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Simultaneous Detection of Dual Biomarkers from Humans Exposed to Organophosphorus Pesticides by Combination of Immunochromatographic Test Strip and Ellman Assay

Mingming Yang[a,b,#], **Yuting Zhao**[a,#], **Limin Wang**[d], **Michael Paulsen**[c], **Christopher D. Simpson**[c], **Fengquan Liu**[b], **Dan Du**[a,*], and **Yuehe Lin**[a,*]

[a]School of Mechanical and Materials Engineering, Washington State University, Pullman, WA 99164, United States

[b]Jiangsu Academy of Agricultural Sciences, Nanjing 210014, People's Republic of China

[c]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA 98195, United States

[d]College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, People's Republic of China

Abstract

A novel sandwich immunoassay based immunochromatographic test strip (ICTS) has been developed for simultaneously measuring both butyrylcholinesterase (BChE) activity and the total amount of BChE (including inhibited and active enzyme) from 70 μLpost-exposure human plasma sample. The principle of this method is based on the BChE monoclonal antibody (MAb) capable of acting as both capture antibody and detection antibody. The BChE MAb which was immobilized on the test line was able to recognize both organophosphorus BChE adducts (OP-BChE) and BChE and provided equal binding affinity, permitting detection of the total enzyme amount in post-exposure human plasma samples. The formed immunocomplexes on the test line can further be excised from the test-strip for subsequent off-line measurement of BChE activity using the Ellman assay. Therefore, dual biomarkers of BChE activity and phosphorylation (OP-BChE) will be obtained simultaneously. The whole sandwich-immunoassay was performed on one ICTS, greatly reducing analytical time. The ICTS sensor showed excellent linear responses for assaying total amount of BChE and active BChE ranging from 0.22 to 3.58nM and 0.22 to 7.17nM, respectively. Both the signal detection limits are 0.10nM. We validated the practical application of the proposed method to measure 124 human plasma samples from orchard workers and cotton farmers with long-term exposure to organophosphorus pesticides (OPs). The results were in highly agreement with LC/MS/MS which verified our method is extremely accurate. Combining the portability and rapidity of test strip and the compatibility of BChE MAb as both capture antibody and detection antibody, the developed method provides a baseline-free, low-cost and rapid tool for in-field monitoring of OP exposures.

Corresponding authors: Yuehe.lin@wsu.edu or annie.du@wsu.edu.
[#]These authors contributed equally to this work.

Keywords

Pesticides exposure; Biomarkers; BChE monoclonal antibody; Enzyme inhibition; Immunochromatographic test strip

INTRODUCTION

Organophosphorus pesticides (OPs) are widely used for crop protection, and have contributed to dramatic increases in crop yields in modern agriculture (Du et al. 2011b). Even at relatively low levels, OPs have been linked to neurodevelopmental deficiencies in young children (Eskenazi et al. 2007). The toxicity of OPs is reflected by covalent binding to the active site of cholinesterase to inhibit the activity of the enzyme.(Fidder et al. 2002). The inhibition of ChE, which includes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), leads to an excess of acetylcholine within the nerve tissue and affects the transmission of the neurotransmitter (Aryal et al. 2012; Williams et al. 2007), causing tremors, lacrimation and bradycardia, even death (Lin et al. 2004). Therefore, development of simple and reliable methods for rapid detection of OPs exposures is necessary and essential.

Biomonitoring is an efficient approach for assessing internal dose and potential risk from exposure to OPs. Currently, four kinds of biomarkers have been used for detection of OPs exposure including assay of active enzyme (Ge et al. 2013b; Liu et al. 2005; Zhang et al. 2014), detection of unbound OPs (Li et al. 2014; Xu et al. 2015), measurement of metabolites (Zhang et al. 2013; Zou et al. 2010) and quantification of phosphorylated enzyme adducts (OP-ChE) (Jiang et al. 2013; Johnson et al. 2015). The majority of the measurements for metabolites and unbound OPs are performed by gas or liquid chromatography coupled with mass spectrometry (GC/LC-MS) (VanDine et al. 2013; Zhu et al. 2015). Although these methods are sensitive and accurate, they are faced by several disadvantages notably use of sophisticated equipment requiring a well-trained technician to operate, time-consuming analysis generally performed off-site at a laboratory, lack of portability and concomitant delay in reporting of results (Zhang et al. 2014).

