and binding to the coating antigen, TCSG-BSA and were also tested in the inhibition assay with the target TCSG.

Purification of antiserum 2621—The presence of a sub-population of antibodies in the rabbit antisera, that were binding to BSA masked the antibody recognition of target, TCSG (for further details, see Antibody Characterization in Results and Discussion). Thus, a magneto-precipitation method was employed for antisera purification. In this method, epoxy-coated magnetic beads (2.8 μm superparamagnetic beads, M-270 Epoxy Dyna Beads, Life Technologies, Chicago, IL) were pre-coated with BSA. The BSA-pre-coated beads were then incubated with the antisera. The beads were separated from the supernatant with a magnet. A functional characterization of the supernatant indicated that the BSA binders were eliminated from the antiserum, improving both signal-to-noise ratio and inhibition by the target analyte. The beads were resuspended in DMF by vortexing for 2 min so that the sample was easily withdrawn from the vial. Ten mg of beads contained in 0.33 mL of DMF were transferred to a tube with a cap. The tube was placed on a magnet for 4 minutes. Leaving the beads undisturbed, the supernatant was carefully removed. The tube was removed from the magnet and the beads were washed with 1 mL of freshly prepared sodium phosphate buffer (0.1 M, pH 7.4), vortexed for 30 s, and mixed for 15 min on a rotor. The tube was then placed on the magnet so that the beads were drawn to the side of the tube (not to the bottom). The buffer was removed and the beads washed again with 1 mL of 0.1 M sodium phosphate buffer, vortexed for 30 s then the supernatant buffer removed. The beads were then resuspended in 1 mL of a 5% BSA-PBS solution and allowed to shake in the rotor for about 2.5 h at 4 °C. Once the beads were coated with the BSA, the supernatant solution was removed. The BSA-precoated beads were then resuspended with a 1:10 dilution of the Ab 2621 in 2 mL of 1X PBS, pH 7.4). The beads were shaken on a rotor for ~ 3 hours. The beads were separated by use of a magnet and the clear supernatant solution was carefully removed.

Standard curve preparation—Standard curves were prepared similar a previous report [35]. Basically, various concentrations of TCSG were prepared by five-fold serial dilution of a 2000 ng/mL stock in a 10% MeOH (methanol)-PBS dilution buffer (final dilution in the plate wells being 5% MeOH-PBS). Antisera obtained from all three rabbits 2619, 2621 and 2623 were tested. Controls consisted of two negative controls that contained no antibody or no coating and a positive control in 5% MeOH-PBS that contained no analyte.. The IC_{10} and the IC_{50} values (limit of detection and assay sensitivity respectively) and the linear range of detection(IC_{20} – IC_{80}), were obtained from the calibration curve data. The data were fitted to a four-parameter logistic equation, using Igor Pro 6.22A (Wavemetrics, Inc., Portland, OR)[35].

Cross-Reactivity Determination—Coated plates (1 μg/mL in coating buffer; 100 μL/well) were incubated overnight at 4 °C. After washing 5× PBST, plates were blocked with 1% skimmed milk-PBS (350 μL/well) for an h at room temperature then washed 5× to remove excess blocking agent. Compounds tested for cross reactivity, TCSG and cross-reactants triclosan, BDE-47, from the class of polybrominated diphenyl ethers, *p*-nitrophenyl-β-D-glucuronide, 3-phenoxy benzyl alcohol-β-D-glucuronide were added (50 μL/well) starting at 2000 ng/mL, followed by a five-fold serial dilution, ending at 2×10^{-4} ng/mL. Each ELISA plate was run with TCSG and one cross-reactant, with quadruplicate wells for each concentration. Purified primary antibody (final dilution of 1:10,000, 50 μL/well) was added and incubated for 1.5 h. After washing, secondary antibody, GAR-HRP (1:5000) was added and incubated for 1.5 h. Excess conjugate was removed by washing 10× with PBST. A 100 μL of substrate solution was added to each microwell. Color development was stopped with the addition of 50 μL of stop solution after a 15 min incubation at room temperature. The cross reactivity was calculated as [(IC_{50} of target)/(IC_{50} of cross-reactant)] × 100 [35].

Matrix Effect Determination—Blank urine samples from two healthy volunteers were used in this study. Once collected, the samples were stored at −20 °C until further use. For the matrix effect study, the samples were thawed to room temperature and were vortexed well before use. Standard solution in 10% MeOH-PBS was spiked into neat urine samples to determine matrix interferences. Calibration curves (five-fold serial dilutions starting from 2000 ng/mL to 2×10^{-4} ng/mL of TCSG in 5% MeOH-PBS, final dilution in plate wells) were run in various dilutions of urine (1/2, 1/10, 1/20, 1/50, 1/100 and 1/200). A curve was also run in urine, without the addition of any solvent or buffer. IC_{50} values obtained from curves generated in each dilution of urine and from that obtained in urine alone were compared with those generated from the assay buffer (5% DMSO-PBS) [35].

