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Pretreatment-free detection of diazepam in beverages based on a thermometric biosensor

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ABSTRACT

Diazepam (DZP) has increasingly appeared in the nonmedical field, posing an ever-growing threat to public-health safety. Methods that accelerate and simplify the on-site detection of DZP are urgently needed. In this study, we investigated the feasibility of applying a flow-injection analysis (FIA)-based thermometric enzyme-linked immunosorbent assay (TELISA) biosensing strategy to rapidly detect DZP in real samples. The scheme involved three key steps: 1) FIA-TELISA was modified to optimize performance in DZP detection; 2) the selected samples that were spiked with a predetermined amount of DZP were respectively determined; and 3) detection results were validated by high-performance liquid chromatography. After calibrations, a linear range of 45.37–726.71 ng mL^{-1} was obtained, with a corresponding detection limit of 33.71 ng mL^{-1}. During the continuous detection, each sample required 15 min to generate an entire heat signal, and the cycle time including regeneration and retrimming was <45 min. Verification results showed that the performance characteristics of FIA-TELISA in practical applications could meet the requirements of DZP detection. Notably, pretreatment did not require elaborate extraction procedures to correct the matrix effect of the beverages. Other potential applications of TELISA technology, including the possibility of developing an in vitro assay model to analyze diverse targets, are being discussed.

1. Introduction

Diazepam (DZP) is a drug that belongs to the most widely prescribed 1,4-benzodiazepines for the therapeutic treatment of anxiety, insomnia, epilepsy, acute alcohol withdrawal, and muscular spasms [1,2]. The family act on the brain and nerves to produce a calming effect by a neurotransmitter chemical (gamma-aminobutyric acid), which nerves use to communicate with one another [3]. As a rule, DZP is administered orally, but the drug can also be administered intramuscularly or intravenously. The therapeutic dose is less than 30 mg as a single dose for hypnotism [4]. Although the short-term use of this medication is generally safe and effective, potential side effects, including the dependence, drowsiness, ataxia, and confusion, have been disputed [5]. According to a previous observation [2], the consumption of DZP along with other depressant substances, such as alcohol, could exacerbate the sedative effects and increased the absorption rate of the drug. Owing to these effects, DZP has been used in drug-facilitated sexual assault [6] and robberies [7]. In such cases, which commonly occur in clubs and bars, victims are unaware that their drink may have been "spiked" with a massive dose of benzodiazepines, such as DZP. In other cases, DZP has been revealed to be used in the illegal adulteration of herbal medicine and functional foods [8,9]. Falling prices and increased availability of the drug have also been linked to an increase in these incidents [10]. More recently, considerable attention has been focused on the presence of DZP in water and sewage effluents [11]. Involuntary ingestion of these media would engender social hazards and potential intoxication of sufferers as a causal factor.

A range of analytical approaches have been published to determine DZP in beverages and related samples. At present, chromatographic techniques, such as high-performance liquid chromatography (HPLC) [8,9], LC/mass spectrometry (MS) [12], gas chromatography/MS [13], and high-performance thin-layer chromatography [14], have been employed and achieved initial success in medical and pharmaceutical analysis. Moreover, the qualitative determination of several different benzodiazepines has been reported by direct electrospray probe/MS [15]. Recently,

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Ribeiro et al. [16] used a multipump flow system that is integrated with a photodegradation system for the fluorometric determination of DZP in different beverages. However, in this case, certain sample ingredients are reported to interfere. A couple of nitro-substituted 1, 4-benzodiazepines in various kinds of drinks have been determined by indirect laser-induced fluorescence through a microfluidic device flowing liquid–liquid extraction [17]. Further reports have utilized capillary zone electrophoresis for the determination of numerous benzodiazepines [18], including DZP, in beverages [19]. Molecularly imprinted polymers have been utilized to modify the surface of a screen-printed carbon electrode (SPCE) for the conductometric determination of DZP [20]. Although high sensitivity and reliability have been achieved, the previously mentioned methods presents a common disadvantage, namely, requirement of intricate sample pretreatment, which involves extraction, cleanup, and pre-concentration. These procedures usually require the use of toxic reagents. Moreover, the commonly used analysis instrument is often inapplicable for continuous monitoring.