Enzyme activity in blood is a good biomarker for biomonitoring of exposure to OPs because it directly provides a quantitative biochemical effect of the exposure (i.e., enzyme inhibition). An important confounding challenge with this assay is the need of a baseline for each individual before a meaningful enzyme activity change can be determined. However, because the ChE activity baseline for individuals is generally not available, this measurement is often based on a population average. In addition, an individual’s level of ChE activity fluctuates over time. This factor creates uncertainty in historically obtained individual ChE activity measurements and thus may provide false results for low level exposure. In this regard, a baseline from individual subject is a better control than that from population average level served as pre-exposure baseline because the former can be obtained in real-time and is more accurate for calculation of ChE inhibition. According to the mechanism involved in phosphorylation of ChE, the inhibition event produces very stable enzyme complexes (OP-ChE) (Liu et al. 2008). One challenge is to find antibodies capable

of specifically recognizing the phosphorylated adducts due to the unique structure of OP-ChE. The unique structure of OP-ChE is that the phosphoserine moiety is in a deep and narrow invagination. Current specific antibody can only recognize ChE moiety, while it is very hard to develop the other pair to recognize the phosphoserine moiety which is only several Å and deep in the gorge of ChE. We found that the same ChE antibody was able to be used as both capture and detecting antibody in sandwich immunoassay, which avoided to use the antibody to recognize the narrow invagination (Quinn 1987).

Different methods, including Michel (Δ pH) ChE assay (Ellin et al. 1973), fluorescence assays (Parvari et al. 1983), colorimetric Ellman assay (Ellman et al. 1961) and radioactive assays (Gordon et al. 1982) have been developed to measure the changes of enzyme activity from a basal level. Due to substantial inter-individual (e.g., age, ethnicity, sex, etc.) variation in the baseline of ChE, an important challenge with these assays is the need to determine pre-exposure ChE activity for each individual before a meaningful post-exposure change in activity can be determined (Gupta). Since the ChE activity baseline for individuals is generally not available, ChE activity measurements may provide false results for low level exposure such as ≤ 20% enzyme inhibition. In order to avoid the need for baseline measurements, we have reported baseline-free approaches to measure OPs exposures, such as reactivation of phosphorylated ChE (Du et al. 2012; Ge et al. 2013a), and parallel detection of the total enzyme and active enzyme separately (Du et al. 2011a).

Comparing to AChE, BChE is more abundant in human plasma and has higher rate of reaction (Aryal et al. 2012; Pantazides et al. 2014), therefore BChE was adopted as model analyte. In this paper, we report a simultaneous measurement of post-exposure BChE enzyme activity together with quantifying the total amount of BChE enzyme by immunoassay. Since the total concentration of enzyme (including active and inhibited) is measured contemporaneously with BChE activity, it can replace the need for measurement of subject-specific pre-exposure enzyme activity. Here, we found that anti-butyrylcholinesterase (BChE) monoclonal antibody (MAb) is able to be used as both capture and detecting antibody in sandwich immunoassay. It is not necessary to adopt diverse antibodies for recognizing an antigen performing distinctive determinants simultaneously. The reason is that BChE in the native human plasma is a tetramer, consists of four identical subunits that can simultaneously be bound by the same monoclonal antibodies, wherein each binding is serving for either capturing or detection. (Haeberli 1992; Li et al. 2008; Ngamelue et al. 2007; Pan et al. 2009). That means each BChE protein has four epitopes which can be recognized by the same MAb. Our approach is based on an immunochromatographic test strip (ICTS) platform. Due to the advantages of portability, rapidity, and low cost, ICTS has become a widely used platform for in-field application (Ge et al. 2014; Kim et al. 2015; Quesada-Gonzalez and Merkoci 2015; Wen et al. 2017). The immobilized BChE MAb on the test line is able to recognize both BChE and OP-BChE in human blood samples (Sporty et al. 2010). Gold nanoparticle labeled anti-BChE MAb (MAb-Au) as a recognition element is used to quantify the total BChE. The captured BChE on the test line is then excised for off-line detection of BChE activity using the Ellman assay. Therefore, such an approach can simultaneously provide results of dual biomarkers of both enzymatic activity and phosphorylation, offering a more accurate and inexpensive tool for assessing exposure to OPs.

