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Short communication

A highly sensitive and class-specific fluorescence polarisation assay for sulphonamides based on dihydropteroate synthase

Zhanhui Wang [a,1], Xiao Liang [a,b,1], Kai Wen [a], Suxia Zhang [a], Chenglong Li [a], Jianzhong Shen [a,*]

[a] College of Veterinary Medicine, China Agricultural University, Beijing Laboratory for Food Quality and Safety, Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, Beijing 100193, People's Republic of China
[b] College of Veterinary Medicine, Qingdao Agricultural University, No. 700 Changcheng Road, Qingdao 266109, People's Republic of China

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ABSTRACT

We describe a fluorescence polarisation assay based on the use of dihydropteroate synthase (DHPS) and a fluorescence probe for multi-sulphonamide detection. Dihydropteridine pyrophosphate (DHPPP) was synthesised and acts as the first substrate for DHPS. Under optimised conditions, the half-maximal inhibitory concentrations (IC_{50}) of the assay were less than 100 ng mL^{-1} for at least 29 sulphonamides and the time needed for the detection was less than 20 min. More importantly, the assay revealed quite uniform affinities for all of the individual sulphonamides tested, which has never before been achieved in an antibody-based assay.

1. Introduction

Antibody-based assays, i.e., immunoassays, are relatively inexpensive and rapid screening methods to detect targets of interest in a variety of samples. Immunoassays are a particularly cost-effective alternative to instrumental methods used in large-scale monitoring programs of chemical contaminants, such as antibiotics or pesticides, in food and environmental matrices (Bovee and Pikkemaat, 2009). Additionally, an immunoassay that can detect one class of analytes in a single run would be preferable for efficient surveillance purposes. Thus, class-specific immunoassays have gained increased attention because of their short assay time, low sample consumption, high sample throughput and reduced detection cost per assay (Han et al., 2012; Wang et al., 2012; Zeng et al., 2012). To achieve this goal, the generation of an antibody with class-specificity is necessary, in addition to the development of multi-label and multiplex techniques (Franek et al., 2006). Until now, extensive effort has been devoted to developing antibodies that are class-specific to chemical contaminants, e.g., sulphonamides (Adrian et al., 2009; Pastor-Navarro et al., 2009), fluoroquinolones (Pinacho et al., 2012; Wang et al., 2007) and organophosphates (Piao et al., 2009; Wang et al., 2010). However, these previously reported class-specific antibodies are specific and only recognise a few analytes because of their high discrimination ability toward slight structural changes of the target molecules; thus, the production of real antibodies with class-specificity is likely impossible. Therefore, the development of alternatives to antibodies for class detection is an active and tremendously important area of research.

Alternatives include those derived from chemical synthesis, such as molecularly imprinted polymers (MIPs), and those obtained by modification of biological materials, such as aptamers, enzymes and some protein scaffolds (Maragos, 2009a). To date, the combination of selectivity and sensitivity needed for class-specific assays has been achieved with the use of enzymes over other materials. An advantage of using naturally occurring enzymes in class-specific assays is their non-specificity and ability to recognise and bind a generic range of analytes that exert their effects by interaction with the enzyme of interest. As an example, the enzyme acetylcholinesterase (AChE) is frequently used to develop class-organophosphate screening methods, although these enzyme-based methods do not exhibit the same sensitivity as chromatographic methods (Van Dyk and Pletschke, 2011).

Sulphonamides are a group of synthetic antibiotics that are widely used in human medicine as well as in veterinary medicine for the treatment of infectious diseases and as growth-promoting feed additives (see Fig. S1). Currently, more than 20 sulphonamides are used in clinical studies (Wang et al., 2013a). For

* Corresponding author. Fax: +86 10 6273 1032.
E-mail address: sjz@cau.edu.cn (J. Shen).
[1] These authors contributed equally to this paper.

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example, at least nine and six sulphonamides have been approved for use in veterinary medicine in the Netherlands and in the Czech Republic, respectively (Cliquet et al., 2003; Franek et al., 2006). To minimise the risk of sulphonamides, the maximum residue limits (MRLs) are set to 100 μg kg^{-1} for total sulphonamides by the EU and China (Bangemann, 1999; Ministry of Agriculture of the People's Republic of China, 2002). To achieve the goal of class-sulphonamide detection, many antibodies class-specific to sulphonamides have been produced; however, each of these antibodies was capable of recognising only a single type of sulphonamide (Cliquet et al., 2003; Franek et al., 2006; Wang et al., 2013a). In our previous study, we prepared dihydropteroate synthase (DHPS) and developed a microplate assay for multi-sulphonamides for the first time (Liang et al., 2013). DHPS is one of several critical enzymes in the de novo biosynthesis of folate cofactors and is the target for sulphonamides, which is a competitive inhibitor of *para*-aminobenzoic acid (PABA, the second substrate). In the assay based on DHPS, 9 sulphonamides were detected at concentrations less than 100 ng mL^{-1} and 28 sulphonamides exhibited half-maximal inhibitory concentration (IC_{50}) values ranging from 426 to 50,000 ng mL^{-1}. Obviously, the sensitivity of the assay is inferior to that of antibody-based assays and chromatographic methods (Salvia et al., 2012; Wang et al., 2013a; Zhou et al., 2014); thus, this method could not satisfy the requirements of regulatory agencies. Levy et al. and other groups have demonstrated that the sulphonamide target for competitive inhibition of DHPS with PABA should be the DHPS–DHPPP binary complex (DHPPP is the acronym for dihydropteridine pyrophosphate, which acts as the first substrate for DHPS), rather than DHPS alone (Levy et al., 2008). In addition, the time required to complete the assay we previously reported was longer than 2 h because of the need to separate the unbound probe in solution before the bound probe could be calculated. Fluorescence polarisation assay (FPA) is a non-separation method based on the differences in the fluorescence polarisation (FP) of the tracer (fluorescent-labelled analyte) in the receptor-bound and non-bound fractions (Hunt et al., 2006). FPA is a simpler and faster analytical method, which makes it better suited for high-throughput screening of large numbers of samples.