As DZP is widely used in clinical and forensic cases, a rapid, convenient, and interference-free approach is necessary for "on-the-spot" detection in practical samples. With the development of sensor technology, many developing non-chromatographic methods have been applied to detect DZP such as electrochemistry [21,22] and optical fiber [23] sensors. However, these schemes both need a certain extent pretreatment and cannot perform continuous monitoring in real samples. The enzyme thermistor (ET), a thermometric biosensor device, has been developed and studied in numerous application areas because of advantages like superior versatility and general operational stability [24]. Previous demonstrations have focused on food analysis [25], bioprocess monitoring [26], determination of heavy metals [27], and so on. Thermometric enzyme-linked immunosorbent assay (TELISA) is based on the conventional enzyme-linked immunosorbent assay (ELISA) but utilizes heat production from the enzyme label, which is measured by an ET unit [24]. This flow injection analysis (FIA)-based TELISA has been shown to be achievable in the literature [28,29]. Recently, Qie et al. [30] have used a Protein G Sepharose^{TM} 4 Fast Flow (PGSFF) integrated with FIA-TELISA system for the detection of atrazine in tap water. Preceding investigations have indicated that TELISA prevents multiple universal drawbacks but offers a variety of advantages in comparison with conventional immunoassays. First, TELISA can be used for the determination of compounds in complex matrices. Utilization of the thermometric detection principle may prevent the influence of optical properties and turbidity to the signal. Second, the immunosorbent in TELISA can be used repeatedly when appropriate reagents are employed for regeneration, and continuous detection is achieved. Moreover, PGSFF provides a considerably broader scope of antibodies (Abs) available for detection of the desired analytes. In consequence, the sensor has been perceived as a feasible tool that is suitable for monitoring adulteration and poison.

In this paper, we constructed a FIA-TELISA method combined with PGSFF for the rapid detection of DZP on the basis of a direct competitive model. DZP–β-lactamase conjugates were prepared and used to produce a thermal signal in the presence of ampicillin trihydrate. During detection, free DZP and DZP–β-lactamase conjugate would compete with each other to bind to the anti-DZP mAbs, which are captured by PGSFF. After optimization, FIA-TELISA is employed to analyze several typical samples, including tap water, juice, rum, and oral liquid, which are spiked with increasing concentrations of DZP. All samples are adjusted to pH 7.0, and filtered, and scarcely needed further sample pretreatment. Meanwhile, these spiked samples are also analyzed via HPLC for validation. As expected, the detection results exhibited satisfactory recoveries without marked difference between samples. Thus, the TELISA method can be seen to achieve DZP quantification both in functional foods and commercial beverages. The detection sensitivity match up to the existing sensors used for DZP [21–23]. In a sense, this work suggests a potential high-development detecting platform for 'on-the-spot' determination.

2. Experimental

2.1. Reagents and materials

Ampicillin trihydrate (anhydrous basis) and β-lactamase (from *Bacillus cereus*) used for the preparation of the enzyme conjugate were purchased from Sigma Chemical Co. (USA). The DZP used to prepare standard solutions in dimethyl sulfoxide (DMSO) and spiked samples, other analogs, and zopiclone were provided by Aladdin (Shanghai, China). PGSFF, the immunosorbent, was acquired from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Protein G took up the Fc region of IgG, and the Fab region of IgG remained available for antigen binding, thus rendering PGSFF extremely useful for the isolation of immune complexes. Moreover, compared to protein A, protein G binds more strongly to mouse IgG_1 [31]. The anti-DZP mAb (subclass, mouse IgG_1; immunogen, carboxylated diazepam coupled with bovine serum albumin) dissolved in phosphate-buffered saline (PBS, pH 7.0) was obtained in our laboratory. N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC—HCl) were purchased from Sigma Chemical Co. (USA). All beverage samples, namely, juice (Masterkong Fresh Orange), rum (Bacardi Gold Rum), and functional foods (President Brand Jingyi Oral Juice), were randomly selected (9 samples in total) and bought locally. All water used for preparing the buffers and samples was collected from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals used were of analytical grade.

2.2. Instrument

The thermometric detection system used in these studies is shown in Fig. 1. The system consisted of a six-port injection valve (Type 50 from Rheodyne, Cotati, USA), an HPLC pump (UC 3281 from Union Optoelectronic Technology, Beijing, China), and an ET unit (Omic Bioscience AB, Sweden). Moreover, a standard water bath for the 37 °C incubation was necessary.

2.3. General principle of the procedure

The principle diagram of enzymatic reaction involved in the process is shown in Fig. 2(a). The figure shows a stable temperature signal response to the change of enzymatic catalysis enthalpy of the corresponding substrate. This reaction occurred in the enzyme column after off-line incubation. A large amount of PGSFF was prepacked in the enzyme column built in the ET system to act as immunosorbent. During ET analysis step, the Analyte–β-lactamase–mAb complexes captured by protein G catalyze the degradation of the added ampicillin trihydrate. The resulting temperature change was directly recorded by a thermal probe with a sensitivity of 10^{-5} °C at the outlet of the enzyme column [32]. Then, temperature rise caused by the reaction was measured and amplified with a Wheatstone bridge. Hence, an increase in the peak signal recorded by the data system could reflect and quantify the Analyte–β-lactamase conjugates immobilized in the column. The quantity is inversely proportional to the concentration of the analyte during incubation. Finally, the analytes in the sample injected into the ET system could be determined by analyzing the change in peak signal after blank elimination.