Detection of sulfamethazine in water, milk and pig manure by dipstick immunoassay

V.B. Kandimalla, N. Kandimalla, K. Hruska, M. Franek

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: During the past few years, there has been an increasing interest in rapid visual tests that could be performed outside the laboratory, for example on farms, in store houses or in food production plants. Hence, cost effective and simple screening methods are required for residual analysis of environmental and food samples on-site. Here, a simple and instrumental independent dipstick immunoassay for sulfamethazine detection is described. The polyclonal antibody was optimised in terms of coating dilution on a nitrocellulose membrane, dilution of peroxidase tracer conjugate, blocking agents and incubation times. Test results assessed by visual measurement can be available within 20 minutes. In buffer, water, skimmed milk and pig manure extract, sulfamethazine fortified at 50 and 100 µg/l has exhibited clear visual differentiation in colour development (lower intensity) in comparison to the control spot intensity (high intensity) of the dipstick.

Keywords: antibody; sulfonamides; residues

Sulfonamides are synthetic bacteriostatic agents that share a common *p*-aminobenzoyl ring with an aromatic amino group at the N4-position and differ in the substitution at the N1-position. Sulfonamides are widely used in veterinary medicine for the treatment of bacterial infections and until recently as feed additives and growth promoters. As a result, sulfonamides can occur in food products of animal origin such as milk, edible tissues and also in the environment. The presence of sulfonamide residue levels above the maximum residue limit (MRL) in food may be considered as harmful to consumers. According to legislation of European Union, the MRL for the total amount of sulfonamides in edible tissues is 100 µg/kg (EU Regulation 508/1999). Moreover, the emergence and spread of antibiotic resistant human pathogens have been directly linked to the use of antibiotics in animal husbandry (Botsoglou and Fletouris, 2001; Novarro et al., 2004).

Traditional analytical methods for the determination of sulfonamides include thin layer and liquid chromatography, microbial assays, immunoassays based usually on the ELISA technique and recently tandem mass spectroscopy methods such as LC-MS/MS. The commercially available surface plasmon resonance immunobiosensor (Biacore^{TM}) was used for the quick detection of sulfonamides in milk, pig bile and chicken serum (Haasnoot et al., 2005). Although instrumental techniques provide low detection limits, they are also time consuming, laborious and expensive. On the other hand, immunochemical methods have advantages such as high selectivity, simplicity and cost effectiveness, which make them particularly useful in routine screening analysis within the laboratory or on-site.

Since the 1990's several sensitive ELISA methods and kits have been developed for individual sulfonamides, most of them are quantitative or semi quantitative (Franek et al., 1999, 2006; Haasnoot et al., 2005; McGrath et al., 2005). Major limitations of on-site ELISA application are long incubation times and the requirement of skilled personnel. In recent years, there has been growing interest in developing low cost dipstick immunoassays for the rapid detection of environmental and food contaminants. These assays were based on the immunological

Supported by the Ministry of Agriculture of the Czech Republic (Grant No. MZE 0002716201).

principle that utilises an antibody coated nitrocellulose membrane for the antigen-antibody reaction. The dipstick immunoassay provides a visual assay result by indication of a colour band on the membrane support. Dipstick formats are user friendly, relatively inexpensive and ideal for on-site testing by minimally trained personnel. The dipstick assays do have major limitation: they generally only deliver a qualitative result, i.e. "yes" or "no" answer detected by the unaided eye. However, for an on-site decision or before expensive instrumental analysis, dipstick assays, as other immunoassays, are particularly advantageous due to the elimination of negative samples from further analysis. A modified format of the membrane dipstick assay is lateral-flow dipstick technology. The lateral-flow dipstick assays are faster than the membrane based dipstick assays and their main advantage is one step assay performance without washing. O'Keeffe et al. (2003) reported a lateral flow immunoassay for the detection of sulfamethazine in urine samples allowing the detection of this sulfonamide within the range of 1–10 ng/ml. Another lateral dipstick assay (Campbell et al., 2007) developed for feed additive nicarbazin showed the detection range of 1–150 ng/ml and a similar sensitivity of the membrane dipstick was achieved for the detection of herbicide 2,4-D (Coung et al., 1999). In recent years, both technologies have been employed for the screening of numerous analytes and pathogens, (Coung et al., 1999; O'Keeffe et al., 2003; Peruski and Peruski 2003; Pal et al., 2004; Delmulle et al., 2005).

