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Reprint of 'Evaluating organophosphate poisoning in human serum with paper'

Tzung-Hai Yen [a,1], Kuan-Hung Chen [b,1], Min-Yen Hsu [b,c], Shu-Ting Fan [b], Yu-Fen Huang [d], Chia-Ling Chang [e], Yu-Ping Wang [f], Chao-Min Cheng [b,*]

[a] Department of Nephrology and Division of Clinical Toxicology, Chang Gung Memorial Hospital, Linkou Medical Center, Chang Gung University and School of Medicine, Taipei, Taiwan
[b] Institute of Nanoengineering and Microsystems, National Tsing Hua University, Hsinchu, Taiwan
[c] Department of Ophthalmology, Taichung Veterans General Hospital, Taichung, Taiwan
[d] Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu, Taiwan
[e] Department of Nephrology, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan
[f] Department of Medical Education, Taichung Veterans General Hospital, Taichung, Taiwan

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ABSTRACT

This manuscript describes the development and clinical testing of a paper-based, metabolic assay designed for rapid, semi-quantitative measurement of organophosphate poisoning. Paper-based platforms, including point-of-care devices and 96-well plates, provided semi-quantitative information regarding the concentration of AchE (a biomarker for organophosphate poisoning). The paper-based 96-well-plate developed and implemented in this study was used to measure the level of organophosphate poisoning in three different clinical patients. Results were comparable to those obtained using conventional hospital methods currently considered the "gold standard". This diagnostic device offers several advantages over conventional methods, including short operating time (twice as fast as conventional methods), procedure simplicity, and reduced fabrication cost. With further commercialization efforts, the methods described in this manuscript could be applied to a wide range of potential diagnostic applications in the field.

1. Introduction

This paper describes the development of a rapid and simple method for *in vitro* detection of organophosphate poisoning for early treatment of self-poisoning patients. Our approach employs a paper-based platform and a simplified procedure to detect AchE (an important biomarker used to diagnose organophosphate poisoning) in human serum. Suicide by pesticide is a serious public health problem in the agricultural communities of low- and middle-income countries [1–4]. Self-poisoning with pesticide is an unfortunate, but common approach to suicide; it accounts for a large number of deaths in the Asia Pacific region, in particular [3]. Gunnell and Eddleston estimated that there are 300,000 annual deaths from pesticide self-poisoning in China and Southeast Asia alone, which is significantly greater than the figure for the rest of the world combined [5]. Highly toxic organophosphoric pesticides are readily available, and the records indicate that organophosphate accounted for two-thirds of all pesticide-related suicides [6]. *In vitro* detection of organophosphate at the earliest stage is critical to lowering the fatality rate, i.e., 15–30% in developing and rural countries when early diagnosis cannot be carried out [1,6].

There are three aspects of diagnosing organophosphate poisoning: (i) Direct observation of clinical symptoms and histories [7], (ii) observation of the reaction of patients to anticholinergic drugs, and (iii) detection of the level of cholinesterase in blood [8]. While clinical syndrome observation for diagnosis is simple and saves time spent handling clinical samples, under-experienced doctors may make inaccurate diagnoses that delay proper treatment, especially when patients lose consciousness and cannot communicate circumstances. In resource-poor settings, it is more reliable and cost-effective to detect cholinesterase level decline in blood. Organophosphate and carbamate pesticides strongly bind with cholinesterase family compounds in human blood. Cholinesterase activity could be used as a parameter to evaluate organophosphate poisoning. Ellman's assay and its modified methods [9,10] have been commonly used in hospitals. Recently, the Walter Reed Army Institute of Research (WRAIR) developed a modified

Abbreviations: AchE, acetylcholinesterase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylbenzidine

* Correspondence to: Institute of Nanoengineering and Microsystems, National Tsing Hua University, No.101, Sec. 2, Kuang-Fu Rd., East Dist., Hsinchu 30013, Taiwan.
E-mail address: chaomin@mx.nthu.edu.tw (C.-M. Cheng).

[1] These authors contributed equally.

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Ellman's method that can simultaneously determine red blood cell cholinesterase and serum cholinesterase activities by comparing enzymatic results with different substrates and inhibitors [11]. Other approaches for detecting cholinesterase include fluorescence assays [12], radioactive assays [13], and the use of biosensors for electrochemical detection [14,15]. Generally, detection of cholinesterase by these methods requires specific equipment and professional operators, and the applicability of these techniques is limited by available resources. While some methods [16,17] have been developed for rapid cholinesterase testing, most are laboratory-relevant only and are not useful for a clinical assay.

The WHO has suggested that diagnostic devices for developing countries should meet a number of criteria in accordance with the acronym, ASSURED, i.e., affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users [18]. Multiple techniques for paper-based platform fabrication have been reported to meet these criteria, including processes using wax printing [19], SU-8 photolithography technique [20], alkyl-ketene-dimer (AKD) inkjet printing [21], and the use of polydimethyl-siloxane (PDMS) with a modified x–y plotter [22]. Compared with other methods, wax printing offers several advantages including low cost of production, easily available equipment, simplicity, and easily prepared substrate [23]. We, and other research groups, have developed various methods for diagnosis and sensing (e.g., colorimetric assays, fluorescence assays, and electrochemistry) to monitor metabolic response in human serum using paper. The first study that used a paper-based diagnostic device to test a serum specimen measured aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to monitor recovery following drug-induced liver injury [24]. This study demonstrated a novel method for rapid, semi-quantitative testing and detection of AST and ALT. Paper has also been employed as a reliable platform for ELISA-based detection of disease activity in patients with human immunodeficiency virus, dengue fever, vascular endothelial growth factor, or autoimmune antibodies [25–28]. Note, paper can be used to detect nucleic acid as a means of diagnosing dengue fever (specific to serotype-2) [29,30]. Here, we have developed an *in vitro* diagnostic device to detect organophosphate poisoning in both buffer and human serum. We have endeavored to make organophosphate poisoning detection easier and faster in order to facilitate early treatment for poisoned patients.