In this study, we prepared DHPPP to use as the first substrate, with the expectation of inducing the conformational change of DHPS before sulphonamide binding, resulting in improved affinity for sulphonamides. On the basis of the DHPS–DHPPP binary complex, we developed a homologous FPA for the detection of class-sulphonamides after selecting an appropriate tracer and optimising the reaction conditions. The developed FPA is simple, rapid, highly sensitive and class-specific for the detection of at least 29 sulphonamides; it exhibited an IC_{50} of less than 100 ng mL^{-1}. The analysis of sulphonamides in milk samples required less than 20 min to complete.

2. Materials and methods

2.1. Materials

All sulphonamides shown in Fig. S1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Haptens SA10, SSS and CS were previously synthesised in our laboratory (Wang et al., 2013a, 2013b). The 6-hydroxymethylpterin pyrophosphate was obtained from Schirks Laboratories (Jona, Switzerland). Fluorescein isothiocyanate (FITC) isomer I and 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF) were supplied by Sigma-Aldrich (St. Louis, MO, USA). The cloning and expression in *Escherichia coli* of DHPS from *Streptococcus pneumoniae* (*S. pneumoniae*) has been previously described (Liang et al., 2013). 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIPEA) were purchased from Aladdin Industrial Corp. (Shanghai, P.R. China). Common solvents and salts were of analytical reagent grade and were supplied by Beijing Reagent Corp. (Beijing, P.R. China).

2.2. DHPPP and tracers synthesis

The detailed synthesis procedures for DHPPP and tracers are described in the Supplementary text.

2.3. Bioassay development and optimisation

To construct a DHPS dilution curve, the protein was diluted with assay buffer to concentrations ranging from 1:25 to 1:800. Then, 70 μL of tracer solution per well was mixed with 70 μL of assay buffer per well in the absence or presence of sulphonamides in the microplate well. Subsequently, 70 μL of the series dilution of DHPS per well was added to the mixtures, which were shaken for 10 s in a microplate reader. After an incubation of 20 min at room temperature, FP was measured at λ_{ex}=485 nm and λ_{em}=530 nm (emission cutoff=515 nm, *G*-factor=1.0).

With DHPS at an optimal dilution, the ionic strength (the concentration of Tris–HCl (pH 8.0) and $MgCl_2$) of the assay buffer and the incubation time were further optimised. Tris–HCl concentrations of 0, 10, 20, 40 and 80 mM were tested with the $MgCl_2$ concentration fixed at 5 mM. The concentration of $MgCl_2$ (0, 10, 20, 40 and 80 mM) was determined in the same manner previously mentioned, with the Tris–HCl concentration fixed at 20 mM. Seven different incubation times (0, 5, 10, 15, 20, 30 and 60 min) were evaluated. In the FPA optimisation, we used sulphamethazine (SMZ) as a reference analyte to construct standard curves.

2.4. Recovery study

Milk sample (4 mL) was fortified with five analytes, SMZ, SDM, SQX, SMM, and SMX, at concentrations of 50, 100, and 200 μg L^{-1}. The samples were allowed to stand for 30 min at RT. An equal volume of 55% saturated $(NH_4)_2SO_4$ solution was added to deproteinise the samples, followed by mixing in a vortex mixer for 2 min and then standing for 15 min at RT. The precipitate was removed by centrifugation at 4 °C for 10 min (8000*g*). Then, 2 mL of the supernatant was diluted in 6 mL assay buffer and three replicates were determined at each concentration using the optimised FPA.

3. Results and discussion

3.1. Assay principles

The general concept of the FPA is illustrated in Scheme 1. FPA is a competitive solution-phase homogeneous assay based on differences in the FP of the fluorescent-labelled analyte (tracer) in the receptor-bound and non-bound fractions. In such a method, DHPPP first binds to DHPS, forming the DHPS–DHPPP complex; the complex is then mixed with various sulphonamides and a fixed concentration of tracer in a microwell. The sulphonamides and tracer (CS-BDF in this case) compete for the binding site of the DHPS–DHPPP complex. With an increase in the sulphonamide concentration, displacement of the tracer from the DHPS–DHPPP complex will result in a decrease in FP values; the reverse is also true (Jameson and Ross, 2010; Maragos, 2009b; Smith and Eremin, 2008).