The present work demonstrates the applicability of a simple dipstick assay for the qualitative screening of sulfamethazine (SMZ) in three different matrices: water, milk and pig manure.

MATERIAL AND METHODS

Chemicals and biochemicals

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) dioctyl sulfosuccinate sodium salt (98%), horseradish peroxidase (HRP), hydrogen peroxide (H_2O_2) and sulfamethazine (sulfadimidine) were procured from Sigma (St. Louis, USA). 3,3'5,5'-tetramethylbenzidine (TMB) was from Serva (Heidelberg, Germany). All chemicals were of analytical grade. The nitrocellulose membrane and (0.45 µ pore size, Product No: N8142-10EA) and syringe filters (32 mm PF filter) were procured from Sigma-Aldrich, USA and the PALL Gelman Laboratory, USA, respectively. Preparation of the rabbit antibody against sulfamethazine and the HRP conjugate are described in a previous paper (Franek et al., 1999). The antibody used in the optimised ELISA exhibited 50% binding inhibition in buffer at approximately 0.15 µg/l as well as 100% cross-reactivity between sulfamethazine and its major metabolite N4 acetyl-sulfamethazine, slight cross reactivity with sulfamerazine (5.2%) and a negligible reaction with other related antimicrobials.

Buffers and solutions

The following buffers and solutions were used: the coating buffer was 50 mmol/l carbonate buffer (pH 9.6), phosphate buffer saline was composed of 10 mmol/l phosphate buffer (pH 7.2) with 145 mmol/l NaCl. The blocking solution was PBS with 1.5% BSA. The HRP conjugate (tracer) was diluted in PBS with 0.5% BSA. The washing buffer was PBS with 0.05% Tween-20 (PBST). The substrate solution contained 4.6 ml TMB solution (6 mg/ml in DMSO), 0.55 ml H_2O_2 (1.0% v/v) and 1.15 ml dioctylsulfosuccinate (100 mg/ml ethanol) in 43.7 ml of 1 mol/l, pH 5.5 sodium acetate buffer.

Preparation of test strips

Nitrocellulose membrane was cut into 0.5 × 1.5 cm pieces, 2 µl of antibody (diluted 1:250 with coating buffer) was coated on a strip at two sites using a micropipette (Figure 1) and incubated at 4°C for three hours. For the blocking of non-specific binding sites, the nitrocellulose membrane was placed in a Petri dish that contained 10 ml of blocking solution and was gently agitated for 1.5 h at room temperature. After washing with PBST (two times) and PBS (once), strips were air dried for 15 min and cut into two equally sized pieces (one for testing and the other for comparison) and stored at 4°C until further use.

Assay performance

Test and control strips were immersed in 1.0 ml of sample solution and PBS, respectively, for 8 min. After incubation, strips were washed with PBST (two times) and PBS (one time). Both strips were

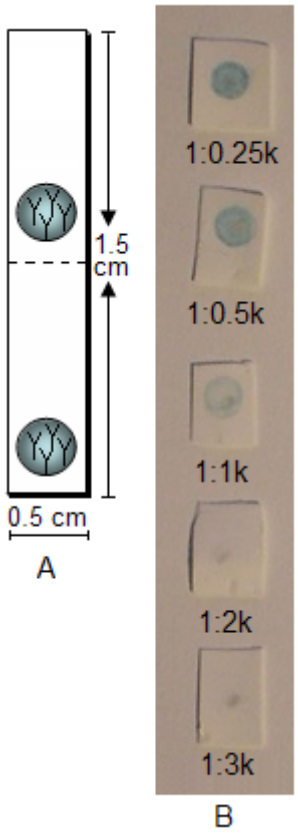

Figure 1. (A) Schematic diagram of dipstick and (B) dot blot intensity at different concentrations of antibody in combination with 1:4 000 HRP-tracer

then dipped into the Eppendorf tube containing 1.0 ml of tracer solution (diluted 1:4 000) with intermittent shaking for 8 min. After washing, both the strips were immersed into 4.0 ml of substrate solution and incubated for colour development for 4 min. The resulting blue colour dot blot/spot intensity is inversely proportional to the concentration of sulfamethazine.