2. Experimental Section

2.1 Materials and Reagents

Human butyrylcholinesterase (ab96367) (>100U), BChE monoclonal antibody (ab17246), goat anti-mouse IgG antibody and HRP-conjugated goat anti-mouse IgG (HRP-Ab_2) was purchased from Abcam Inc. (Cambridge, MA). Paraoxon-ethyl was purchased from Chem Service, Inc. (West Chester, PA). Acetylthiocholine chloride (ATCh), 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), phosphate buffer saline (PBS), Tween-20, sulfuric acid, acetone, 3,3′,5,5′-Tetramethylbenzidine (TMB), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, U.S.A.). 40nm gold nanoparticles (Au NPs) was purchased from nanoComposix (San Diego, CA). Microplates for enzyme-linked immunosorbent assay (ELISA) were purchased from Becton (Franklin Lakes, NJ). Fiber conjugate pad, fiber sample pad, nitrocellulose membrane and absorbent pad were obtained from Millipore (Bedford, MA).

2.2 Instrumentation

The separation of gold nanoparticle labeled BChE MAb (MAb-Au) was performed by centrifugation (Eppendorf 5417C; Eppendorf, US). The 10–140 incubator was purchased from Quincy Lab, Inc. The test strip was fabricated using a Guillotine Cutting System (BioDot LTD; Irvine, CA). The Peak Area on the test line was recorded by a portable test strip reader ESE-Quant GOLD (DCN Inc.; Irvine, CA). The absorbance on the test line was measured with a Tecan Safire^2 microplate reader (Tecan, Switzerland).

2.3 Preparation of Au labeled BChE MAb

The principle of Au labeled antibody is based on electrostatic adsorption between antibody and gold nanoparticles. (Geoghegan and Ackerman 1977). Briefly, 0.2 M K_2CO_3 was added to 1.0 ml Au NPs (OD_{528} = 0.508) solution to achieve a pH of 8.5, followed by adding 30 μL BChE MAb solution (1mg/ml) and keepingthe mixture at room temperature (about 25°C) for 1h after mixing it thoroughly. At pH 8.5, Au and BChE MAb showed the most effective conjugation (Figure S1A and S1B). Then, BSA was added to the above solution and allowed to stand for another 1 h. After that, the mixture was centrifuged at 10,000 rpm for 25 min and washed with 2 % BSA solution twice. The resultant precipitate was re-dispersed in deionized water which containing 2% BSA and 3% sucrose to 100 μL.

2.4 Preparation of ICTS

Scheme 1 illustrates the mechanism of the ICTS. The ICTS is comprised of four sections: sample pad, conjugate pad, nitrocellulose membrane (NC membrane) and absorbent pad. The four sections were assembled on a plastic scale board, as shown in Scheme 1. The conjugate pad was pretreated with pH 7.4 PBS buffer containing 2% BSA. On NC membrane, the Test line and Control line were prepared by dispensing BChE MAb (1 mg/mL) and goat anti-mouse IgG antibody (0.5 mg/mL) using a BioDot BioJet BJQ 3000 dispenser with the lining rate 1.5 μL/mm. Then the ICTS were dried overnight at room temperature and cut at the width of 4.0 mm using a Bio-Dot Paper Cutter module CM4000. Finally, the test strips were stored in Ziploc bags under dry conditions at room temperature.