Results and Discussion

Rationale for design of immunogens

Although TCSG can be directly coupled to a protein through the carboxylic acid group, direct coupling may pose some disadvantages. Steric hindrance from a bulky carrier protein is a concern because there is little space between the hapten and the carrier protein. As a result, efficient antibody recognition of TCSG may be hampered due to steric hindrance by the bulky protein. Thus, the immunogens were designed with two different commercially available heterobifunctional cross-linkers (AEDP and TFCS) to link TCSG to the large, bulky protein, Thy. In order to generate an antiserum that is selective for an analyte, the site for attachment to the protein should be distal to the portions of the structure that make it unique. Thus, the −COOH group on the glucuronide was chosen for attachment and the AEDP and TFCS linkers were used as spacers between the hapten and the protein. This precludes the formation of antibodies with high cross reactivity to other urinary phenolic glucuronides. Both AEDP and TFCS contain an amine group that can be covalently linked to the –COOH group of TCSG, and a carboxyl group that can then be conjugated to the free amino groups of the lysine residues on Thy.

Antibody characterization

Antisera collected from three rabbits 2619, 2621 and 2623 were subjected to checkerboard titration using the coating antigen. All three antisera showed significantly high titers (data not shown) indicative of the response of the rabbits to the immunogen. Next, checkerboard titrations were performed using the heterologous coating antigens shown in Fig. 3 and TCSG coupled directly to BSA. A 2-fold serial dilution of the antibody, starting from a 1000X dilution up to a 128,000X dilution, screened against a five-fold serial dilution of coating antigen (2×10^4 ng/mL to 1.3 ng/mL) was performed.

There was a low signal-to-noise ratio detected in the negative control wells that had no coating antigen but coated with 1% BSA-PBS. This was pinpointed to a sub-population of antibodies in all three rabbits that preferentially bound to the BSA. An approach to achieve target detection by the assay was to selectively eliminate the BSA binders from the rabbit sera. This was done by incubating the sera in 1% BSA-PBS for 30 min at room temperature or by purification by a magneto-precipitation technique. Once the BSA binders were removed and background was subsequently reduced, checkerboard titrations were repeated.

The optimal concentrations of antibody and the TCSG-BSA coating antigen in the assay were similar for rabbits 2619 and 2621. However, antisera from rabbit 2623 did not show any binding to TCSG except at a high coating antigen concentration of 20 g/mL (see Electronic Supplementary Material (ESM) Fig. S4). Inhibition assays involving Abs 2619 and 2621 with TCSG-BSA and target analyte TCSG gave poor assay sensitivities. For Ab 2619, no inhibition was observed. For Ab 2621, an IC_{50} of 511 ng/mL was obtained (Fig. 4A) As a result of this, antisera from rabbits 2619, 2621 and 2623 were tested for recognition and binding to other heterologous coating antigens, *5*–*8* conjugated to BSA (see Fig. 3). A coating antigen hapten more structurally dissimilar from the target was needed that could potentially enhance assay sensitivity. Of the four antigens tested, *5-BSA*, *6-BSA* and *8-BSA* showed reasonably good binding to antisera from rabbits 2619 and 2621, while antigen *7-BSA* showed no binding at all (see ESM Figs. S5 and S6). However, binding assays indicated that antisera 2623 did not bind to antigens *5*-, *7*- and *8BSA*-, but strongly bound with antigen *6-BSA*, especially at relatively high coating antigen concentrations of 100 and 10 g/mL in wells (ESM Fig. S7).

For both antisera 2619 and 2621, an inhibition screening study was performed with concentrations (100 ng/mL, 10 ng/mL and 0 ng/mL) of the target analyte TCSG with four different antisera dilutions 5000X, 7500X, 10,000X and 15,000X screened against a ten-fold serial dilution of coating antigen concentrations (from 10^4 ng/mL to 0 ng/mL). The combination of antibody and coating antigen *5* that had over 80% inhibition at 100 ng/mL target analyte concentration were screened using eleven concentrations of target TCSG. These concentrations ranged from 2×10^{-4} ng/mL to 2000 ng/mL. This dilution series was also used to assess the linear range of the assay. Wells with zero coating antigen and with zero primary antibody served as the negative controls. Wells with zero analyte concentration served as the positive controls.

Accordingly, inhibition assays run with both Abs 2619 and 2621 employing heterologous coating antigens *5-BSA*, *6-BSA* or *8-BSA* indicated that antigen *5-BSA* was the most suitable

one, giving assay sensitivities (IC_{50}) of 6.06 and 2.85 ng/mL for Ab 2619 and Ab 2621, respectively (see ESM Fig. S8 for standard curve of Ab 2619 and Fig. 4B for Ab 2621). The corresponding slopes were nearly the same for both standard curves. Following this, the antisera obtained from rabbit 2621 and coating antigen *5-BSA* were used for further immunoassay development because it was slightly more sensitive than the assay utilizing 2619. Table 1 summarizes binding and inhibition testing of the different antigens and antisera.

