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Structural considerations on the selectivity of an immunoassay for sulfamethoxazole

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ABSTRACT

Sulfamethoxazole (SMX), a sulfonamide, is a widely used bacteriostatic antibiotic and therefore a promising marker for the entry of anthropogenic pollution in the environment. SMX is frequently found in wastewater and surface water. This study presents the production of high affinity and selective polyclonal antibodies for SMX and the development and evaluation of a direct competitive enzyme-linked immunosorbent assay (ELISA) for the quantification of SMX in environmental water samples. The crystal structures of the cross-reacting compounds sulfamethizole, N^4-acetyl-SMX and succinimidyl-SMX were determined by x-ray diffraction aiming to explain their high cross-reactivity. These crystal structures are described for the first time. The quantification range of the ELISA is 0.82–63 μg/L. To verify our results, the SMX concentration in 20 environmental samples, including wastewater and surface water, was determined by ELISA and tandem mass spectrometry (MS/MS). A good agreement of the measured SMX concentrations was found with average recoveries of 97–113% for the results of ELISA compared to LC-MS/MS.

1. Introduction

Among the numerous pharmaceutical compounds found in groundwater, surface water and wastewater, antimicrobially active compounds [1,2] elicit more concern due to rising antibiotic resistance observed in hospitals [3,4]. Moreover some compounds may cause allergic reactions in predisposed individuals [5]. The high consume of antibiotics and their prevalence in the water cycle support the development of resistances against SMX [6]. Sulfamethoxazole is a bacteriostatic antibiotic and often used in combination with trimethoprim (e.g. Cotrimoxazole, "Cotrim forte"). It is prescribed mostly to treat urinary tract infections. SMX is a frequently used sulfonamide. Although its concentrations vary between countries, the detection frequency is often 100% for effluent wastewater [7–9]. This demonstrates the high stability of SMX and thus SMX has been proposed as a marker for the entry of pharmaceuticals in the environment. Sulfamethoxazole is detectable in different waters like surface, ground, drinking and wastewater [10]. The measured concentrations vary for influents of wastewater treatment plants (WWTPs) in a range of 8–3180 ng/L [9,11] and for effluents from 243 to 2000 ng/L [8,11]. The removal rate in the WWTPs is influenced by the type of wastewater treatment [11] and ranges from −7.5% to 88% [9,11]. Due to the incomplete removal in WWTPs, it can be also detected in surface waters. The measured concentrations are generally lower, 3.6 ng/L have been reported for France [12] and 30–85 ng/L for Germany [13], but can exceed 4870 ng/L, as in China [7]. Kolpin and co-workers found SMX in 13 of 104 water streams in the United States with a mean of approximately 150 ng/L and a maximum concentration of 1900 ng/L [14]. SMX was also detected in ground water [15] and in a concentration of 12 ng/L in drinking water [16].

About 85% of ingested SMX molecules are metabolized in the human body and thus in urine and faeces only 15% of the parent compound remains unaltered [1]. Known metabolic products are N^4-hydroxy-, 5-methylhydroxy-, N^4-acetyl- and N^1-glucuronide-sulfamethoxazole [17].

