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Research Article
Simple Colorimetric Method for Cholinesterase-inhibitor Screening in Gastric Content by Using Phytoesterase Enzyme from Kidney Bean

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Abstract

Background and Objective: Diagnosis of cholinesterase inhibitor insecticide ingestion is based on clinical suspicious and should be confirmed by cholinesterase essay. However, serum cholinesterase activity test requires specific instruments and procedure. This study aimed to develop simple colorimetric test to detect cholinesterase inhibitors in the gastric content, using phytoesterase and alpha naphthyl acetate as a chromogenic substrate. **Materials and Methods:** Methomyl and chlorpyrifos were selected for the phytoesterase enzyme inhibition assay. The experiment was conducted using pooled insecticide-free gastric content sample from ten cadavers. The gastric content samples were prepared by simple filtration procedure or liquid-liquid extraction procedure with dichloromethane or ethyl acetate. The inhibitor concentrations measured by the developed phytoesterase enzyme inhibition assay were compared with those analyzed by the LC-MS/MS and the GC-FPD. **Results:** Different sample preparation procedures, sensitivity and specificity and specificity of the test were investigated. Sample extracted with dichloromethane reduced the effect of matrix in gastric content as same as ethyl acetate. The developed color test method of detection showed 56.52% sensitivity and 100% specificity for methomyl, 100% sensitivity and 96.30% specificity for chlorpyrifos. The limit of detection of the assay was 422.6 ng mL^{-1} for methomyl and was 339.8 ng mL^{-1} for chlorpyrifos. **Conclusion:** This developed method could be used an alternative diagnostic test for methomyl and chlorpyrifos self-ingestion.

Key words: Phytoesterase inhibition, cholinesterase inhibitor, methomyl, chlorpyrifos, gastric content, dichloromethane, carbamates, organophosphates

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Self-harm by pesticide ingestion, especially insecticides, is one of suicide methods which remains worldwide public health concerns. Recent systematic review estimated that pesticide self-poisoning deaths account for 13.7% of all suicide-cases globally each year and this number was believed to be underestimated[1]. The prevalence was predominant in rural region of Asia, due to their ease of pesticide accessibility in the agricultural society[1,2]. The cholinesterase (ChE) inhibitor insecticides, organophosphates (OP), were reported to be the major causative agent of self-poisoning fatality in developing countries. Less toxic ChE inhibitors known as carbamates, were also mentioned in those cases of self-poisoning[3]. Improving medical management is a suggested strategy to counter pesticide self-poisoning issue, as well as restriction on highly-lethal pesticides[1,4]. To reduce the fatality rate, the correct diagnosis based on clinical suspicion, early resuscitation and administration of specific antidote must be made[5,6]. Confirmation of OPs and carbamates poisoning should also ideally be done with the ChE assays, either in plasma, serum, whole blood or stomach contents[5,7]. Ellman's method is the enzymatic-colorimetric method widely used for this purpose[8]. However, this method required a spectrometer which may not be available in hospitals located in remote area. Apart form Ellman's method for OPs and carbamate poisoning diagnosis, there are several enzymatic based methods for ChE inhibitors detection. Alkaline phosphatase, acid phosphatase and tyrosinase were the examples of the enzymes that were utilized for this purpose[9]. Phytoesterase or the plant-extracted esterase enzyme is an interesting novel esterase enzyme source for ChE inhibitor pesticides detection. Among those, phytoesterase extracted from kidney bean was reported to be inhibited by OPs and carbamates insecticide and was used for rapid method to determine OP residues in vegetable samples, yielding high sensitivity and acceptable accuracy[10]. In those cases of intentional pesticide ingestion, the gastric content samples were found containing high concentration of the remaining causative agents[11-13]. Therefore, gastric content seems to be a suitable sample for ChE inhibitor insecticides detection. When using Ellman's method for insecticide detection in gastric content, serum samples with normal ranges of ChE are required as source of ChE. However, the normal serum samples may not be available in every hospital, therefore, an alternative enzyme method such as alpha naphthyl acetate (ANA)-phytoesterase colorimetric method may possibly by more practical for the diagnosis at the emergency room. Unfortunately, gastric content itself was reported to contain ChE enzyme[14], which can potentially react with ANA and interfere this color test[15]. Directly use of gastric content samples for ANA-phytoesterase method to detect insecticides might give a false negative result due to the excessive gastric intrinsic esterase enzyme activity. This study aimed to investigate the suitable solvent for a simple liquid-liquid extraction procedure that can reduce the effect of intrinsic esterase enzyme in gastric content using ANA-phytoesterase colorimetric method. Moreover, the sensitivity and specificity of this developed qualitative method was compared with the standard chromatographic methods.