Assay optimization

The optimum concentrations of coating antigen *5*-BSA and Ab 2621 were 1 μg/mL and 1:5,000 dilution in the well, respectively. The optimized concentration of the secondary antibody, was 1:5000 in wells. A series of co-solvents were screened and methanol was selected to improve solubility. PBS buffer containing methanol at different concentrations was tested to assess changes in assay sensitivity. There was not much variation between the tested concentrations of methanol in PBS (5, 10, 20, 30, and 40% methanol-PBS final dilution in wells) suggesting that methanol did not affect the binding of the antibody (see Fig. S1 in the ESM). Thus, a 10% methanol-PBS in 0.15 M PBS, pH 7.4 buffer was selected to prepare the standard solutions and spiked urine samples for assay optimization (5% methanol-PBS as final dilution in wells). The linear range of detection ($IC_{20} - IC_{80}$) was 2.6 – 24.8 ng/mL, as determined from the calibration curve.

Other solvents screened were PBS, DMSO and acetonitrile. For PBS, the target analyte was dissolved in 10X PBS and then diluted to 1X PBS before running the assay. The calibration curve obtained in PBS closely matches that in 5% methanol-PBS (Fig. S2a in in the ESM). There was only partial solubility of TCSG in acetonitrile. With DMSO in PBS as assay buffer, there were greater variations between replicates and a low signal-to-noise ratio (different percentages of DMSO in PBS were tested: Figs. S3a and S3b in the ESM).

Cross reactivity

In order to determine antibody selectivity, the following compounds *9*–*14* with structural similarity to target TCSG were investigated for relative cross-reactivity: the parent aglycone, TCS, *9* and the other predominant metabolite of TCS found in human urine, TCS-*O*-sulfate, *10*; BDE-47, *11* (a congener of the flame retardant, polybrominated diphenyl ether) and TCC (triclocarban), *12*. Both *11* and *12* co-occur with TCS in the environment; *p*-nitrophenyl-β-D-glucuronide, *13* owing to the fact that the latter is also a glucuronide like the target. With *5*-BSA as the competing coating antigen, Ab 2621 showed no cross-reactivity to the tested compounds.

Ionic strength and pH

An optimum ionic strength and pH are vital for a good working assay, aiding in improving the antibody-antigen binding and minimizing interference from interfering components in the urine matrix. Two key characteristics of a urine sample are pH and urine specific gravity. An increase in urine specific gravity, which is highly solute-dependent, implies an increase in ionic strength. An inter-sample variation of these two components is normally substantial. The desired assay should be such that it is insensitive to large variations in either pH or ionic

strength. Fig. 5A and Fig. 5B illustrate the effect of ionic strength and pH, respectively, on the assay sensitivity. A broad range (ionic strength 1.6 mM to 813.5 mM PBS and pH 2 – 10) was selected for the test. A decrease in the maximum absorbance is observed with an increase in ionic strength, although this inverse relationship is not apparent with changes in pH. As is evident, in both figures, parallelism is maintained between the curves as there is no significant variation in the slopes of the curves. Correspondingly, there are no sizeable variations in the IC_{50} values. This directly suggests that the assay is quite robust, but the ideal conditions of pH and ionic strength have to be selected in order to adjust for the variations in sample conditions.

Matrix effects and assay validation

Variations in analyte reactivity in the presence of the matrix were tested by measuring the standard curves in urine spiked with the target compound TCSG. The standard curves in spiked urine obtained at different dilutions of the urine were compared with that obtained in the assay buffer (5% MeOH-PBS final dilution in wells). Fig. 6A shows the calibration curve along with various dilutions of urine in 10% MeOH-PBS (1/10, 1/20, 1/50, 1/100 and 1/200). As is evident from these curves, there is parallelism between the curves because there is a very minimal variation between their slopes. Accordingly, the corresponding IC_{50} values also show the least variation. This implies a lack of interference from the urine matrix. Also shown in Fig. 6B are the standard curve in assay buffer, 1/2 dilution of urine and a standard curve in urine itself. There is a clear parallelism between the curves, indicating that the assay is robust and is unaffected by interfering substances in the urine matrix. The limit of detection (calculated from the IC_{10}) in urine was determined to be 2.5 ng/mL.

For the recovery study, TCSG dissolved in MeOH was spiked at 2×10^{-4} to 2000 ng/mL in 100 μL of blank urine (1 in 10 dilution), and analyzed in triplicate. Table 1 shows the mean percent recovery data for the target given for three different analyte concentrations. A recovery of > 80% points to minimal interference from the urine matrix.

Conclusion

The new competitive indirect, polyclonal antibody-based ELISA presented here is useful for the selective detection of the target TCSG, a major human urinary metabolite of the biocide, triclosan. The optimized assay has an IC_{50} value of 2.85 ng/mL. A theoretically estimated value of the concentration of TCSG found in human urine is ~ 300 ng/mL, taking into account that at least three-fourths of TCS is metabolized into its major metabolites in the human body, from an oral dose, as exemplified in the study by Sandborgh-Englund et al. [30]. Thus, the sensitivity of the assay is more than adequate for use in monitoring triclosan exposure in humans. The immunoassay can be utilized for the direct detection of the glucuronide metabolite, thereby successfully eliminating the need for acid or enzymatic hydrolysis to the parent TCS for the sake of detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by the National Institute of Environmental Health Sciences (NIEHS) Superfund Basic Research Program (P42 ES004699). The heterologous haptens were generously provided by Dr. Ki Chang Ahn and Dr. Hyoungee Baek.