2.5 Preparation of OP-BChE

5 μL paraoxon-acetone solution (75 mM) (the volume of acetone less than 5% of total volume) was dropped into 1 mg/ml BChE solution, then diluted with PBS buffer, followed by standing the mixture at room temperature for 72 h. Meanwhile, BChE solution without paraoxon-acetone was used as control. The inhibition of BChE was measured by Ellman assay according to Hossain et al with a little modification. (Hossain et al. 2009).

2.6 ELISA test for the affinity of BChE MAb to BChE and OP-BChE

The affinity of BChE MAb to BChE and OP-BChE, respectively was detected by one-site ELISA. 50 μL BChE and OP-BChE solution with a series of different concentrations (0, 0.72, 1.43, 2.87, 5.74 nM) were added into 96 well microplate with three replicates and incubated at 37°C for 2 h. After washing the wells with PBST for several times, 200 μL of 3% BSA in PBS was added into each well and allowed to stand for 1.5 h. Thereafter, 50 μL of 2 μg mL^{-1} BChE MAb was added into each well after the plates were washed several times. Afterwards, 50 μL of 20,000-fold diluted HRP-Ab_2 was added, and incubated for 40 min, and re-washed. Finally, 50 μL substrate TMB was added to the respective wells and left to keep at room temperature for 20 min. Then, HRP reaction with TMB was stopped by adding 25 μL of 2.0 M H2SO4. The plate was rapidly tested on a microplate reader.

2.7 Assay procedure

70 μL sample was dropped in the sample pad and it flowed along the strip via capillary action. First, the sample interacted with the MAb-Au in conjugate pad to form a BChE-MAb-Au complex. Then it continued flowing onto the NC membrane until captured by BChE MAb in the test line. The excess MAb-Au continued to migrate and was captured in the control line. The total BChE signal intensity was recorded by test strip reader. Then, the test line was excised and transferred into a centrifuge tube containing 100 μL ATCh solution and incubated for 15 min. The active BChE was measured by colorimetric analyses with Tecan Safire^2 microplate reader after adding DTNB.

2.8 Human plasma samples

The human plasma samples were collected from workers in Washington State and in Pakistan who were occupationally exposed to OPs. The samples were diluted 100-fold with PBST (phosphate buffer saline solution containing 0.05% Tween-20) before detection. Then, 70 μL of the diluted solution was dropped onto the sample pad to complete the test.

3. Results and Discussion

3.1 Principle of Immunoassay on ICTS

Plasma-derived BChE is a tetramer that comprises four glycoprotein subunits that are identical and a polyproline-rich peptide. The same monoclonal antibodies bind to different BChE subunit hence can be used to capture and detect BChE and OP-BChE. Our approach is based on a one-step ICTS for parallel measurements of active enzyme and total amount of enzyme from post-exposure samples. As shown in Scheme 1, both OP-BChE and active BChE in the sample solution first interacted with gold nanoparticles labeled with anti-BChE

(MAb-Au) on the conjugate pad to form OP-BChE/MAb-Au and BChE/MAb-Au complexes. The formed complexes kept flowing and were captured by anti-BChE MAb immobilized on test line to form sandwich immunocomplexes namely BChE MAb/OP-BChE/MAb-Au and BChE MAb/BChE/MAb-Au. The excess MAb-Au continued to migrate and was captured in control line. The color intensity on test line was recorded by test strip reader. This signal reflects the total amount of BChE (including active and phosphorylated). Then the test line was excised from test strip and analyzed for BChE activity via the Ellman assay. This response is only related to the active amount of BChE in the sample. Moreover, phosphorylation can be calculated by subtracting active BChE from total amount of BChE. In comparison to the electrochemical sensing platform (Du et al. 2011a), the measurement in this paper is more applicable. ICTS is less expensive and easier to used compared with the flow injection system.