MATERIALS AND METHODS

The study was carried out at Environment and Health Research Unit Laboratory, Research Institute for Health Science, Chiang Mai University, from March-November, 2019.

Chemicals: Kidney bean (*Phaseolus vulgaris* L.) was obtained from Chiang Mai Province, Thailand. Alpha naphthyl acetate (ANA), fast blue B salt and sodium dodecyl sulphate (SDS) were purchased form Sigma-Aldrich.

ChE inhibitor pesticide standards: The pesticide standards were purchased from Dr. Ehrenstorfer GmbH. In this study, methomyl and chlorpyrifos were selected as representatives for carbamate and OP insecticide due to the fact that both of them were Thailand's common pesticides according to their highly imported amount among all pesticides[16].

Gastric content specimen: Ten gastric-content samples were obtained from the Forensic Toxicology Laboratory, Department of Forensic Medicine, Chiang Mai University. The samples were included by two criteria-negative screening for insecticides by thin-layer chromatography and normal serum cholinesterase activity (laboratory reference, male >4,900 U L^{-1}, female >4,300 U L^{-1})[17] from the same cadavers. Those samples with a suspected history of any poisoning and/or bloody appearance samples were excluded. All samples were pooled and mixed, then filtered by Whatman® No. 1 filter pater. The prepared gastric sample was stored at -20°C.

Liquid-liquid extraction (LLE) procedure: The LLE protocol of this study was designed to divide one gastric content sample into a set of two sample extracts. This was for 2 different methods for methomyl and chlorpyrifos determination,

ANA-phytoesterase colorimetric method and the standard chromatographic method. For the study of the suitable solvent for the developed colorimetric method, only one extracted sample was tested for esterase activity. The 250 mL of gastric sample and 500 mL of selected solvent-ethyl acetate or dichloromethane were mixed and then shaken for 5 min. The solvent layer was separated from the mixture into 2 tubes, each for 200 mL was left under fume hood until completely dry. Twenty milliliters of methanol was used to re-dissolve one tube of the extracted sample, followed by adding 180 mL of PBS. Other extracted samples were kept in a 4°C refrigerator for standard chromatographic analysis.

Kidney bean crude esterase enzyme extract: The phytoesterase extraction was modified from Jiang *et al.*[18]. The kidney bean was blended into fine powder. Twenty grams of kidney bean powder was added into 80 mL of distilled water. At 4°C, the mixture was stirred for 16 h, following by centrifugation at 4,000 rpm for 20 min. The clear supernatant was collected, subsequently filtered by microporous filter membrane. The crude enzyme extract was kept in a -20°C freezer.

Esterase activity assay: The esterase enzyme activity was performed based on spectrophotometer method modified from Yang *et al.*[10] In brief, 50 mL of ANA and 150 mL of sample were mixed in 96-well plate. After 15 min incubation at room temperature, 25 mL of 1% fast blue B salt in 5% SDS solution (1:2.5) was added into each well. The Kinetic change of the blue color was determined at 595 nm by microplate reader at 5 min.

Solvent study for LLE procedure: In order to investigate whether LLE method could eliminate the intrinsic esterase enzyme in stomach content, the LLE procedure using dichloromethane and ethyl acetate was performed on pesticide-free gastric content samples. These extracted samples, simple filtrated samples and the diluted kidney bean crude enzyme (1:200 in PBS) were measured for esterase activity (n = 3). The solution of 10% methanol in PBS was served as a negative control.

Determination methomyl and chlorpyrifos from gastric sample: The simple filtrated gastric content sample was spiked with methomyl and chlorpyrifos at the concentration of 40×10^3, 10×10^3, 1×10^3, 100 and 10 ng mL^{-1}. Each of concentrations, including insecticide-free gastric content, were divided into 9 samples and then extracted by LLE procedure (total number of 54 samples, divided into 2 sets of extract). The first extract set of samples were reinstituted as described in the LLE protocol, then were mixed with 1:200 diluted crude enzyme in PBS for 15 minutes before measuring the esterase activity. Ten percent of methanol in PBS was used as a control solvent. The percentage of enzyme-activity inhibition was calculated according to the equation[19]:

$$I = \frac{E_0 - E_1}{E_0} \times 100$$

where, I is the inhibition percentage of enzyme activity, E_0 is the enzyme activity of the control group and E_1 is the enzyme activity of the test group.

