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Determination of sodium benzoate in food products by fluorescence polarization immunoassay

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ABSTRACT

A rapid and sensitive fluorescence polarization immunoassay (FPIA), based on a polyclonal antibody, has been developed for the detection of sodium benzoate in spiked samples. The immunogen and fluorescein-labeled analyte conjugate were successfully synthesized, and the tracer was purified by TLC. Under the optimal assay conditions, the FPIA shows a detection range of 0.3–20.0 μg mL^{-1} for sodium benzoate with a detection limit of 0.26 μg mL^{-1} in the borate buffer. In addition, the IC_{50} value was 2.48 μg mL^{-1}, and the cross-reactivity of the antibodies with ten structurally and functionally related analogs were detected respectively. Four kinds of food samples (energy drink, candy, ice sucker, RIO™ cocktail) were selected to evaluate the application of FPIA in real systems. The recoveries were 96.68–106.55% in energy drink; 95.78–100.80% in candy, 86.97–102.70% in ice sucker, and 103.58–109.87% in benzoate contained sample RIO™ cocktail, and coefficients of variation of this method were all lower than 11.25%. Comparing with the detection results of HPLC, the developed FPIA has comparative performance in the real sample determination. The results suggest that the FPIA developed in this study is a rapid, convenient and simple method, which is suitable to be used as a screening tool for homogeneous detection of sodium benzoate in food products.

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1. Introduction

Sodium benzoate, the sodium salt of benzoic acid, is generally used as a chemical preservative to prevent alteration or degradation caused by microorganisms during storage [1]. Both sodium benzoate and benzoic acid exhibited inhibitory activity against a wide range of fungi, yeasts, molds and bacteria [2,3]. Sodium benzoate is more widely used in a great variety of foods and beverages because of its good stability and excellent solubility in water. However, excessive intake of these preservatives might be potentially harmful to the consumers, because they have the tendency to induce allergic contact dermatitis, convulsion, hives and hepato cellular damage and others [4]. Moreover, with the growing use of the additives, the preservative residues can be considered as environmental contaminants [5]. For these reasons, benzoic acid and its salt are limitedly used as preservatives in some food products. In China, the maximum permitted utilization of benzoates in different types of food ranges from 0.2 to 2.0 g kg^{-1} (GB2760-2011). However, benzoates might be still excessively added in foods, because they are inexpensive and easily available. Therefore, developing a simple, rapid and economic analytical method for benzoates monitoring and controlling is important for food safety.

There are various techniques studied for benzoates determination. Traditionally, benzoates are analyzed mainly by thin layer chromatography (TLC) [6,7], gas chromatography (GC) [8,9], capillary electrophoresis (CE) [10–12], and micellar electro kinetic chromatography [13,14]. So far, the dominant way to detect benzoates is high-performance liquid chromatography (HPLC) [15–19]. Other methods are also reported for benzoates analysis, including second-order derivative spectrophotometry [20,21], chemometrics enhanced spectrophotometry [22,23], near-infrared reflectance spectroscopy [24], polarography [25] and enzymatic determination [26]. Nevertheless, these methods do not allow an easy analysis, because the instruments are expensive, the assays are relatively time-consuming and in some cases, require steps of extraction, laborious manipulation or sample pretreatment. It is necessary to develop a rapid, simple, economic and easy-to-use detection method as an alternative for benzoates determination.

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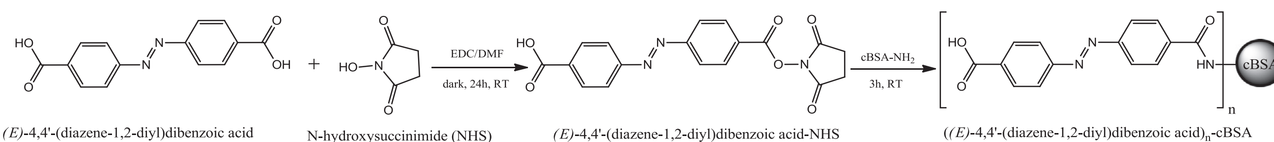

Fig. 1. Synthesis of immunogen ((*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA).

Immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), provide simple, sensitive, specific and inexpensive tools for analysis of various targeted analytes [27]. However, ELISA is a heterogeneous method which involves repeated washing and a certain degree of reaction time (1–2 h). Fluorescence polarization immunoassay (FPIA) is a homogeneous technique (no separation or washing), which is an excellent screening tool in food and environmental analysis because of its rapidity, reliability and ease of use [28]. It bases on the different fluorescence polarization between antibody bound tracer (fluorescein-labeled analyte) and the nonbound form. If the sample contains free (unlabeled) analyte, it will compete with the tracer for antibody-binding sites, which will cause a decrease of the polarization signal. To our knowledge, development of FPIA for sodium benzoate or benzoic acid in real samples has not been reported yet. In this study, the polyclonal antibody against sodium benzoate was prepared by the well-designed immunogen. Based on that, an FPIA for sodium benzoate was developed and the accuracy, specificity and sensitivity of the method were studied.

