Development of a nano-SiO₂ based enzyme-linked ligand binding assay for the determination of ibuprofen in human urine

Qian-Long Wangₐ,₁, Jing Xieᵇ,ᶜ,₁, Xing-De Liᵈ, Li-Sheng Dingᵃ,ᵈ, Jian Liangᵃ,ᵈ, Pei Luoᵇ,⁎, Lin-Sen Qingᵇ,⁎

⁎ Corresponding authors.
E-mail addresses: pluo@must.edu.mo (P. Luo), qings@cib.ac.cn (L.-S. Qing).
₁ These authors contributed equally to this work.

a CAS Key Laboratory of Mountain Ecological Restoration and Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China
b School of Pharmacy, Chengdu Medical College, Chengdu 610083, China
c State Key Laboratory for Quality Research in Chinese Medicines, Macau University of Science and Technology, Macau, China
d Key Laboratory of Natural Medicine and Translational Medicine, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China

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A B S T R A C T
The application domains of classic enzyme-linked ligand binding assay (ELBA) is relatively narrow due to the high cost and hardly available binding receptor. In here, we described for the first time the possibility of developing a new ELBA based on silica nanoparticles (nano-SiO₂) to assess the ibuprofen in human urine. Nano-SiO₂ with a large surface area was introduced as stationary phase to improve the analytical performance. In the experiment, a competitively binding procedure with human serum albumin (HSA) was performed between the ibuprofen presented in sample and horseradish peroxidase labeled ibuprofen (HRP-ibuprofen) subsequently added. After centrifugal separation, the HRP/ibuprofen/nano-SiO₂ composite catalyzed the substrate solution (TMB/H₂O₂) with a color change from colorless to yellow for quantitative measurement via an ultraviolet spectrophotometer. As a validation of the new principle, the developed nano-ELBA method was applied in the determination of ibuprofen excreted in human urine with excellent performance. This detection range only depends on the solubility of ligand and sensitivity of UV spectrophotometer. Our results indicate that this new method demonstrated to be able to rapidly and adequately determine the concentration of components in biological samples and advocate its effectiveness for various applications.

1. Introduction
Ibuprofen, as one of the important nonsteroidal anti-inflammatory drugs is a commonly used for treatment of acute and chronic pain and in a variety of rheumatic and musculoskeletal disorders by inhibiting the activity of enzyme cyclooxygenase [1]. The efficacy, safety, and tolerability of ibuprofen are in line with the benefit and risk evaluation done for underlying arthritis disease conditions in clinical practice and the therapeutic fact. Therefore, monitoring of ibuprofen in biological samples is of great importance for public health.

Historically, a variety of complex instrumental methods have been utilized to determine ibuprofen, including chromatographic method [2–11], electrochemical method [12–17], optical methods [18–20], and others [21–23], combined with some effective sample preparation techniques, such as ultrasound-assisted magnetic dispersive solid-phase microextraction [24], hollow fiber-based liquid-phase microextraction [25], solid-phase extraction by molecularly imprinted polymer [26], hyperbranched polyglycerol/graphene oxide nanocomposite reinforced hollow fiber solid/liquid phase microextraction [27]. In spite of their accuracy and reliability, all these methods have practical limitations that come into play in experiment design, including expensive instrument, sophisticated sample preparation, time consuming, high detection limit, poor specificity, and/or toxic reagents. Therefore, there is an immediate need for relative simple and rapid methods with improved selectivity, simplicity, sensitivity and low-costing for the determination of ibuprofen from biological samples.