Abbreviations

Ab	antibody
AEDP	3-[(2-aminoethyl)dithio]propionic acid hydrochloride
BSA	bovine serum albumin
CR	cross-reactivity
DCC	dicyclohexyl carbodiimide
DMSO	dimethyl sulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
GAR-HRP	goat anti-rabbit IgG peroxidase conjugate
HRP	horseradish peroxidase
MeOH	methanol
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline plus Tween 20
TCS	triclosan
TCSG	triclosan-*O*-glucuronide
TFCS	N-[ε-trifluoroacetylcaproyloxy]succinimide ester
TMB	3,3',5,5'-tetramethylbenzidine
Thy	thyroglobulin

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Biographies

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Bruce D. Hammock is a Distinguished Professor of Entomology and Nematology, and in the University of California Davis Comprehensive Cancer Center. He is principal investigator of the UC Davis Biotechnology Training Grant and the Director of the NIEHS UC Davis Superfund Research Program. Besides leading research in advancement of immunoassay technology, he also conducts active research to understand the endogenous role of soluble epoxide hydrolase using well designed, selective substrates and inhibitors, examining mechanism of action and metabolomics as tools.

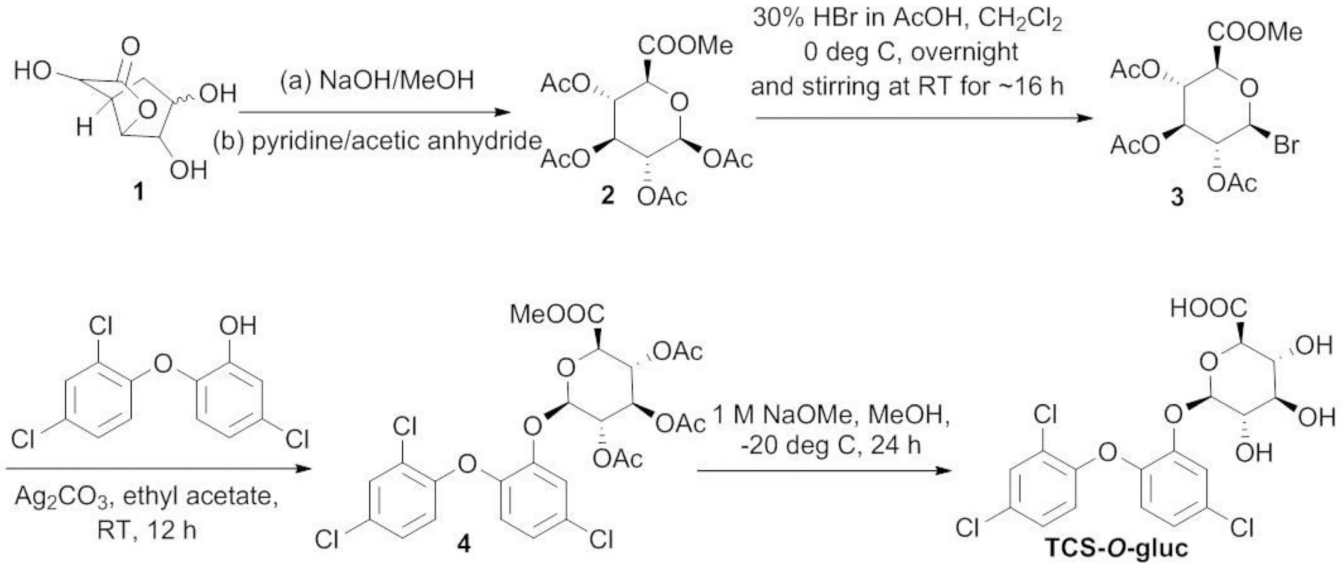

Fig. 1.
Synthetic route for triclosan-*O*-glucuronide (TCSG)