Sample preparation

Milk. Skimmed milk was purchased from a local milk shop. The milk was centrifuged (1 250 × g, 10 min, 4°C), the upper fat layer was removed and the defatted milk was used for fortification. The stock solution of sulfamethazine in methanol (0.1 mg/ml) was mixed with the defatted milk to obtain the concentrations of 0, 20, 50,100, 1 000 and 5 000 ng/ml. The solutions were thoroughly stirred, allowed to stand for at least 1 h and subjected to the dipstick analysis.

Water. Water samples were collected from the River Svratka, Brno-Modrice, Dubnany-Agro Krasno and Brno Dam, Brno, Czech Republic. After filtration through a syringe filter, the pH of water samples was adjusted to 7.5. The samples were spiked with sulfamethazine to obtain the concentrations of 0, 20, 50, 100, 1 000 and 5 000 ng/ml and subjected to dipstick analysis.

Pig manure. Pig manure samples were collected from a pig house in the Veterinary Research Institute, Brno. Sulfamethazine free manure was spiked with sulfamethazine to obtain the concentrations of 0, 20, 50, 100, 1 000 and 5 000 ng/g. The samples were kept at room temperature for one hour. Then, 1 ml of methanol-water (50:50 v/v) was added and samples were incubated at room temperature for 1 h while shaking. After incubation, samples were centrifuged (2 800 × g, 10 min) and the supernatant was subjected to dipstick analysis.

RESULTS AND DISCUSSION

Attempts to establish a group specific (generic) dipstick assay for sulfonamides were not successful when generic antibodies, previously developed in this laboratory (Franek et al., 2006), were used for the antibody coated nitrocellulose membrane preparation. The sensitivity of the generic antibody on the membrane toward sulfonamides was reduced by 2–3 orders of magnitude in comparison to conventional competitive ELISA. Therefore, a more sensitive antibody against sulfamethazine was employed to achieve the desired assay parameters. The improved sensitivity of the dipstick was reached using a combination of the immunoreagents developed for the sulfamethazine ELISA (Franek et al., 1999). The dipstick assay was optimised in terms of the immunoreagent concentration, blocking agents and incubation times in order to develop intense dot blots on a nitrocellulose membrane for the visual detection test for sulfamethazine.

A three hour incubation at 4°C was sufficient to achieve the optimised antibody immobilisation on the nitrocellulose membrane (data not presented). Additional prolongation of incubation time (up to 12 h) did not significantly influence the immobilisation yield. With an increase of antibody and tracer concentrations, the dot blot intensity was increased to yield the maximum colour development. The optimal antibody and tracer dilutions

for the maximum colour intensity were 1:250 and 1:4 000, respectively.

In order to achieve maximal differentiation of the spot intensity between different analyte concentrations, non-specific binding on the membrane surface must be minimised. Therefore, the effectiveness of glycine, skimmed milk and BSA was studied as blocking solutions to reduce the non-specific interactions. It was found that glycine at the concentrations of 2, 4 and 6% w/v exhibited poor blocking efficiency at room temperature, whereas BSA and skimmed milk were shown to be effective blocker agents with good blocking efficiency. However, skimmed milk treated strips at 3%, 5% and 10% concentrations resulted in lower colour development when compared with BSA. It can be noted that BSA might be influencing the enzymatic reaction between the HRP and substrate by providing some favourable conditions to the HRP. Previous reports also suggested that some of the inert proteins could stabilise the active enzymes (Okawa et al., 1999; Kandimalla et al., 2006). Okawa et al (1999) reported that BSA can improve the thermostability of the enzymes. The thermostability of glucose oxidase activity was improved after co-immobilisation with inert proteins inside the sol-gel (Kandimalla et al., 2006). Based on the comparison of blocking efficiency in PBS, 1.5% BSA was chosen as a suitable blocking solution. Optimal incubation time for the blocking step was 1 h at room temperature.