The enzyme-linked ligand binding assay (ELBA) relies on the binding of ligand molecules to receptors, antibodies or other macromolecules, such as enzyme-linked immunosorbent assay (ELISA) and enzyme-linked receptor assay (ELRA). ELBA with highly sensitivity could avoid aforementioned drawbacks by trace analytic extraction and decreasing the matrix interference obviously. However, the conventional indirect ELISA method for ibuprofen determination needs expensive primary and secondary antibodies with high cost, tedious
and time-consuming procedures [28]. And ELRA method has been so far only used for the analysis of estrogens [29] and β-lactam antibiotics [30] as it is difficult to obtain the corresponding receptor. Human serum albumin (HSA), the most abundant protein in human blood plasma, plays a major role in transporting endogenous and exogenous ligands [31]. HSA could interact with ibuprofen with high affinity to provide a reservoir for a long duration of action and ultimately affect the ADME (absorption, distribution, metabolism, and excretion) profiles in vivo [32]. As HSA is not a pharmacological target of ibuprofen, it is an excellent transport protein to develop a ligand binding assay for ibuprofen determination in human urine samples. Compared with ELISA, utilization of HSA could replace the expensive antibody and significantly reduce the test costing. It is wildly accepted that assay efficiency could be largely improved in virtue of the strong absorption ability and high surface-to-volume ratio [33]. Therefore, nanoparticles have been gradually used as stationary phase in enzyme-linked assays [34] and also show us an emerging and promising direction in development of new methods of HSA-ligand binding assay.

Herein, we developed a newly nano-SiO2 based direct competitive ELISA method for ibuprofen determination using HSA as receptor and horseradish peroxidase (HRP) as a marker enzyme. As far as we know, the proposed method was the first time to use HSA as a receptor protein for ELRA analysis with advantages of high accuracy, low costs, fast speed and little requirement on laboratory equipment, thus would greatly expand the coverage and provide more flexibility in small compound determination of biological fluids.

2. Materials and methods

2.1. Instrumentation

The UV absorbance of samples was measured by UV-L5S spectrophotometer (Shanghai INESA Scientific Instrument Co., Ltd.). The morphologies and sizes of SiO2 nanoparticles were tested by transmission electron microscope (TEM, FEI Tecnai G20, FEI, Co, United States). The thermo-gravimetric analysis (TGA) was obtained by heating powdered samples from room temperature to 800 °C under nitrogen atmosphere using TGA Q500 V20.13 Build 39 thermo-analysis system (TA, Co, United States). MALDI-TOF mass data was obtained from AB Sciex 4800 Plus MALDI TOF/TOF analyzer (AB Sciex, CA) in linear high internal mode.

2.2. Reagents and materials

Ibuprofen standard (CAS 15687-27-1) was purchased from Shanghai Titan Chemical Co., Ltd. (China). Human serum albumin (HSA) was purchased from Shanghai Xinyu Biological Technology Co., Ltd. (China). Horseradish peroxidase (HRP), 3-aminopropyltrimethoxysilane (APTMIS), 3,3′,5,5′-tetramethylbenzidine (TMB), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich Co., Ltd. (United States). Tetraethyl orthosilicate (TEOS), ethanol, ammonia solution (NH3·H2O), hydrogen peroxide (H2O2) and other chemicals of AR grade were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The dialysis bag (MWCO 14000, nominal flat width 44 mm, diameter 28 mm, vol/length 6.15 mL/cm) was bought from USA Viskase Corporation. Ibuprofen granule (batch number H2116D02) was purchased form Hainan Zambon Pharmaceutical Co., Ltd. (Haikou, China). Urine samples donated by volunteers.

2.3. Preparation of SiO2-HSA

Nano-SiO2 coupled with HSA (SiO2-HSA) was prepared by the carbodiimide crosslinking method through amino-functionalized silicon dioxide nanoparticles (SiO2-NH2) and HSA. Firstly, SiO2-NH2 was prepared by TEOS and APTMS according to our procedures reported previously. Then, 1 g of SiO2-NH2 and 50 mg of HSA were dispersed in 50 mL PBS buffer solution (10 mM, pH 7.4), gently mixed well, and dripped slowly by 2 mL reaction reagent containing 400 mg of EDC and 250 mg of sulfo-NHS in PBS. After gently shaking for 12 h at room temperature, the reaction mixture was washed twice by PBS buffer and 100 mL aqueous solution of 5% skimmed milk was used to seal up these unoccupied binding sites. SiO2-HSA was obtained by centrifugal separation at 4000 rpm for 5 min and stored at 4 °C for the further use. The preparation procedures were illustrated in Fig. 1.