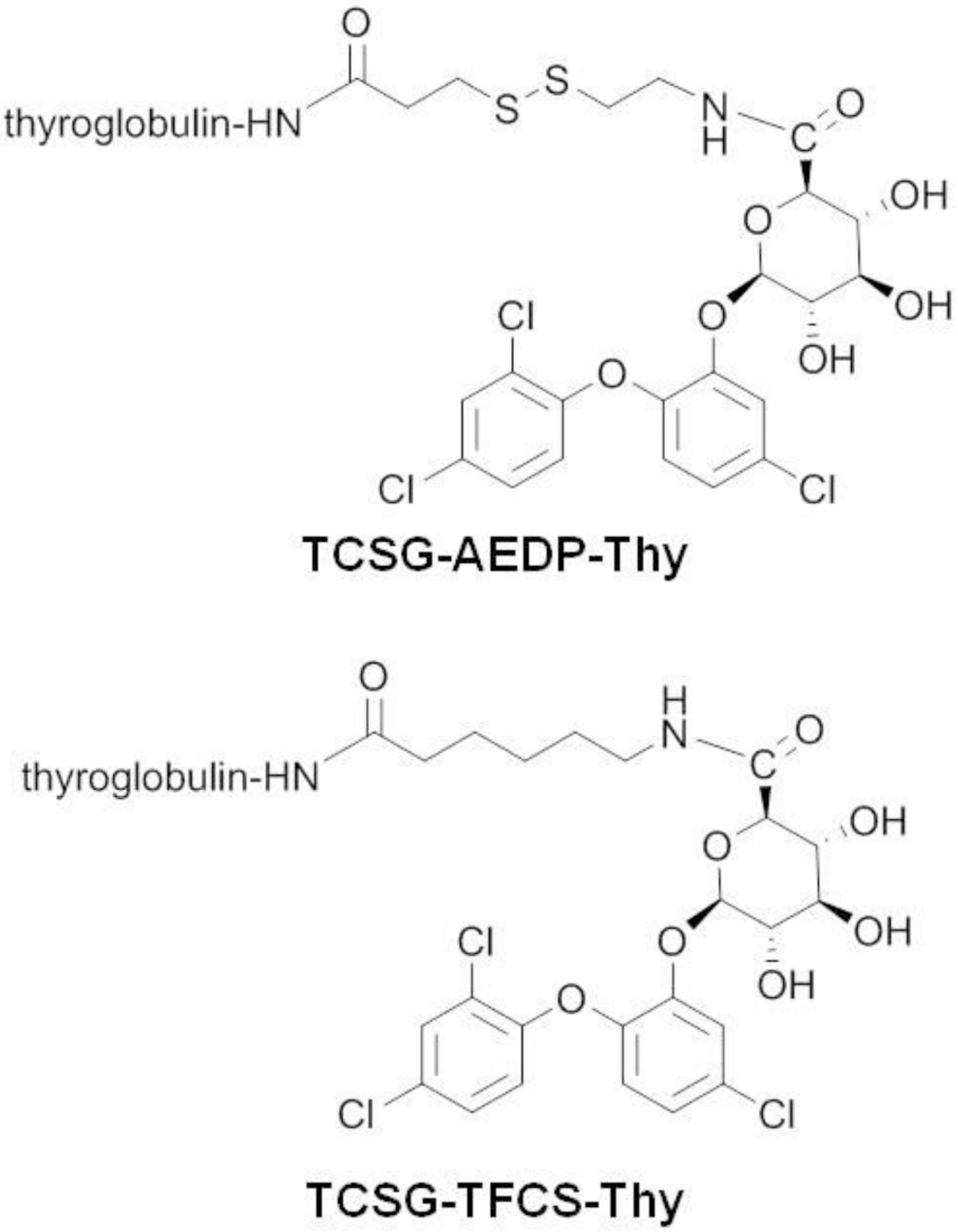

Fig. 2.
Structures of immunogens TCSG-AEDP-Thy and TCSG-TFCS-Thy.

