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Rapid and sensitive determination of clenbuterol residues in animal urine by surface-enhanced Raman spectroscopy

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ABSTRACT

A rapid, selective, and quantitative method to determine clenbuterol content in animal urine based on Surface-Enhanced Raman Spectroscopy (SERS) by aggregated re-oxidized graphene oxide/Au nanoparticle (roGO/AuNPs) hybrids and liquid-liquid extraction has been proposed in this study. To realize the quantitative calculation, melamine was optimized as the internal standard. The peak intensity of melamine observed at $\triangle\nu$ = 709 cm^{-1} was first set to 1000 as basis, and the peak at $\triangle\nu$ = 1474 cm^{-1} was selected as the characteristic peak of clenbuterol used for the quantitative calculation. Sufficient linearity was obtained in the range of 1–20 ng mL^{-1} (R^2 = 0.997). The limit of detection and quantification in urine matrix were as low as 0.5 and 1 ng mL^{-1}, respectively. The recovery rates were 78.6–89.4% with coefficients of variation less than 9.8%, and the storage time of the substrates is approximately 180 days under 4°C. The detection results of the developed SERS detection method agree well with those determined by LC–MS/MS, while the cost time significantly reduced to 8 min/sample. This new protocol could be easily developed for the routine monitoring of the illicit use of clenbuterol in animal farming.

1. Introduction

Clenbuterol (CL) is a representative compound of β2-adrenergic agonist, which has been known as a bronchodilator and decongestant in the human medicine [1]. However, this kind of β2 adrenergic has been abused as a feed additive for livestock, due to its capacity to improve growth rate and reduce carcass fat [2]. Due to the long half-life in animal blood, CL easily remains in the tissues of animals such as in the meat, liver, eyes and so on. The residual CL in animal products may pose severe threat to humans, causing acute poisoning with symptoms of muscular tremor, cardiac palpitation, vomiting, nausea, nervousness and chills, particularly to the people with asthma or cardiovascular disease [3]. It has been banned for use as growth promoters in livestock in China [4] and European Union [5]. Generally, the method to monitor the illegal use of CL in animal farming is to detect the drug content in animal urine based on several analytical methods such as gas chromatography-mass spectrometry (GC–MS) [6,7], gas chromatography-tandem mass spectrometry (GC–MS/MS) [8], and liquid chromatography-tandem mass spectrometry (LC–MS/MS) [9,10]. These accurate methods usually require time-consuming sample pretreatment steps, expensive instrumentations and sophisticated technical operators. The employment of some bioanalytical screening methods based on the enzyme linked immunosorbent assay (ELISA) [11,12], immunochromatographic assay based on up-conversion phosphors [13], fluorescent multi-component immune-chromatography [14] are the strategies to reduce the cost of monitoring. However, there still exist many challenges to improve the current techniques. The above techniques depend mainly on the specific interaction between antibody and antigen. The high rate of “false positive” is the prevalent phenomenon. Therefore, some novel screening methods such as fluorescent detection [15], colorimetric detection [16], electrochemical biosensor [17], and quartz crystal microbalance sensor [18] have also been developed recently.

Surface-enhanced Raman Scattering (SERS) technique has been developed by leaps over the past two decades and have attracted increasing attention in recent years. The intensity of Raman signal would be significantly amplified when the target analyte was adsorbed or in proximity to the roughened surface of metal nanostructures, which was first observed in the early 1970s [19]. Compared to conventional Raman spectroscopy, SERS can enhance the normal Raman signal of the

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target analyte at the extremely low concentration up to 10^6-10^{12} times even down to a single molecule level [20]. As one of the most powerful analytical tools for trace detection, the SERS technique has been increasingly used in many areas, such as drugs [21], food safety [22,23], environment pollutants [24,25], and pesticide or veterinary drug residue detection [26]. Recently, some research about the CL detection in animal urine base on SERS-based competitive immunoassay have been reported. Wei's group developed the CL detection method based on the integration of magnetic nanoparticles and SERS. A facial liquid magnetic competitive immunoassay was developed to detect CL with high sensitivity and specificity [27]. Afterwards, the similar method has also been employed to separate CL and salbutamol (SAL) in mixed solutions simultaneously and realized the SERS detection with the limit of detection (LOD) as low as fg mL^{-1} level [28]. Moreover, based on the competitive binding between free CL, CL-BSA immobilized on the substrates with its antibody labeled on SERS nanoprobes, Yu and his coworker realized the competitive SERS detection of CL [29]. As a consequence, SERS-based competitive immunoassay has been employed as a popular candidate for determining the content of CL in the effect of antigen-antibody bindings.

One important challenge limiting the practical application of SERS is reproducibility. Metal nanoparticle colloids are widely used as SERS substrates; however, the aggregation of metal nanoparticle is difficult to control, leading to the random formation of "hot spots". In the case, the graphene-based substrates have been attracted more and more attention. The sp^2-hybridized carbon atoms constituted a large π-bond, which made the target molecules homogeneously adsorbed onto the surface through π-π interactions, thereby improving the stability of the composite substrates. In addition, the graphene-based substrate can also provide Raman enhancement, known as the graphene-enhanced Raman scattering (GERS) effect [30]. For instance, graphene oxide nanosheets have been utilized to design sensors for the detection of a biomarker survivin [31,32]. And a novel nano-biosensor based on the reduced graphene oxide has also been designed for the assessment of DNA modification/damage [33].