The three objectives of our study were as follows: (1) develop procedures to detect AchE activity in buffer and human serum; (2) demonstrate the use of paper to estimate the concentration of AchE in serum from a patient at two time points (immediately after poisoning and after recovery); and, (3) compare our results with those obtained from conventional techniques in a clinic (Chang Gung Memorial Hospital, Linkou Medical Center, Taiwan). We observed the influence of organophosphate on AchE concentration and the effect of treatment by tracing the variation in AchE activity before and after patients were treated, unlike most studies to date, which have only detected the level of organophosphate in the time frame just after poisoning. Our study included examination of organophosphate poisoning level after patients were cured, so that we could not only compare our results to clinical standards (i.e., the methods that have been used in the medical center), but also to track post-treatment recovery (i.e., clinical follow-up) with our paper-based approach.

2. Materials and methods

2.1. Materials and reagents

A wax printer (No.: Phaser 8560, Xerox, Norwalk, CT, USA) was used to manufacture hydrophobic barriers on Whatman filter paper no. 1 (Whatman, Maidstone, UK) in the form of a 96-well plate. PBS (Sigma Aldrich, St. Louis, MO, USA) was used to dissolve reagents and dilute samples. AchE, choline oxidase, horseradish peroxidase (HRP) Type VI, and acetylcholine chloride (Sigma Aldrich, St. Louis, MO, USA) were used for AchE concentration detection, and 3,3′,5,5′-Tetramethylbenzidine (TMB) (Komabiotech, Seoul, Korea) was used as a coloring reagent to measure HRP oxidization. We used one of the carbamate pesticides, 2,3-dihydro-2,2-dimethyl-7-benzofuranol *N*-methylcarbamate (carbofuran) (Sigma Aldrich, St. Louis, MO, USA), for *in vitro* AchE inhibition.

2.2. Preparation of paper-based platforms

The proposed design of the paper-based diagnostic device incorporated two plastic films, a serum separation membrane and a patterned paper [24]. A commercial serum separation membrane allows for the collection of a patient's serum (from whole blood), and a wax-patterned paper (e.g. 96-well plates), fabricated as described previously [29], was used to constrict fluids inside hydrophilic channels via hydrophobic, i.e., wax barriers (see Fig. 1A).

2.3. Level of organophosphate poisoning in both buffer and human serum using paper-based 96-well plates for measuring

The activity of AchE was estimated by a three-step enzymatic reaction as shown in Fig. 1B: (1) AchE catalyzes the hydrolysis of acetylcholine into choline and acetic acid; (2) choline is then oxidized by choline oxidase to betaine and hydrogen peroxide; and (3) TMB reacts with hydrogen peroxide to develop an intense blue color that can be read directly.

We used a five-step procedure to carry out our diagnosis on paper in both PBS buffer and human serum as follows: (1) acetylcholine (1 mM), choline oxidase (1 U mL^{-1}), and HRP (1 U mL^{-1}) were mixed together in a tube to make a mixed reagent solution; (2) 2 μL of human serum was placed in the solution (diluted up to ten-fold); (3) 2 μL of mixed reagent solution was placed onto the test zones of our paper-based 96-well plate, and the device was incubated at room temperature for 10 min (test zones were dried slightly); (4) 2 μL of AchE at concentrations at 0.12, 0.23, 0.47, 0.94, 1.88, and 3.75 U mL^{-1} were placed onto our paper-based 96-well plate to set up a standard curve; and, (5) 2 μL of TMB was placed onto each of these paper-based test zones to obtain colorimetric-based output signals (from colorless to blue). As the volumes of reagents are fixed, droppers with a particular volume are suitable for testing. The colorimetric results on paper-based 96-plates can be read by an inexpensive scanner, thus a costly plate reader is not needed. Images were recorded at a resolution of 600 dpi using a commercial scanner (No.: MADF-C50, Microtek, Hsinchu, Taiwan). The recorded images were turned into RGB values (inverted value, specific to the red channel) and then analyzed using ImageJ (public software from NIH) to measure the intensity of each test zone. The signal from a test zone that did not receive any AchE was used as the baseline; for serum testing, the signal of a test zone with the diluted serum sample was used as the baseline. This process is shown in Fig. 1C.

2.4. In vitro AchE inhibition following spiking of pesticides via paper-based 96-plates

For buffer testing, a carbamate pesticide, carbofuran, was dissolved in distilled water at a concentration of 100 mg L^{-1} as stock solution. AchE (3.75 U mL^{-1}) was then mixed with a series of carbofuran concentrations (0, 0.0006, 0.0024, 0.0098, 0.0391, 0.0156, and 0.625 mg L^{-1}) and incubated for one hour. Following this, acetylcholine (1 mM), choline oxidase (1 U mL^{-1}), and HRP