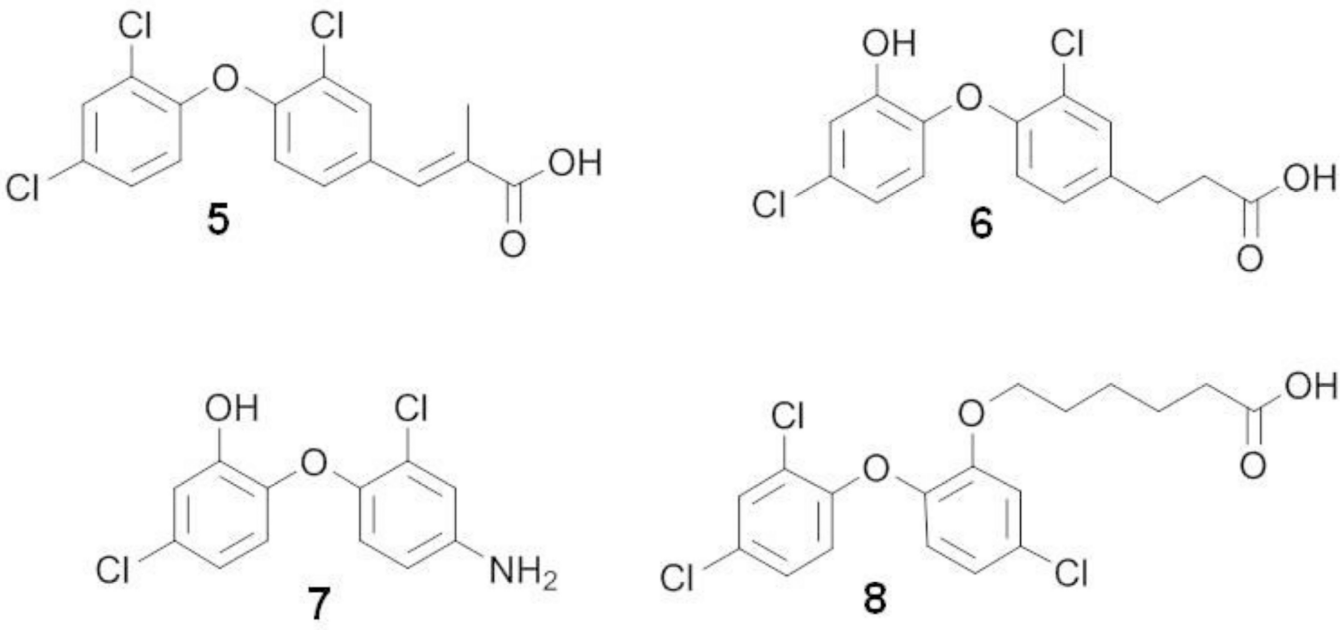

Fig. 3.

Chemical structures of heterologous haptens *5* – *8*: (*E*)-3-(3-chloro-4-(2,4-dichlorophenoxy)phenyl)-2-methylacrylic acid (*5*), 3-(3-chloro-4-(4-chloro-2-hydroxyphenoxy)phenyl)propanoic acid (*6*), 2-(4-amino-2-chlorophenoxy)-5-chlorophenol (*7*) and 6-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)hexanoic acid (*8*)

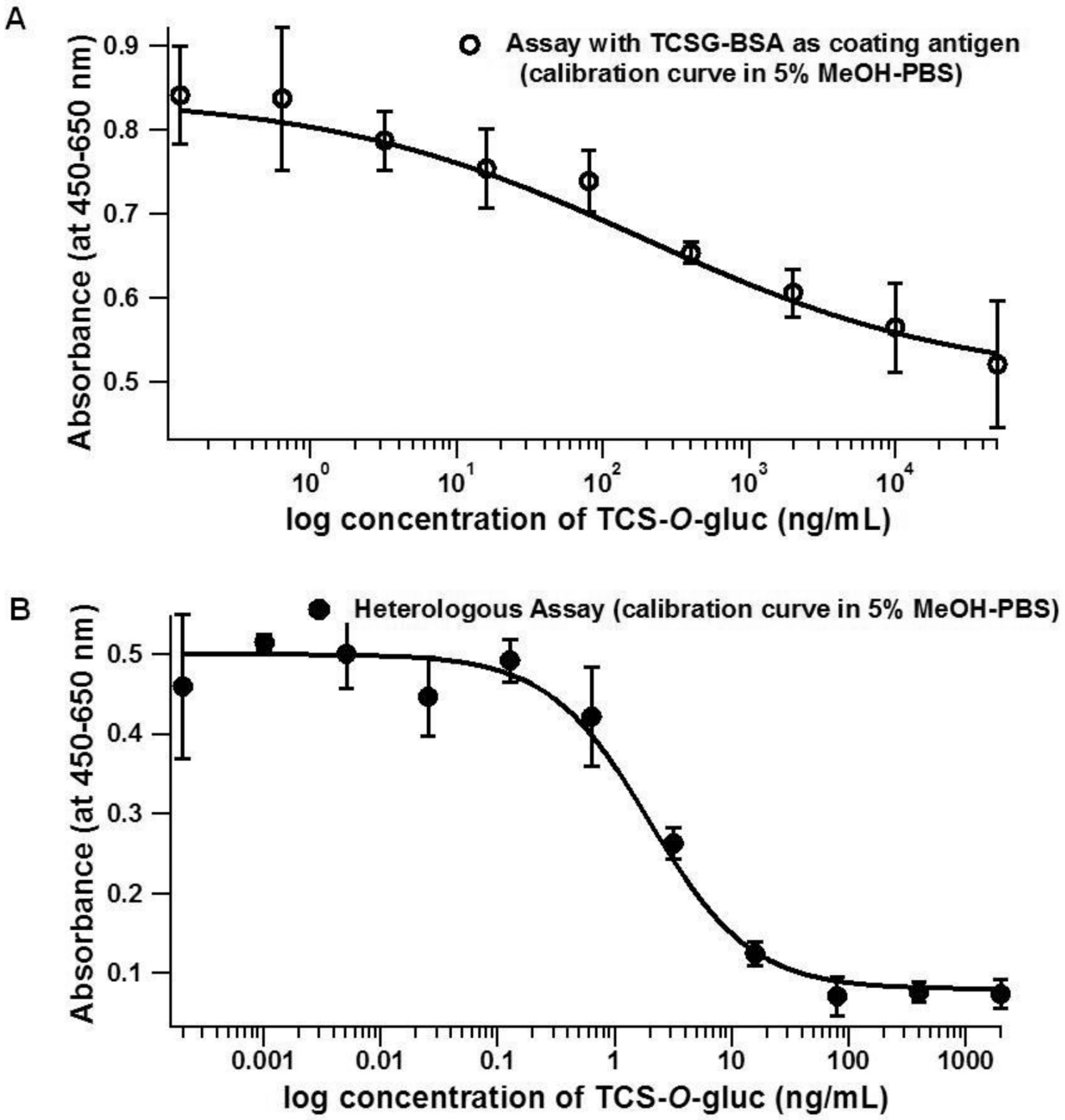

Fig. 4.