2.4. Preparation of HRP-ibuprofen

HRP labeled ibuprofen (HRP-ibuprofen) was prepared by the following procedures: 21 mg (0.1 μM) of ibuprofen was dissolved in 4 mL DMF containing 23 mg DCC (0.11 mM). The mixtures were stirred for 15 min, added of 22 mg sulfo-NHS (0.11 mM), and reacted for 8 h at 0 °C. Then, the supernatant obtained by centrifugal separation at 4000 rpm for 5 min was dropped into 10 mL HRP solution (1 mg mL−1) for reaction of 8 h. Finally, the reaction solution was placed in a dialysis bag with a molecular weight cut off of 14,000 and then suspended in 100 mL PBS buffer solution (10 mM, pH 7.4) with gently stirring at 4 °C. The PBS buffer solution was changed every 6 h. After dialyzing for 24 h, HRP-ibuprofen was obtained and stored at 4 °C for the further use.

2.5. Optimization of HRP-ibuprofen dilution

As a direct competitive method, optimization of the ratio of SiO2-HSA/HRP-ibuprofen need to make sure that the amount of HRP-ibuprofen is more than that of SiO2-HSA. Method optimization was carried out as follows: accurately weighed 9 batches of SiO2-HSA with a fixed amount of 5 mg respectively, dispersed in 1 mL PBS buffer solution (10 mM, pH 7.4), mixed with 0.5 mL HRP-ibuprofen solution with a series of gradient dilution (1:1, 1:5, 1:10, 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600, respectively), incubated at 20 °C for 15 min. Then, the nanoparticles were washed three times with 1 mL PBS buffer and separated with centrifuge. Finally, the precipitation was incubated with 1 mL of TMB substrate solution (0.5 mL of 2 mg mL−1 TMB, 42 μL of 0.75% H2O2, 9.5 mL of substrate buffer (0.1 M citrate-phosphate buffer, pH 5.0)) for 15 min at 20 °C and terminated with 0.5 mL of 2 M H2SO4. The absorbance of the supernatant was measured by ultraviolet spectrophotometer at 450 nm.

2.6. Nano-ELBA measurement

The Nano-ELBA analytical method for ibuprofen determination in urine was based on the direct competitive principle, which was similar to direct competitive ELISA described earlier. Firstly, 5 mg SiO2-HSA nanoparticles were dispersed in 1 mL sample solution. Subsequently, 0.5 mL HRP-ibuprofen solution was charged into the above milky liquid under gentle vortex and reacted for 15 min. The reactive mixture was centrifuged at 4000 rpm for 5 min, washed with PBS buffer and repeated three times. Finally, the precipitation was incubated with 1 mL of TMB substrate solution (0.5 mL of 2 mg mL−1 TMB, 42 μL of 0.75% H2O2, 9.5 mL of substrate buffer (0.1 M citrate-phosphate buffer, pH 5.0)) for 15 min at 20 °C. After terminated with 0.5 mL of 2 M H2SO4, the absorbance of the supernatant was measured by ultraviolet spectrophotometer at 450 nm. The procedures described above were illustrated in Fig. 2.

![Illustration of procedures in the preparation of SiO2-HSA.](image)
2.7. Method validation

A stock solution of ibuprofen was prepared by dissolving 100 mg ibuprofen standard into a 10 mL volumetric flask with N,N-dimethylformamide to volume. Work solutions I-VI were then diluted step by step to the necessary concentrations (5, 10, 20, 35, 50, 70 ng mL\(^{-1}\)). All the standard solutions were kept in a refrigerator at 4 °C before use. After analyzed in quintuplicate by the nano-ELBA procedures aforementioned, the calibration graph was plotted based on linear regression analysis of the UV absorbance (y) versus concentrations (x, ng mL\(^{-1}\)) of the work solutions I-VI at six different concentrations. The LOD was defined as the result of the average value minus three times of the standard deviation value. The precision was evaluated by the intra- and inter-assay variation. The intra-assay variation was determined by three different concentrations of ibuprofen standards (15, 30, 60 ng mL\(^{-1}\)) within 12 h according to the reported analytical method in nonuplicate. The inter-assay variation was determined within seven days under the same conditions. The repeatability was evaluated by a real urine sample solution (sample 4, sampling time-point at 6 h after a single oral dose of 100 mg of ibuprofen granule). Six independently determinations of sample 4 were performed under the nano-ELBA measurement procedures described in Section 2.6. The recovery was investigated by spiking three different amounts of ibuprofen standard (80%, 100% and 120% of ibuprofen content in sample) to sample 4, and then analyzed under this proposed method. Each sample was analyzed in triplicate. The total amount of each analysis was calculated from the corresponding calibration curve. The recovery was defined as the ratio of detected amount and added amount.