3.2 Characterization of ICTS

In our approach, having the same binding affinity of BChE-MAb to both BChE and OP-BChE plays a crucial role to detect the total amount of BChE (including OP-BChE and active BChE). We verified it by both ELISA and test strip. Figure 1A shows the absorbance values from ELISA using a series of concentrations of the BChE and OP-BChE. The result showed that anti-BChE-MAb had almost equal binding affinity to BChE and OP-BChE at the same concentrations. Figure 1B shows the results on the test strip assay. It was observed that the test line had almost the same signal intensity and color density when using the same concentration of BChE and OP-BchE (3.44 nM). This demonstrates that the BChE-MAb can equally recognize BChE and OP-BChE, which is important for the principle of detection of total enzyme amount from post-exposure samples. Conversely, when we changed the antigen to AChE (3.44 nM) instead of BChE, the signal intensity on test line disappeared, which further demonstrated that the BChE-MAb can specifically recognize BChE and OP-BChE. Furthermore, PBST as a control did not show any color and the obvious signal, which proved that the responses came from antigen-antibody interaction as opposed to non-specific reaction.

3.3 Optimization of analytical parameters for BChE detection

There are three main parameters that affected the sensitivity of immunoassay: the pH of working solution, the volume of MAb-Au used on ICTS, and immunoreaction time of the ICTS. The influence of the aforementioned parameters was studied using 3.44 nM BChE. The pH of the working solution directly affected the stability of the antigen-antibody interaction. To ascertain the optimal pH of the working solution, pH was varied from 6.0 to 9.5, and the signal from the test strip was measured. In Figure 2A, the peak area on the test strip increased with increasing pH and reached a maximum at pH 7.5. So pH 7.5 was used for the subsequent experiments. Since MAb-Au was also used as detection antibody, the volume of MAb-Au is another important issue for the sensitivity of immunoassay. It can be seen from Figure 2B that the signal intensity increased with the increase in volume of MAb-Au within 2.0 μL and then plateaued. The immunoreaction time (i.e. time elapsed between applying sample to the test strip and reading response at the test line) is another important issue which can affect the sensitivity of immunoassay. As show in Figure 2C, the signal intensity increased rapidly with the increase of immunoreaction time during the first 25 min

and eventually stabilized, indicting completion of immunoreaction. Therefore, 25 min was adopted for the experiments.

3.4 Detection of total amount and activity of standard BChE

To test the performance of ICTS for simultaneous detection of the total BChE and uninhibited BChE, the measurements were conducted with various concentrations of standard BChE in PBST. As can be seen from Figure 3A, the color intensity on the test line weakened along with the decrease of total amount of BChE (from 1 to 8). The signal corresponding to the total amount of BChE was recorded by the test strip reader after 25 min. The peak area on the test line was found to be directly proportional to the concentration of standard BChE. The calibration curve is y=1058.427x+843.219 which was obtained at the concentration range from 0.22 to 3.58 nM (Figure 3B). After detecting total amount of enzyme, the test line was excised and dropped into a centrifuge tube containing 100 μL ATCh solution and incubated for 15 min, followed by adding 100 μL DTNB. The UV absorbance of the mixture solution was recorded at 412nm. As shown in Figure 3C, the calibration curve of active BChE is y=0.1056x+0.1546 which was obtained at the concentration range from 0.22 to 7.17 nM. The detection limits of total amount of BChE and active BChE were both 0.1nM, respectively. Then the OP-BChE can be calculated by subtracting active BChE from total amount of BChE.

3.5 Evaluation of ICTS for measurement of OPs exposure

To demonstrate this method for quantitative detection of OPs exposure based on ICTS, standard BChE samples were used for this purpose. Briefly the standard BChE samples were firstly inhibited with different concentrations of paraoxon and tested with ICTS. Then the total amount of enzyme and enzyme activity were calculated from the calibration curve described above. As shown in Table 1, a broad percentage of phosphorylation (9–99%) was achieved by varying the concentrations of paraoxon (0.002–0.2 mg kg^{-1}). As expected the BChE activity decreased accordingly. Those results were further compared with the Ellman assay, the relative deviations were ± 5% and the correlation coefficient is 0.9983 (Fig. S2), indicating that this approach is reliable and accurate.