ELISA inhibition curves for TCSG using A) Reagent concentrations: antiserum 2621, 1:8,000 (final dilution in wells); coating antigen TCSG-BSA, 4 μg/mL (100 μL/well), secondary Ab – GAR- HRP, 1:10,000 dilution. Coefficient values: A_{max} 0.88, A_{min} 0.44, IC_{50} 510.9, slope 0.31 B) 5-BSA as the coating antigen: Heterologous format; reagent concentration: antiserum 2621, 1:5,000 (final dilution in wells); coating antigen - 1 g/mL (100 μL/well), secondary Ab – 1:5,000 dilution. Coefficient values: A_{max} 0.82, A_{min} 0.07,

IC_{50} 2.85, slope 1.18. Standards prepared in 10% MeOH-PBS (5% MeOH-PBS as final concentration in wells). Each data point represents the mean of quadruplicates.

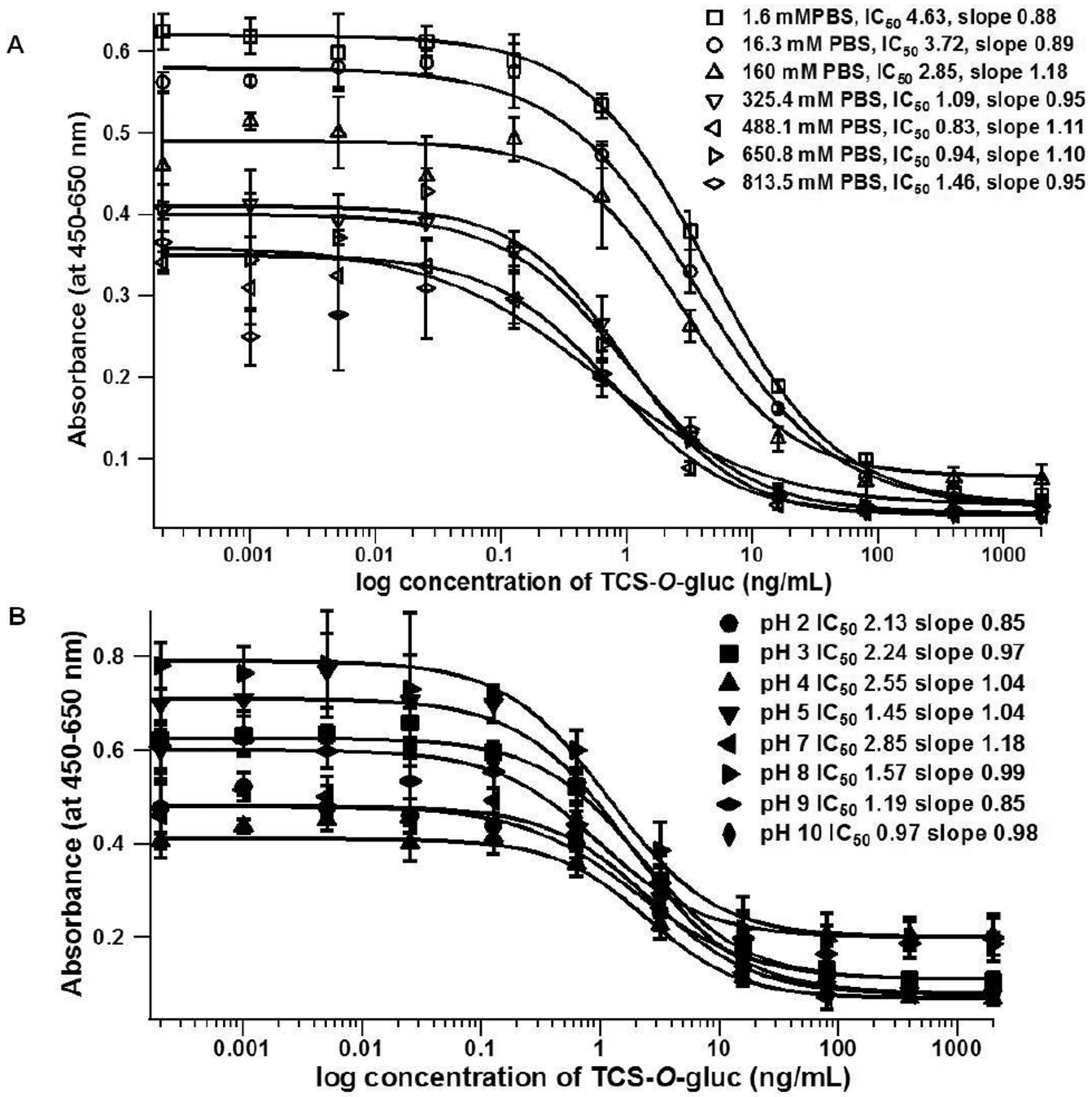

Fig. 5.

