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Flow cytometric immunoassay for sulfonamides in raw milk

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ABSTRACT

Sulfonamide antibiotics are applied in veterinary medicine for the treatment of microbial infections. For the detection of residues of sulfonamides in milk, a multi-sulfonamide flow cytometric immunoassay (FCI) was developed using the Luminex MultiAnalyte Profiling (xMAP) technology. In this automated FCI, a previously developed biotinylated multi-sulfonamide mutant antibody (M.3.4) was applied in combination with fluorescent beads, directly coated with a sulfathiazole derivative, and streptavidin–phycoerythrin (SAPE) for the detection. With this FCI, at least 11 different sulfonamides could be detected (more than 50% inhibition at the 100 ng mL^{-1} level) and, after an incubation of 1 h, measurements were rapid (10 s per sample). For the application with raw milk, a 96-well microplate-based filtration step was included into the protocol to remove disturbing milk fat particles. Because of differences in sensitivity towards different sulfonamides, the FCI was considered and validated as a qualitative screening assay. For sulfadoxine, the most applied sulfonamide in Dutch dairy cattle, the detection capability (CCβ) was <50 μg L^{-1} and this level seems feasible for five other sulfonamides. For sulfadiazine, the CCβ was <200 μg L^{-1} and this level seems feasible for four other sulfonamides. A major advantage of the applied xMAP-technology, with its 100 different color-coded bead sets, is the possibility to develop multiplex immunoassays for the simultaneous detection of several antibiotics.

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1. Introduction

Sulfonamide antibiotics (sulfonamides) are used in veterinary and human medicine for the treatment and prevention of microbial infections. For the medication of cows, five sulfonamides, including sulfachloropyridazine, sulfamethoxazole, sulfamethazine, sulfadoxine and sulfadiazine, are approved in The Netherlands of which sulfadoxine and sulfadiazine for medication of lactating cows [1–3]. If withdrawal periods are disobeyed after administration, milk from medicated animals may contain sulfonamide residues and the European Union (EU) established a maximum residue limit (MRL) of 100 μg L^{-1} for the total amount of sulfonamides in milk [4].

For the detection of sulfonamides, a number of different methods based on different technologies (e.g. microbial inhibition assays, immunoassays, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS)) have been described [5]. The chromatography-based methods are laborious and/or expensive and more suitable for confirmation

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(especially LC–MS). The microbial inhibition assays and immunoassays are screening methods of which the former are cheap, have a broad specificity but a relatively large assay time (4–6 h) and the latter are more (group) specific and faster. Nowadays, one of the most rapid immunoassay technologies applies a commercially available automated surface plasmon resonance (SPR)-based biosensor (Biacore). For the fast detection of sulfonamides (5–10 min per sample), applications were described for the determination of sulfamethazine residues in milk [6,7], the detection of sulfamethazine and sulfadiazine in pig bile [8,9] and for the multi-sulfonamide detection in chicken serum [10–12] in which a recombinant multi-sulfonamide antibody (M.3.4) was selected as most sensitive towards most of the sulfonamides tested [11–13]. However, these biosensors are expensive, limited in high volume testing (6–12 samples per hour) and limited in multiplex detection (simultaneous measurements can be performed in 1–4 flow channels).