In following experiments, the cross-reactivity among sulfamethazine and six related sulfonamides (Figure 2) was tested in the optimised dipstick system. However, only visual evaluation of the colour development among selected sulfonamides was used to estimate cross-reactivity responses among tested sulfonamides (Figure 3). The slight cross-reactivity (colour interference) was observed for sulfamerazine whereas additional tested sulfonamides did not exhibit any visible reaction. It should be noted that the antibody used for immobilisation on the polystyrene surface of the microtitre plate exhibited around 100% cross-reactivity between sulfamethazine and its major metabolite N^4-acetyl derivative and a relatively slight cross-reaction with sulfamerazine (cca 5%, Franek et al., 1999). The ELISA cross-reactivity pattern is comparable with the presented dipstick using the same antibody; therefore, a strong response towards acetyl sulfamethazine can also be expected for this membrane based assay. However, experimental data based on this assumption has not been obtained due to a lack of the compound.

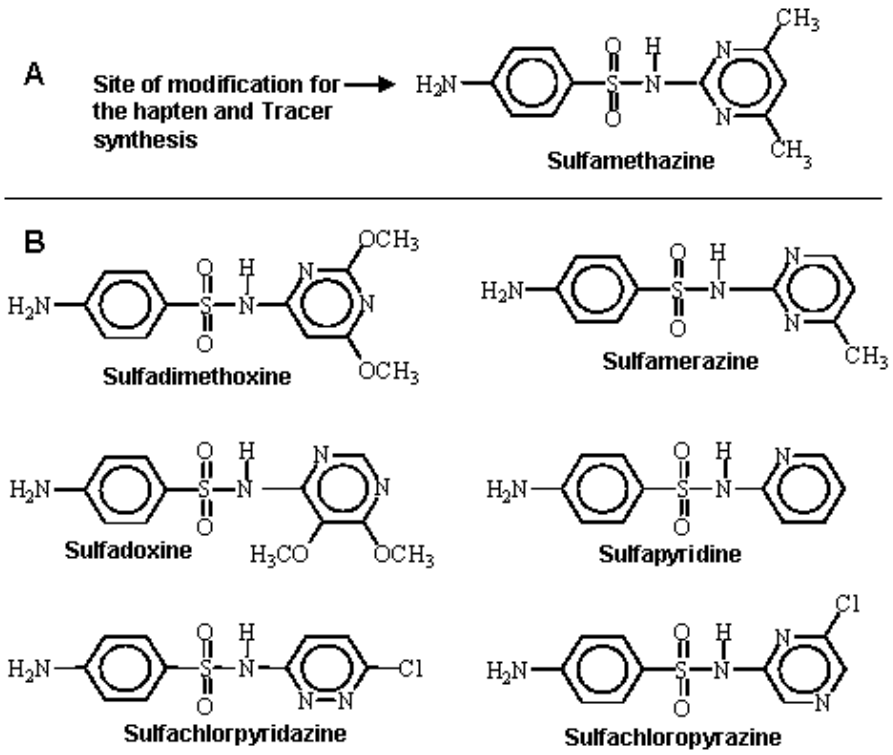

Figure 2. Structures of (A) sulfamethazine (hapten) and (B) compounds used for cross-reactivity testing