2.8. Statistical analysis

Descriptive statistical parameters such as mean value, standard deviation (SD), and coefficient of variation (CV) were calculated by Microsoft Excel 2007.

3. Results and discussion

This work represented a bio-recognition-linked approach for the analysis of ibuprofen based on the ELBA of ibuprofen to HSA. Ibuprofen could interact with the principal binding sites of HSA in subdomain IIIA with high affinity, which had been studied by biophysical and computer modeling methods [32]. As illustrated in Fig. 2, ibuprofen in sample and ibuprofen labeled by HRP (HRP-ibuprofen) competitively bind to HSA molecules, which were immobilized on the surface of nano-SiO\(_2\) particles. After centrifugal separation, HRP/ibuprofen/nano-SiO\(_2\) composite catalyzed the coloring substrate solution (TMB/H\(_2\)O\(_2\)) from colorless to blue, and finally to yellow when stop solution (2 M H\(_2\)SO\(_4\)) added. As the ibuprofen existed in samples and HRP-ibuprofen subsequently added were able to competitively bind with HSA, so the higher contents of ibuprofen in the sample, the less HRP-ibuprofen bindings on the SiO\(_2\)-HSA, resulting in less obvious color reaction of substrate solution. The properties of nano-SiO\(_2\) used as stationary phase, such as large surface area, made the binding interaction between ibuprofen and HSA more substantially which led to a significantly improved performance with shortened assay time and enhanced reproducibility.

3.1. Material characterization

The TEM image of SiO\(_2\)-HSA with diameter less than 100 nm is shown in Fig. 3. The thermogravimetric analysis (TGA) was used to confirm the functionalization of SiO\(_2\)-NH\(_2\) HSA nanoparticles. As shown in Fig. 4, the variation of weight loss curves between SiO\(_2\)-NH\(_2\) and SiO\(_2\)-NH-BSA nanoparticles was different due to the decomposition of organic substance on the surface of SiO\(_2\). The weight loss of SiO\(_2\)-NH-HSA was more than that of SiO\(_2\)-NH\(_2\), which thus indicated that HSA was successfully functionalized on the silica nanoparticles. The coupling ratio of HRP-ibuprofen was determined by MALDI/TOF. As shown in Fig. 5, the molecular weights of HRP and HRP-ibuprofen were 43291.9805 and 44098.9545 respectively. Accordingly, the coupling ratio was calculated to be about 4:1.
3.2. Establishment and optimization of the ELBA

Optimizing of the ratio of SiO$_2$-HSA/HRP-ibuprofen not only contributed to a high detection sensitivity, but also a reduction of material consumption. As shown in Fig. 6, a graph was plotted based on the OD$_{450}$ values (y) versus the logarithm of HRP-ibuprofen dilution from 1:1 to 1:1600. The graph showed an obvious turning point at ($x_{1.70}$, $y_{1.53}$), corresponding to the dilution 1:50. Based on conservative selection, the dilution 1:40 was used as optimal parameters for ELBA analysis.

3.3. Analytical figures of merit

Through determination the OD$_{450}$ values of blank (B$_0$) and standard solutions (B) at concentrations ranging from 5 to 70 ng mL$^{-1}$, a linear calibration curve was established as $y = -0.013x + 0.988$ with good correlation ($r^2=0.998$). The LOD was determined as 1.516 ng mL$^{-1}$.