3.6 Validation of ICTS with human blood samples

To further validate the practical application of the proposed method, blood collected from orchard workers in Washington State (89 samples), and chlorpyrifos manufacturing workers and cotton farmers in Pakistan (35 samples) with long-term exposure to OPs were measured. Before detection, those samples were diluted 100-fold with PBST. Percent phosphorylation was also determined independently using an LC/MS/MS procedure that directly measures the BChE-OP adduct (Riutta 2012). As shown in Figure 4, these samples covered a wide range of % phosphorylation, between 0–92%. Due to the complexity of human plasma, ICTS might have the possibility of false positive results. However, the mean absolute difference between the ICTS and LC/MS/MS measurements was 7 % (range 0–47%), and the agreement between the two methods was excellent (Pearson correlation 0.85, $p<0.001$). We believe that the proposed ICTS method will offer a more inexpensive tool for rapid and accurate monitoring of exposure to OPs.

4. Conclusion

In summary, we have developed a simple immunochromatographic test strip capable of detecting OPs exposures in human blood samples. This approach is based on simultaneous determination of enzyme activity and total amount of enzyme in one post-exposure sample. The novelty of this method includes: (1) the anti-BChE monoclonal antibody which binds both BChE and OP-BChE, which is a breakthrough for the sandwich immunoassay of total BChE; (2) a single test line can provide results for both BChE activity and total amount of BChE. The proposed method offers a baseline-free, low-cost, rapid and accurate tool for in-field assessing of OP exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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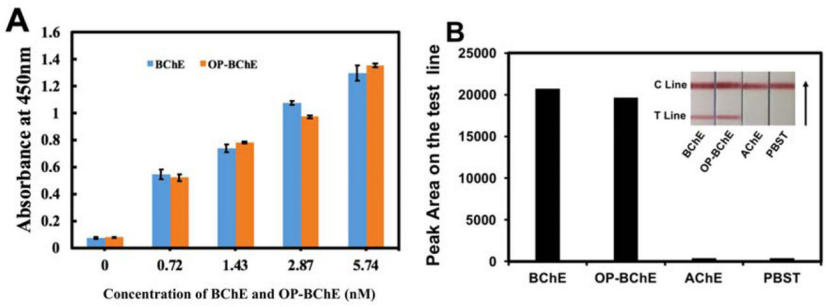


Figure 1.

(A) ELISA of the binding affinity of BChE MAb to BChE and OP-BChE. (B) Results of different samples on test strip reader analysis. The inset shows the visual color results on the test strip.

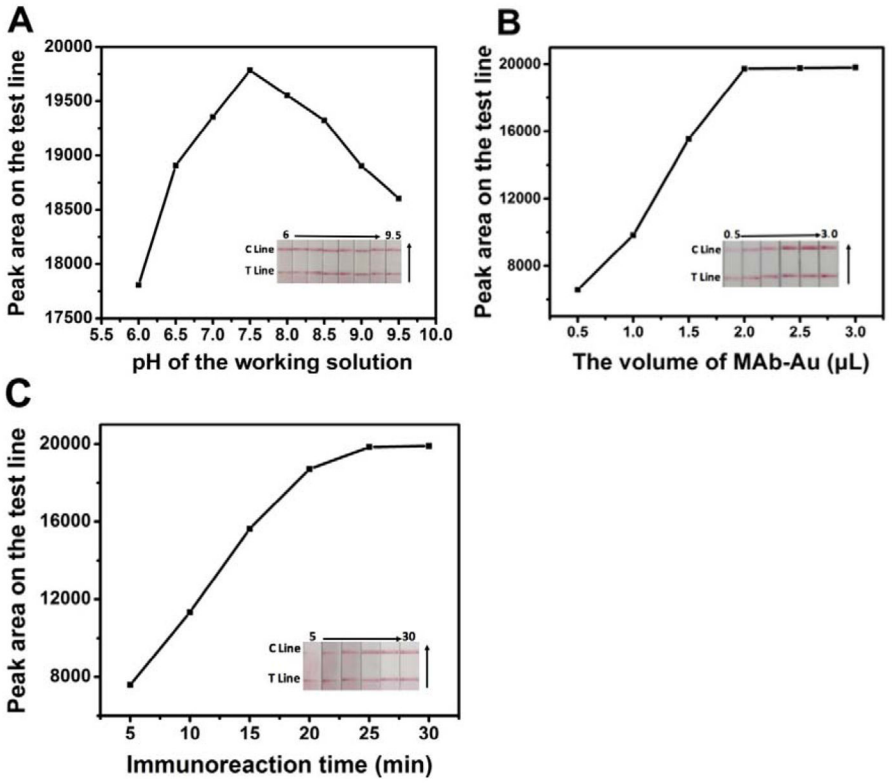


Figure 2.