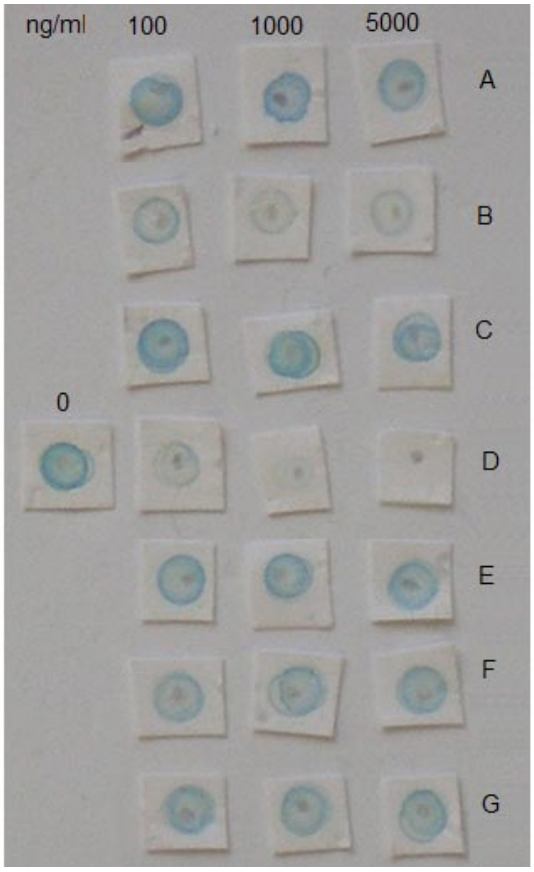

Figure 3. The dot blots on the nitrocellulose membrane shows the degree of the cross-reactivity among seven different sulfonamides (A) sulfadimithoxine, (B) sulfamerazine, (C) sulfadoxine, (D) sulfamethazine, (E) sulfachloropyridazine, (F) sulfachloropyrazine, and (G) sulfapyridine. Dilutions: coating antibody (1:250), HRP tracer (1:4 000)

Dose responses in various matrices were examined to obtain starting data for the dipstick application to the detection of sulfamethazine in environmental and food screening. Whereas water samples did not require any sample pre-treatment, centrifugation of milk samples was needed in order to obtain fat free milk for analysis. When the milk fat is present in a sample, a dark background colour is produced due to the adhering of the fat to the nitrocellulose membrane. Pig manure samples also need an extraction/centrifugation step prior to dipstick detection due to matrix interference whereas the corresponding ELISA, based on the same antibody, allowed the trace detection of sulfamethazine in manure without any pre-treatment operations (Diblikova et al., 2005). Figure 4 shows responses to sulfamethazine in buffer compared with those obtained for matrices spiked in the range of 0–5 000 ng/ml. No significant differences in colour intensity between the buffer and matrices are apparent within the range of the tested concentrations. Additionally, it is apparent that the presence of added sulfamethazine at levels of 20–100 ng/ml progressively reduced the colour intensity in all matrices approaching the background in control spots (Figure 4). Detection ability of the dipstick assay was verified using naturally contaminated field manure. The concentration of the 570 ng sulfamethazine in 1 g of the pig manure sample was determined by LC-MS/MS and compared with the dipstick response. No colour development, indicating a positive response, was observed when the manure extract was spotted onto dipstick strip.

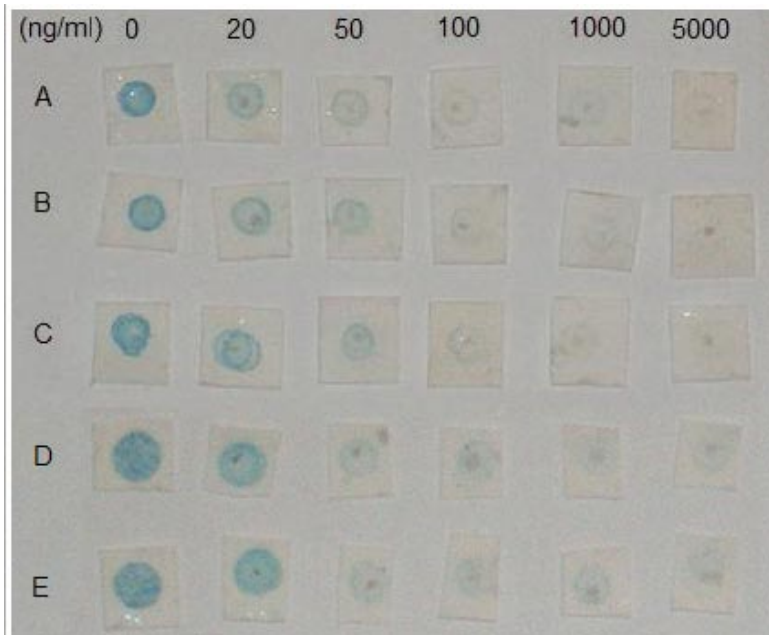

Figure 4. Comparison of the dipstick dose responses for sulfamethazine in (A) PBS, (B) tap water, (C) milk, (D) river water, and (E) pig manure. Dilutions: coating antibody (1:250), HRP tracer (1:4 000)

The analysis time and detection range of the developed dipstick system could further be improved by employing the lateral-flow dipstick immunoassay as an additional stage of this development. The main advantage of the lateral-flow dipstick is the one step assay performance without washing.