Precision of the nano-ELBA method was determined by the intra- and inter-assay coefficient of variation (CV) as shown in Table 1. The intra-assay precision was calculated by detecting three different concentrations (15, 30, 60 ng mL$^{-1}$) within 12 h in nonuplicate, and their CVs were 2.3%, 3.9% and 2.08%, respectively. The inter-assay precision was calculated by detecting the three different concentrations in different days, and their CVs were 3.4%, 3.5% and 2.5%, respectively. The intra- and inter-assay CVs were both below 5%, which indicated that the reported analytical method exhibited high precision. A good repeatability was confirmed by six independently determinations using a real urine sample solution (sample 4). The average content of sample 4 was 187.23 ± 3.41 ng mL$^{-1}$. To identify the recovery, a certain amount of ibuprofen standard (150, 190 and 225 ng mL$^{-1}$) was spiked to real urine sample 4 (sampling time-point at 6 h after a single oral dose of 100 mg of ibuprofen granule). As shown in the Table 2, the mean recoveries were ranged from 94.49% to 97.95%. Thus, the results indicate that the reported analytical method can be applied to detect ibuprofen in urine, and has potential for the detection of biological samples.

3.4. Nano-ELBA determination of ibuprofen in urine

Urine samples were collected within 36 h after a single oral dose of 100 mg of ibuprofen granule. The present nano-SiO$_2$ based ELBA method was applied to the determination of ibuprofen in urine samples. The quantification was carried out using calibration curve.

Table 1

|         | Actual (ng mL$^{-1}$) | Detected (ng mL$^{-1}$) | CV (%) |
|---------|-----------------------|-------------------------|--------|
| Intra-day | 15            | 14.92 ± 0.24           | 2.3    |
| (n=9)       | 30            | 30.23 ± 0.89           | 3.9    |
|           | 60            | 60.51 ± 1.10           | 2.0    |
| Inter-day | 15            | 14.77 ± 0.41           | 3.4    |
| (n=7)       | 30            | 29.75 ± 0.86           | 3.5    |
|           | 60            | 59.74 ± 1.06           | 2.5    |
Some samples out of the range of the calibration curve were diluted by blank urine to be within. As illustrated in Fig. 7, the amount of the ibuprofen detected at levels ranging from 5.01 ± 2.95 to 389.23 ± 12.37 ng mL⁻¹ in the interval from 0 to 36 h.

4. Conclusions

In the present study, a simple and rapid nano-SiO₂ based enzyme-linked ligand binding assay (ELBA) was developed for quantitative determination of ibuprofen in human urine samples. It is the first report to utilize HSA in place of pharmacological receptor for the development of ELBA method, which could significantly reduce test cost, expand the coverage and provide more flexibility in small compound determination of biological fluids. Through introducing nano-SiO₂ as stationary phase with larger surface area, a substantial interaction between ligand and receptor significantly improved the analytic performance with shortened assay time and enhanced reproducibility. As a proof of principle, this novel assay was validated to be suitable for the quantification of ibuprofen in urine with low detection limits, consistent reproducibility, and high recovery. It can be expected that the proposed method could be a potential application in the determination of other small-molecule compounds from various biological samples.

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Table 2

| No. | Content/ng mL⁻¹ | Added/ng mL⁻¹ | Detected/ng mL⁻¹ | Recovery/% | Mean recovery/% | SD | CV/% |
|-----|-----------------|---------------|-----------------|------------|----------------|----|------|
| 1   | 187.23          | 150.00        | 330.12          | 95.26      | 97.95          | 0.029 | 2.91 |
| 2   | 150.00          | 333.69        | 100.94          | 97.64      |                |      |      |
| 3   | 150.00          | 338.64        | 95.34           |            |                |      |      |
| 4   | 190.00          | 368.38        | 96.87           |            |                |      |      |
| 5   | 190.00          | 371.29        | 91.25           |            |                |      |      |
| 6   | 190.00          | 360.60        | 88.11           |            |                |      |      |
| 7   | 225.00          | 407.97        | 96.32           |            |                | 0.016 | 1.70 |
| 8   | 225.00          | 403.12        | 94.89           |            |                |      |      |
| 9   | 225.00          | 400.73        | 94.95           |            |                |      |      |

Fig. 7. Mean concentration-time profile for ibuprofen in urine after a single oral dose of 100 mg.