Optimization of parameters of the test strip. (A) Effect of the pH of working solution on detection of total enzyme. (B) Effect of the volume of MAb-Au on detection of total enzyme. (C) Effect of immunoreaction time on the test strip.

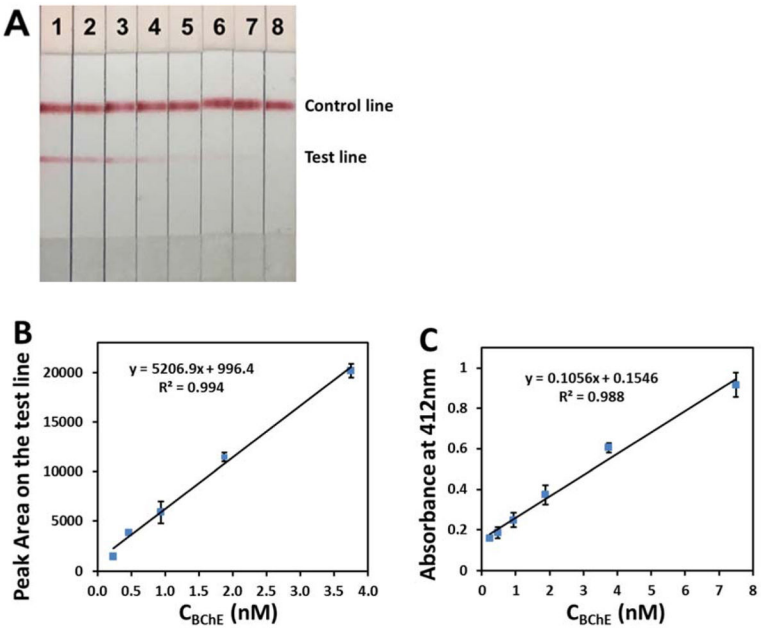


Figure 3.

(A) Detection of different concentrations of total amount BChE in PBST with ICTS. Concentrations of BChE from 1–8 were 14.34, 7.17, 3.58, 1.80, 0.90, 0.45, 0.22, 0nM. (B) The calibration curve of the total amount of enzyme recorded by strip reader. (C) The calibration curve of active enzyme recorded by colorimetric analysis.