Effect of A) ionic strength (1.6 – 813.5 mM) and B) pH (pH 2 – 10) on the assay sensitivity. Standards prepared in 10% MeOH-PBS (5% MeOH-PBS as final concentration in wells). IC_{50}S and slopes indicated in the legends.

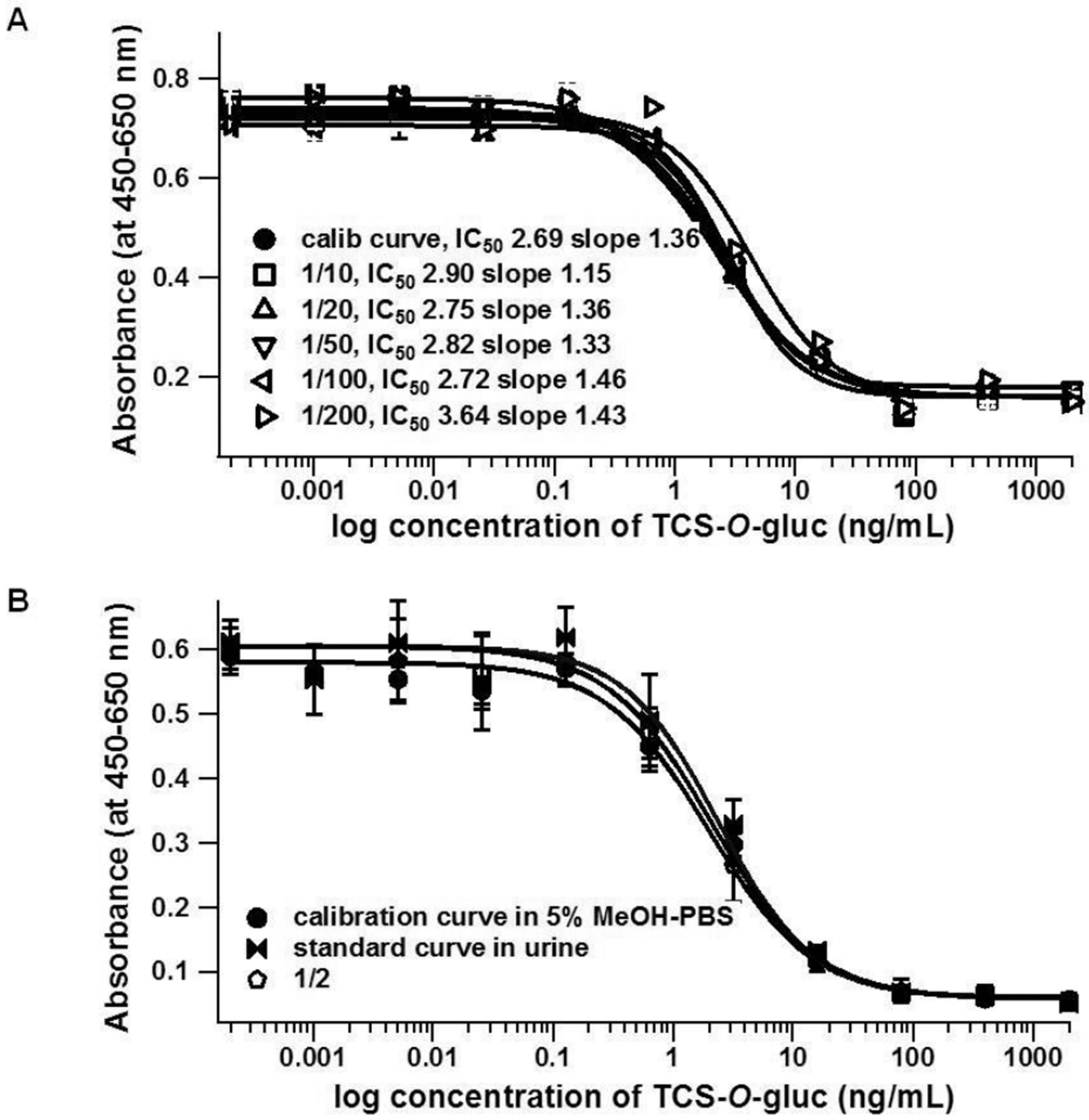

Fig. 6.

Matrix effects evaluation for blank urine spiked with target TCSG. The plate-coating antigen was 5-BSA with Ab 2621. Each data point represents the mean of quadruplicates. The calibration curve was generated in 5% MeOH-PBS (final concentration in wells). Tested dilutions were A) 1/10, 1/20, 1/50, 1/100, 1/200 and B) 1/2 and standard curve generated in the urine matrix itself.

Table 1

Recovery of TCSG from spiked urine sample

Spiked concentration[a] (ng/mL)	Detected (ng/mL)	Mean percent recovery (%) (SD)
16	20.5	128.2 (11.6)
3.2	3.2	99.6 (4.7)
0.64	0.6	93.6 (4.3)

[a] Different amounts of TCSG (in MeOH) were added to the urine matrix. The spiked urine samples were diluted 10-fold with PBS (5% MeOH) (n = 3).