2. Materials and methods

2.1. Reagents and apparatus

Sodium benzoate, benzoic acid, 4-aminobenzoic acid, sodium salicylate, potassium sorbate, phenol, 4-aminobenzenesulfonic acid, sodium 2-hydroxypropanoate, calcium propionate, phenylalanine, phenol, *N*,*N*-dimethylformamide (DMF), methanol, trichloromethane, ethylenediamine, and triethylamine of analytical grade were supplied by Guangfu Fine Chemical Research Institute (Guangfu Chemical Co., Tianjin, China). 2-Phenylacetic acid, bovine serum albumin (BSA), Freund′s complete adjuvant (cFA), and Freund′s incomplete adjuvant (iFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *N*-hydroxysuccinimide (NHS) was from Academy of Military Medical Sciences (Military Medical Institute, Beijing, China). (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid and 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl) were obtained from TCI (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). 3′,6′-Dihydroxy-5-isothiocyanato-3H-spiro[isobenzofuran-1,9′-xanthen]-3-one (5-FITC) of ultrapure grade was from Amyjet Scientific Inc. (AAT Bioquest Inc., Sunnyvale, USA). Energy drink, candy, ice sucker, RIO^{TM} cocktail, redbull, and sports drink were purchased from a local supermarket (Tianjin, China). Thin-layer chromatography (TLC) plates (Silica gel GF254, $5 \times 10\ cm^2$) were from SILIDA Science and Technology Co., Ltd. (Tianjin, China).

The buffers used in this work include: (1) borate buffer (BB, pH 8.5) consisted of 2.5 mM sodium tetraborate and 0.1% NaN_3; (2) phosphate-buffered saline (PBS, pH 7.4) composed of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4 and 2.7 mM KCl.

FPIA analyses were performed by the Sentry® 100 portable system (Diachemix Corporation, Milwaukee, WI, USA) using $10 \times 75\ mm^2$ glass culture tubes (VWR Internationals.r.l., Milan, Italy) and excitation and emission wave lengths (λ_{ex}, λ_{em}) of 485 and 535 nm, respectively. UV–vis spectra of conjugates were obtained by SHIMADZU UV-1800 (SHIMADZU, Kyoto, Japan). Determination of molecular weight was acquired by Liquid Chromatograph Mass Spectrometer (LC/MS, Agilent 6520 Q-TOF, USA). Ultimate 3000 liquid chromatography system (Dionex, Sunnyvale, CA, USA) was used in real samples determination for comparison, with an Inert Sustain C_{18} column ($150 \times 4.6\ mm^2$, i.d., 5 μm, Shimadzu-GL, Shanghai, China) and a mobile phase of methanol – 0.02 mol L^{-1} ammonium acetate (5:95) at 1 mL min^{-1}. The samples were detected at 230 nm with an injection volume of 20 μL.

2.2. Preparation of immunogen

Firstly, carboxylic acid groups of the carrier protein of BSA were converted into primary amine groups with an excess of ethylenediamine as described previously [29]. Briefly, 30.0 mL of PBS (0.1 M, pH 7.4) containing 15.0 mL of ethylenediamine was neutralized by 35.0 mL HCl (12 M). One gram of BSA and 0.639 g EDC was added subsequently. The mixture was incubated for 4 h at room temperature and dialyzed against PBS as well as distilled water respectively. The solution was lyophilized and stored at −20 °C.

In order to expose sodium benzoate for antibody recognition, (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid was used as the hapten (Fig. 1). The immunogen ((*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA) was prepared by carbodiimide-modified active ester method [29,30] as follows. A solution of 3.58 mg of (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid (13.25 μmol), 2.54 mg of EDC (13.25 μmol), and 1.52 mg of NHS (13.25 μmol) in 3.0 mL of DMF was incubated for 24 h at room temperature in dark. Then the mixture solution was added slowly into 3.0 mL of PBS (0.1 M, pH 7.4) containing 30.0 mg of cBSA (0.44 μmol), followed by incubation at room temperature for 3 h. At last, the reaction mixture was dialyzed under stirring against PBS (0.1 M, pH 7.4) as well as distilled water for 3 days respectively to remove the uncoupled free hapten. The purified resultant was lyophilized and then stored at −20 °C.

2.3. Production of polyclonal antibodies

The polyclonal antibodies were obtained by immunizing two male New Zealand white rabbits (2.0 kg). They were subcutaneously immunized at multiple sites in the back with (*E*)-4,4′-(diazene-1,2-diyl)dibenzoic acid–cBSA conjugate. Before the immunization, 1.0 mL of blood was taken from the ear of each rabbit and used as the negative group. The initial immunization was subcutaneously injected with 0.5 mg of conjugate in 0.5 mL of NaCl (0.9%) emulsified with 0.5 mL of cFA. Subsequent booster injections (0.25 mg of conjugate in 0.5 mL of NaCl (0.9%) plus 0.5 mL of iFA) were performed 21 days later and then at 14-day intervals. At the fifth immunization, 0.25 mg of immunogen was dissolved in 1.0 mL of NaCl (0.9%) and injected. One week after the last immunization, the blood was collected and centrifuged. At last, the antiserum was obtained and stored at −20 °C until use.

2.4. Synthesis of the fluorescent conjugates

The fluorescent tracer should be prepared by conjugating sodium benzoate with fluorescein. However, sodium benzoate has no active groups to enable coupling reactions with commonly used fluorescein, FITC. Therefore, an analog of sodium benzoate,

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