Here, the mixture solvent di-(2-ethylhexyl) phosphate/methylene chloride, v/v, 1:800) was first reported as extraction reagent and two liquid-liquid extractions by adjusting the pH values was performed to separate and enrich CL from the urine matrix based on the different distribution of CL between the water and the organic phase. A novel hybrid re-oxidized graphene oxide/Au nanoparticle (roGO/AuNPs) SERS probe for CL determination from urine sample has been designed. The hybrid substrate was stable within 180 days under 4 °C storage temperature. In order to realize quantitative calculation, melamine was selected as internal standard. The limit of detection (LOD) and the limit of quantitation (LOQ) for real urine sample were as low as 0.5 and 1 ng·mL^{-1}, respectively. A sufficient linearity (R^2 = 0.997) was found in the concentration range of 1–20 ng·mL^{-1} in urine, and the recoveries of CL from spiked urine samples were in the range of 78.6–89.4% with relative standard deviation (RSD) values in range of 4.4–9.8% (n = 6). The total detection time was approximately 8 min/sample. The study offers a rapid, sensitive, and reliable detection method that can be used as a great tool suitable for on-site screening of CL in animal urine.

2. Preparation and measurement method

2.1. Chemical reagents

CL and melamine were purchased from Sigma-Aldrich Co., Ltd. (Shanghai, China). Chloroauric acid tetrahydrate ($HAuCl_4{\cdot}4H_2O$, > 99.9%), sodium citrate tetrahydrate, hydrogen peroxide, methylene chloride, sodium hydroxide, nitric acid, di-(2-ethylhexyl) phosphate (BEHP) were analytical grade and obtained from the Sino-pharm Chemical Reagent Co., Ltd. (Beijing, China). The solvent mixture was prepared from BEHP and methylene chloride (v/v = 1:800). Ultra-pure water (Milli-Q, Millipore corp., Bedford, MA) was used to prepare all aqueous solutions. Stock solutions of CL and melamine (1 mg mL^{-1}) were stored in the dark at −18 °C for 3 months prior to analysis. 15 different real urine samples and the non-contaminated animal urine (control sample) from sheep, pig, and cow were obtained from the Chinese National Feed Supervision from 2016 to 2018, previously tested by LC–MS/MS. These urine samples were kept frozen at −20 °C for no more than 1 month before being used for further analysis. The sample glass vials were cleaned with aqua regia and dried in an oven at 100 °C before use.

2.2. Synthesis and characterization of re-oxidized graphene oxide /AuNPs hybrids (roGO/AuNPs)

The synthesis of roGO/AuNPs was similar with the method reported before by our group [34]. Briefly, (1) 90 mL 2 mg L^{-1} GO aqueous was mixed with 10 mL 30% hydrogen peroxide, followed by stirring for 4 h under ultraviolet light excitation (Portable ultraviolet radiation light, WFH-204B, 254 nm, 50HZ). In this step, the GO aqueous was re-oxidized. (2) 35 mL of distilled water was mixed with 3.50 mL re-oxidized GO and 0.35 mL aqueous sodium citrate. The mixture was stirred and heated to reflux. (3) 500 μL of 1 wt % $HAuCl_4$ was quickly added to the mixture. The solution was heated under reflux for another 10 min. (4) The heating source was removed and the solution was continuously stirred until it cooled to room temperature. The resultant roGO/AuNPs were stored at 4 °C for no longer than 180 days. The absorption spectrums of roGO/AuNPs hybrids were tested using UV–vis spectrometer (Shimadzu UV-2700). The morphology of the hybrids substrates was characterized by transmission electron microscopy (TEM, Hitachi H-7500). Before use, the roGO/AuNPs were separated from the solution by centrifugation at 13,500 rpm for 10 min (a portable centrifuge, DAIHAN WiseSpin®CF^{-10}, South Korea) and washed with ultrapure water twice before resuspended in the water phase.

2.3. Sample pretreatment and SERS detection

A 2 mL urine sample was mixed with 10 μL NaOH aqueous solution (C = 1 M) and 2 mL solvent mixture. After being shaken for 30 s, the mixture was centrifuged at 13,500 rpm for 30 s. Then the subnatant was mixed with 2 mL nitric acid solution (pH = 3–4), shaken for 30 s, and centrifuged at 13,500 rpm for 30 s. Next, 2 mL of the supernatant was added into 10 μL NaOH aqueous solution (C = 1 M) and the methylene chloride (2 mL), which was then shaken for 30 s and centrifuged at 13,500 rpm for 30 s. The supernatant was mixed with 2 mL nitric acid solution (0.1 mol·L^{-1}), shaken for 30 s, and centrifuged at 13,500 rpm for another 30 s. Finally, 180 μL of the subnatant was mixed with 600 μL roGO/AuNPs and 2 μL melamine (0.1 mol·L^{-1}) for 10 s. The mixture was transferred into the sample glass vials (12 × 32 mm, Thermo scientific Co., Ltd., Shanghai, China) and analyzed by a portable Raman spectrophotometer (NFQCC-2, assembled in our lab) at 200 mW power and 10 s exposure time with an incident laser wavelength of 785 nm and a spectral resolution of 8 cm^{-1}. Each spectra was an average of three scans ranging from 500–2500 cm^{-1} and processed with Raman Analyzer software from Leap-SCI Technologies, Inc. The Savitzky-Golay second derivative transformation was used to remove the background signal and other pre-processing algorithms, such as smoothening and polynomial subtraction, were also used [35]. To eliminate the effects of the matrix and other factors such as temperature, humidity, and focal distance, the intensity of the internal standard peak of melamine observed at $\triangle\nu$ = 709 cm^{-1} was first set to 1000 as basis for the samples, and then the Raman peak intensity of CL at $\triangle\nu$ = 1474 cm^{-1} was calculated.