In the present study, the multi-sulfonamide mutant antibody was used to evaluate a new multiplex screening technology which consists of flow cytometry in combination with the MultiAnalyte Profiling (xMAP) technology of Luminex [14]. This technology uses small carboxylated polystyrene microspheres (5.6 μm beads), which are internally dyed with a red and an infrared fluorophore. By varying the ratio of the two fluorophores, up to 100 different color-coded bead sets can be distinguished, and each bead set can be coupled to a different biological probe which, in principle, makes it possible to simultaneously measure up to 100 different biomolecular interactions in a single well. The carboxylated bead surface allows simple chemical coupling of capture reagents such as antibodies, oligonucleotides, proteins, peptides or receptors. For the coating of the beads with proteins, standard procedures are available [15]. This procedure to prepare protein-coated beads was previously applied during the development of a triplex inhibition immunoassay for the simultaneous detection of cheaper plant proteins (soy, pea and soluble wheat proteins) as potential fraudulent adulterants in milk powder [16]. For the coating with low molecular weight compounds, the compound is usually conjugated to a carrier protein. Such procedures are described for the detection of pesticides [17] and the thyroid hormone thyroxine [18]. In the present sulfonamide assay, the beads were coated with a sulfathiazole derivate (N^1-[4-(carboxymethyl)-2-thiazolyl] sulfanilamide (TS)) conjugated to ovalbumin (Oval-TS). The performance of these beads was compared with beads directly coated with TS (without the carrier protein). Previously, such a direct coupling to carboxymethylated biosensor chips resulted in an improved assay with a more stable surfaces, suitable for hundreds of cycles, and with a higher hapten density [11]. The direct coupling via the carboxyl group of TS to the carboxylated bead surface was performed as described for the coupling to the carboxylated biosensor chips [11] using ethylene diamine (EDA) as spacer.

The coated beads were used to capture the biotinylated mutant antibodies (M.3.4) of which the binding was inhibited by the presence of analytes (sulfonamides) in the sample (inhibition immunoassay format). Detection and quantification of the immunocomplex was obtained via a reporter molecule (a fluorophore). A dual laser instrumentation system allowed both the identification of the color-coded bead set by its characteristic color (red laser) and the quantification of reporter molecules bound to the beads (green laser). In this model assay, only one bead-set was used for the detection of one group of analytes, the sulfonamides. However, in the future, multiplex assays with multiple bead-sets can be developed for the simultaneous detection of other antibiotics. The performance of the sulfonamide assay in this new format was evaluated for application in raw milk, most challenging and relevant for the dairy industry, and validated as a qualitative screening method according to the validation procedures prescribed in the European Commission Decision 2002/657 [19].

2. Materials and methods

2.1. Materials and instruments

Sulfatroxazole was a gift from Leo Pharmaceutical Products (Weesp, The Netherlands). All other sulfonamides as well as the aminoglycosides, quinolones and trimethoprim were obtained from Serva (Heidelberg, Germany) or Sigma–Aldrich (Zwijndrecht, The Netherlands). The HBS-EP buffer and an amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)) were supplied by GE Healthcare (Uppsala, Sweden). Ethylenediamine (EDA), dimethyl sulfoxide (DMSO), sodium carbonate (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$), Tween-20 and sodium azide (NaN_3) were obtained from VWR International (Amsterdam, The Netherlands). Streptavidin *R*-phycoerythrin conjugate (SAPE) was from Molecular Probes (Leiden, The Netherlands), bovine serum albumin (BSA) from Sigma–Aldrich and EDC, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were from Pierce (Rockford, IL, USA).

Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK) delivered the Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY Platform, which was programmed to analyze a 96-well plate. The system was operated with StarStation System control software. This company also supplied Luminex carboxylated microspheres no. 015 (beads), in a stock bead suspension (1.25×10^7 beads mL^{-1}) which was stored at 4–6 °C, and so-called sheath fluid to run samples on the analyzer.

Protein LoBind Tubes (1.5 mL) were supplied by Eppendorf (Hamburg, Germany). Flat-bottom 96-well filter-bottom microplates equipped with non-sterile, 1.2 μm hydrophil low protein binding Durapore® Membrane, were obtained from Millepore (Billerica, USA). Acrodiscs equipped with 1.2 μM Versapor® Syringe Filter Membrane were purchased from Pall (Ann Arbor, USA). The Snijder test tube rotator was obtained via Omnilabo International (Breda, The Netherlands). The Eppendorf centrifuge 5810R and the ultrasonic cleaner were from VWR International and the microtiter varishaker from Dynatech Lab. (Guernsey, UK).

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