Table 2 Results of the recovery evaluation.
chelate, J. Pharm. Biomed. Anal. 15 (1997) 1805–1811.

[23] S.C. Turk, E. Satana, H. Basan, N.G. Goger, Determination of ibuprofen and parabens in pharmaceutical formulations using flow-injection and derivative spectrophotometry, J. Anal. Chem. 70 (2015) 50–54.

[24] M. Ghorbani, M. Chamsaz, G.H. Rounagh, Ultrasound-assisted magnetic dispersive solid-phase microextraction: a novel approach for the rapid and efficient microextraction of naproxen and ibuprofen employing experimental design with high-performance liquid chromatography, J. Sep. Sci. 39 (2016) 1082–1089.

[25] M.R. Payan, M.A.B. Lopez, R. Fernandez-Torres, M.V. Navarro, M.C. Mochon, Hollow fiber-based liquid-phase microextraction (HF-LPME) of ibuprofen followed by FIA-chemiluminescence determination using the acidic permanganato-sulfite system, Talanta 79 (2009) 911–915.

[26] L.M. Madikizela, L. Chimuka, Determination of ibuprofen, naproxen and diclofenac in aqueous samples using a multi-template molecularly imprinted polymer as selective adsorbent for solid-phase extraction, J. Pharm. Biomed. Anal. 128 (2016) 210–215.

[27] Z. Rezaeifar, Z. Es’haghi, G.H. Rounaghi, M. Chamsaz, Hyperbranched polyglycerol/graphene oxide nanocomposite reinforced hollow fiber solid/liquid phase microextraction for measurement of ibuprofen and naproxen in hair and waste water samples, J. Chromatogr. B 1029 (2016) 81–87.

[28] K.A. Grafe, H. Hoffmann, Development and validation of an indirect enzyme-linked immunosorbent assay (ELISA) for the nonsteroidal anti-inflammatory drug S-ibuprofen, Pharmazie 55 (2000) 286–292.

[29] M. Seifert, S. Haindl, B. Hock, Development of an enzyme linked receptor assay (ELRA) for estrogens and xenoestrogens, Anal. Chem. Acta 386 (1999) 191–199.

[30] J. Adrian, D.G. Pinacho, B. Granier, J.M. Diener, F. Sanchez-Riera, M.P. Marco, A multianalyte ELISA for immunochromatographic screening of sulfonamide, fluoroquinolone and beta-lactam antibiotics in milk samples using class-selective bioreceptors, Anal. Bioanal. Chem. 391 (2008) 1703–1712.

[31] K. Yamasaki, T. Marnyama, U. Kragh-Hansen, M. Otagiri, Characterization of site I on human serum albumin: concept about the structure of a drug binding site, Biochim. Biophys. Acta 1295 (1996) 147–157.

[32] F. Zsila, Z. Bikadi, D. Malik, P. Hari, I. Prechan, A. Bercs, E. Hazai, Evaluation of drug–human serum albumin binding interactions with support vector machine aided online automated docking, Bioinformatics 27 (2011) 1806–1813.

[33] L. Wu, W. Yin, K. Tang, K. Shao, Q. Li, F. Wang, Y. Zuo, X. Lei, Z. Lu, H. Han, Highly sensitive enzyme-free immunosorbent assay for porcine circovirus type 2 antibody using Au-Pr/SiO2 nanocomposites as labels, Biosens. Bioelectron. 82 (2016) 177–184.

[34] Q.L. Wang, J. Li, X.D. Li, L.S. Ding, J. Xie, L.S. Qing, A simple nano-SiO2-based ELISA method for residue detection of 2,4-dichlorophenoxyacetic acid in bean sprouts, Food Anal. Method (2016), http://dx.doi.org/10.1007/s12161-12016-10709-x.