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In view of the SMX concentration range of ng/L up to μg/L in environmental samples, sensitivity is an important issue. Conventional chromatographic or electrophoretic methods minimize matrix effects due to a separation of sample components by high performance liquid chromatography (HPLC) [18] or capillary electrophoresis (CE) [19]. Detection is then performed by amperometry [19], mass spectrometry (MS) [1,20], fluorescence [21] or UV absorbance measurements [22,23]. By using LC-UV the limit of detection (LOD) in pure water, after enrichment by using solid-phase extraction (SPE), is 7.5 μg/L [24], which is typically unsatisfactory. The most common method, due to high sensitivity and selectivity, is the coupling of HPLC to MS/MS. LODs of 0.2 ng/L in pure water, 0.6 ng/L in river water samples and 1.7 ng/L SMX in tap water have been reached [11,12]. This sensitivity presupposes a pre-concentration step such as SPE performed separately or in-line [12]. For SPE different sorbent materials with good recovery rates for SMX have been identified. Examples are Oasis® Hydrohilic-Lipophilic-Balance (HLB) (71–72%) [24], Spark Holland C18 High Density (HD) (94–98%) [12] or Chromabond® EASY (90%) [25] phases. A pre-concentration is often used for quantification of SMX in surface or drinking water [26–29]. Expensive instrumentation, high maintenance costs and the requirement of specifically trained analysts make common methods like mass spectrometry unattractive due to financial restrictions. Immunochemical methods such as enzyme-linked immunosorbent assay (ELISA) have been described as cheap and easy-to-perform methods along with their usefulness in environmental screening and monitoring [30,31]. ELISAs based on the selectivity and affinity of an antibody for its respective antigen can be a valid alternative approach for the quantification of environmental analytes. The advantages of immunochemical methods are high specificity, short analysis time, high throughput of samples, low detection limits and cost effectiveness [32–34]. Immunoassays for sulfonamides including sulfamethoxazole and their application for environmental samples have been described before [35–39]. Liu et al. [35] have described a rabbit monoclonal antibody, which was used to measure sulfathiazole, sulfadiazine, sulfapyridine and sulfamethoxazole in swine urine and milk samples. The cross-reactivities for these four sulfonamides were 100%, 58.8%, 61.1%, 12.8% and for sulfamethizole 0.2%, respectively. The concentration of SMX which inhibited the signal development half (IC_{50}) was 5.27 μg/L. To show the performance of the ELISA, urine and milk samples were spiked with the four sulfonamides to levels of 25–100 μg/L, centrifuged and diluted 20-fold with PBS buffer to minimize matrix effects. The measured recoveries were in a range of 61–111%. It should be mentioned that these concentration levels are very high compared to levels in environmental samples. Results of real water samples from the Czech Republic have been presented by Černoch et al. [37], who used polar organic chemical integrated samplers (POCIS) for sample taking. POCISpest, a triphasic sorbent consisting of Isolute ENV+ polystyrene divinylbenzene and Ambersorb 1500 carbon dispersed on S-X3 Biobeads was employed. By using this kind of pre-concentration, a good correlation between measured SMX concentrations by ELISA and LC-MS/MS in wastewater samples was observed. However a slight overestimation occurred in ELISA. The results of measurements of surface waters showed a bad correlation (Pearson $R^2=0.11$ and 0.18) for ELISA and LC-MS/MS results. An example for direct quantification of SMX in water samples was given by Shelver et al. [36]. In that study an ELISA for SMX was presented with an IC_{50} of 0.255 μg/L for SMX. This high sensitivity allowed to quantify SMX in wastewater from an US American WWTP directly. The measured concentrations in influent wastewaters measured by ELISA were in a range of 1.4–2.5 μg/L and in effluent wastewaters of 1.1–3.0 μg/L. The measured concentrations by ELISA were, compared to LC-MS/MS, on average 1.98-fold higher for influent wastewater and 1.55-fold higher for effluent wastewater. Zhang et al. [38] presented an ELISA with an IC_{50} of 0.163 μg/L for SMX. Obtained concentrations by ELISA were compared to LC-MS/MS on average 1.3-fold higher for influent wastewater and 1.65-fold higher for effluent wastewater. In this study only filtration was applied for sample preparation. Several studies demonstrated that the hapten structures used for the synthesis of immunogens were critical for the selectivity and sensitivity of these assays [40–43]. A comprehensive work, giving credit to previous works by other authors, was presented by Wang et al. [44]. They showed that single-ring sulfonamide hapten conjugates provided in the mouse lower antibody response and less selective antibodies than two-ring hapten conjugates with benzyl or heterocyclic rings at the N1-position. The immunization with an SMX-bovine serum albumin conjugate, synthesized by the diazo method, provided antibodies with a high affinity against sulfamethoxazole and sulfathiazole, resulting in recognition of sulfachloropyridazine, sulfadiazine, sulfamethoxypyridazine and sulfa-merazine.

2. Material and methods

2.1. Materials

The following chemicals and materials have been used: ammonium acetate (NH_4Ac, Fisher Chemicals, Schwerte, Germany, analytical reagent grade, 99.3%), acetic acid glacial (AcOH, Fisher Chemicals, analytical reagent grade, 99.83%), methanol (MeOH, J. T. Baker, HPLC gradient grade), 3,3′,5,5′-tetramethylbenzidin (TMB, Serva, research grade), Tween 20 (Serva, Heidelberg, Germany), 4-methylmorpholine (98%, Fluka), peroxidase from horseradish (HRP, > 90% isoenzyme C, Roche, Mannheim,), tris(hydroxymethyl) aminomethane (Tris, > 99.8%, Merck, Darmstadt, Germany), humic acid sodium salt (HA, 45–70%, Roth, Karlsruhe, Germany), succinic anhydride (> 97%, Fluka), *N*-hydroxysuccinimide (NHS, Merck), sodium hydrogen carbonate (> 98%, Fluka), SMX-d4 (Campro Scientific, Berlin, Germany), untreated, transparent 96 flat-bottom well microtiter plates (Nunc, Thermo Scientific, Rockford, IL, USA), transparent 96 flat-bottom well microtiter plates with high binding capacity (Greiner Bio-One, Frickenhausen, Germany), folded filters (qual., grade 1288, Sartorius stedim, Göttingen, Germany), PD-10 columns (Healthcare Bio-Sciences, Freiburg), Strata™-X 33 μm Polymeric Reversed Phase SPE cartridges (500 mg, 6 mL, Phenomenex, Aschaffenburg, Germany), anti-rabbit IgG antibody (R1364P, LOT 27,160, 2 mg/mL, Acris Antibodies GmbH, Herford, Germany). Hemocyanin from keyhole limpet (KLH, from *Megathura crenulata*), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 99%), sodium phosphate dibasic dihydrate (> 99%), sodium phosphate monobasic dihydrate (> 99%), sodium chloride (> 99.5%), sodium citrate monobasic (> 99%), potassium sorbate (> 99%), potassium phosphate dibasic (> 99%), glycine (> 99%), tetrabutylammonium borohydride (TBABH, > 98%), *N*,*N*-dimethylacetamide (DMA, > 99.8%), *N*,*N*-dimethylformamide (DMF, > 99.8%), isobutyl chloroformate (98%) and sulfamethoxazole (analytical standard) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Instruments were a Milli-Q water purification system (Milli-Q Synthesis A10, Millipore, Schwalbach, Germany) for ultrapure water, a microplate UV/VIS reader (SpectraMax® Plus 384, Molecular Devices, Biberach an der Riss, Germany), an automatic washer (BioTek ELx405 Select™, Bad Friedrichshall, Germany) and a plate shaker (Titramax 100, Heidolph, Schwabach, Germany).