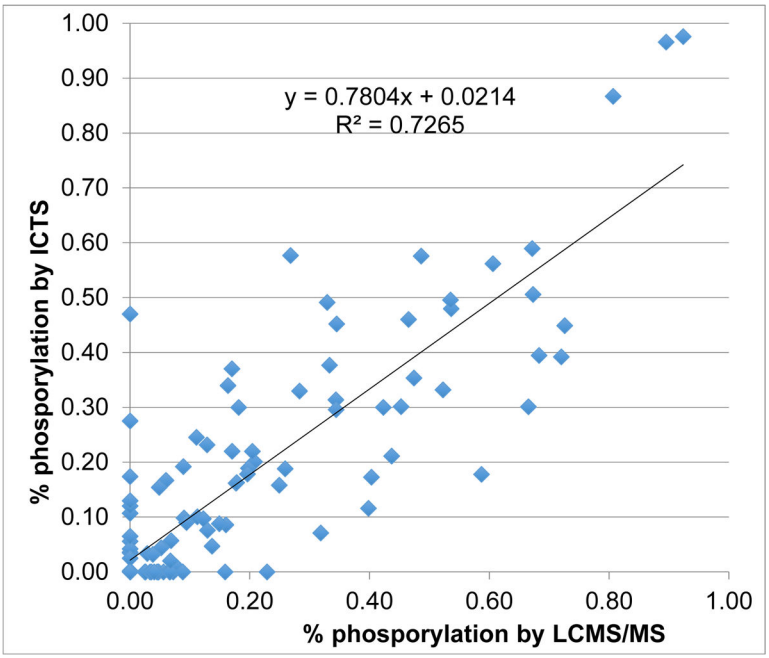
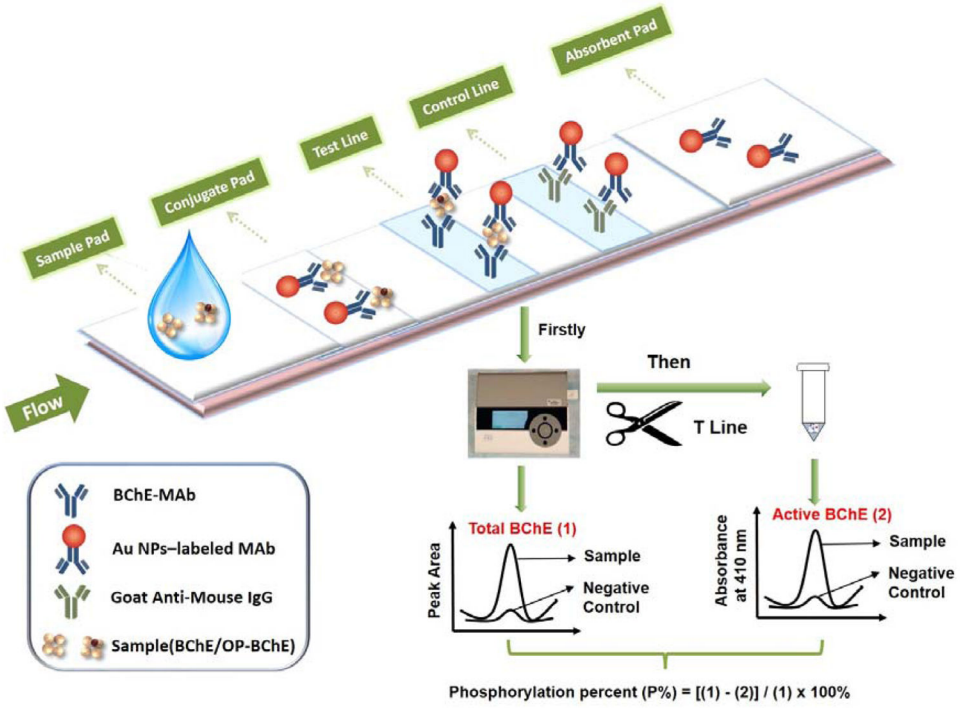


Figure 4.
Comparison of % phosphorylation with human plasma sample by ITCS and LC/MS/MS methods.



Scheme 1.
Illustration of the principle of one-step ICTS

Table 1

Comparison of the Proposed Method with Ellman Assay in Artificially Prepared BChE Samples

Sample No.	Control	1	2	3	4	5	6	7
Exposure to paraoxon (mg kg^{-1})	0	0.001	0.002	0.004	0.005	0.008	0.01	0.02
Known BChE(nM)	4.78	4.78	4.78	4.78	4.78	4.78	4.78	4.78
Active BChE after exposure(c_1, nM)	4.76	4.20	3.89	2.68	2.20	1.38	0.94	0.22
Total BChE(c2, nM)	4.79	4.64	4.79	4.77	4.78	4.80	4.79	4.74
phosphorylation % of BChE by ICTS[1]	0.63%	9.48%	18.79%	43.81%	53.97%	71.25%	80.38%	95.36%
phosphorylation % of BChE by Ellman assay (P′%)	0.00%	9.02%	18.24%	46.08%	56.92%	72.75%	80.69%	94.46%
Relative deviations[2]	+0.63%	+5.10%	+3.00%	−4.93%	−5.18%	−2.06%	−0.38%	0.95%

[1] (P%=(c2-c1)/c2 × 100%)

[2] ((P%-P′%)/P′% × 100%)