REFERENCES

Botsoglou N.A., Fletouris D.J. (2001): Drug Residues in Foods: Pharmacology, Food Safety and Analysis. Macel Dekker, New York. 1194 pp.

Campbell K., Fodey T., Flint J., Danks C., Danaher M., O'Keeffe M., Kennedy D.G., Elliott C. (2007): Development and validation of a lateral flow device for the detection of Nicarbazin contamination in poultry. Journal of Agricultural and Food Chemistry, 55, 2497–2503.

Coung N.V., Bachmann T.T., Schmid R.D. (1999): Development of a dipstick immunoassay for quantitiative determination of 2,4-dichlorophenoxyacetic acid in water, fruit and urine samples. Fresenius Journal of Analytical Chemistry, 364, 584–589.

Delmulle B.S., De Saeger M.D.G., Sibanda L., Vetero I. B., Peteghem C.H.V. (2005): Development of an immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B_1 in pig feed. Journal of Agricultural and Food Chemistry, 53, 3365–3368.

Diblikova I., Cooper K.M., Kennedy D.G., Franek M. (2005): Monoclonal antibody-based ELISA for the quantification of nitrofuran metabolite 3-amino-2-oxazolidinone in tissues using a simplified sample preparation. Analytica Chimica Acta, 540, 285–292.

EU Regulation 508/1999: Official Journal of the European Communities, 9.3. 1999, L60/16, 16–52.

Franek M., Kolar V., Deng A., Crooks S. (1999): Determination of sulfadimidine (sulfamethazine) residues in milk, plasma, urine and edible tissues by sensitive ELISA. Food and Agricultural Immunology, 11, 339–349.

Franek M., Diblikova I., Cernoch I., Vass M., Hruska K. (2006): Broad specificity immunoassays for sulfonamide detection: Immunochemical strategy for generic antibodies and competitors. Analytical Chemistry, 78, 1559–1567.

Haasnoot W., Bienenmann-Ploum M., Lamminkaki U. (2005): Application of a multi-sulfonamide biosensor immunoassay for the detection of sulfadiazine and sulfamethoxazole residues in broiler serum and its use as a predictor of the levels in edible tissue. Analytica Chimica Acta, 552, 87–95.

Kandimalla V.B., Tripathi V.S., Ju H.X. (2006): Conductive ormosil encapsulated with ferrocene conjugate and multiwall carbon nanotubes for biosensing application. Biomaterials, 27, 1167–1174.

McGrath T., Baxter A., Ferguson J., Haughey S., Bjurling P. (2005): Multi sulfonamide screening in porcine muscle using a surface plasmon resonance biosensor. Analytica Chimica Acta, 529, 123–127.

Novarro N.P., Bover C.G., Maquieira A., Puchades R. (2004): Specific polyclonal-based immunoassays for sulfathiazole. Analytical and Bioanalytical Chemistry, 379, 1088–1099.

Okawa Y., Nagano M., Hirota S., Kobayashi H., Ohno T., Watanabe M. (1999): Tethered mediator biosensor. Mediated electron transfer between redox enzyme and electrode via ferrocene anchored to electrode surface with long poly(oxyethylene) chain. Biosensors and Bioelectronics, 14, 229–235.

O'Keeffe M., Crabbe P., Salden M., Wichers J., Peteghem C.V., Kohen F., Pieraccini G., Noneti G.J. (2003): Preliminary evaluation of a lateral flow immunoassay device for the screening urine samples for the presence of sulphamethazine. Journal of Immunological Methods, 278, 117–126.

Pal A., Acharya D., Saha D., Dhar T.K. (2004): Development of a membrane – based immunofiltration assay for the detection of T-2 toxin. Analytical Chemistry, 76, 4237–4240.

Peruski A.H., Peruski L.F. (2003): Immunological methods for detection and identification of infectious disease and biological warfare agents. Clinical and Diagnostic Laboratory Immunology, 10, 506–513.

Received: 2007–05–24
Accepted after corrections: 2007–07–22

Corresponding Author:

Dr. Milan Franek, DrSc., Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel. +420 533 331 901, fax +420 541 211 229; e-mail: franek@vri.cz