In addition to the enzyme activity inhibition, the visible color change was applied as the qualitative cut-off. Blue color change appearing in the negative control group, as a result of phytoesterase activity. Positive result for ChE inhibitor pesticides was indicated by fader or no color change, comparing to control. The other identical sample extracts were analyzed with standard chromatographic method to determine insecticide concentrations. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for methomyl determination. The other extracted samples prepared for standard chromatographic analysis were reconstituted with 100 µL 5 mM ammonium formate and 0.1% formic acid in water. Detection was performed with electrospray ionization operating in positive ion mode and the tandem spectrometer was operated in the multiple reactions monitoring (MRM) mode. The m/z = 88 was selected as the quantifier mass and the m/z = 106 was used as a qualifier mass. Carbamazepine was used as an internal standard. The quantifier and qualifier mass were m/z = 195 and m/z = 194.1, respectively. The analysis was performed under the ion source parameters as follows: the capillary voltage, 3,500 V, nozzle voltage, 500 V; and the nebulizer, 45 psi. Drying gas temperature and flow rate were 320°C and 9 L min^{-1}, respectively, for the sheath gas these parameters were 350°C and 11 L min^{-1}. The electron multiplier voltage was set at 3,500 V. Separation was archived using Eclipse plus C18 2.1×100 mm, 1.8 um column (Agilent Technologies, USA). The mobile phase was composed of 0.1% formic acid in water and acetonitrile with a gradient at a flow rate of 0.3 mL min^{-1}. The following gradient composition was applied: At 5(%B),

then switched to 50 (%B) at 6 min and 80 (%B) at 9 min. The total chromatographic analysis time was 9 min. The limit of detection (LOD) for methomyl was 1.25 ng mL^{-1}. For chlorpyrifos, gas chromatography with flame photometric Detector (GC-FPD) was used for confirmation. The extracted samples for standard chromatographic analysis were reinstituted in 50 µL of ethyl acetate, 1 µL of which was injected into the GC-FPD. A capillary column DB-1701, 0.25 mm, I.D. 30 m, length 0.25 µm film thickness, Agilent J and W column, Agilent Technologies, USA. was used. Temperature was 250°C for the injection port (splitless mode). Temperature programming of the oven was as follows: Initial temperature of 100°C (1 min hold), first ramp at 5°C min^{-1} to 180°C (2 min hold), second ramp at 2°C min^{-1} to 200°C (1 min hold) and final temperature maintained at 300°C for 4 min. The carrier gas was helium 99.999%. The LOD for chlorpyrifos was 10 ng mL^{-1}.

Statistical analysis: In the section of solvent study for LLE procedure, One-way ANOVA (Tukey's multiple comparison test) was used to compare the effect of different sample preparation methods on esterase activity. The inhibition standard curve of methomyl and chlorpyrifos were created. The half maximal inhibitory concentration (IC_{50}) and 15% of maximal inhibitory concentration (IC_{15}) were evaluated using a four parametric nonlinear regression model. The sensitivity and specificity of the developed method compared to standard chromatographic method were calculated in the section of determination of methomyl and chlorpyrifos from gastric sample. The minimum of 48 samples were required for the 80% power, with a 95% confidence interval of this study.

RESULTS

Solvent study for LLE procedure: The mean esterase activities of the samples prepared by different methods were as shown in Fig. 1. There was significant difference between groups as determined by one-way ANOVA ($F(4, 10) = 27.96$, $p<0.01$). The absorbance level of the simple filtrated samples (0.5423±0.0357 AU, $p<0.01$) and the kidney bean crude extract (0.5053±0.1654 AU, $p<0.01$) were significantly greater than negative control (0.0960±0.0046 AU). On the other hand, the Tukey's multiple comparison test showed no statistically significant different absorbance level of the samples extracted with dichloromethane (0.1050±0.0066 AU, $p = 0.99$) and ethyl acetate (0.1043±0.0081 AU, $p>0.99$) from the negative control. There was also no significant difference between samples extracted with dichloromethane and ethyl acetate ($p>0.99$).

Determination methomyl and chlorpyrifos from gastric sample: Only dichloromethane was selected as an extraction solvent in this section (Further explanation in the discussion). The enzyme inhibition standard curve of methomyl and chlorpyrifos were as shown in Fig. 2 and 3. The IC_{50} and the IC_{15} of methomyl were 1,288 ng mL^{-1} (95% CI, 1.094-1.526 ng mL^{-1}) and 422.6 ng mL^{-1} (95% CI, 303.7-553.9 ng mL^{-1}), respectively. For chlorpyrifos, the IC_{50} and IC_{15} were 1.265 ng mL^{-1} (95% CI, 1.006-1.603 ng mL^{-1}) and 339.8 ng mL^{-1} (95% CI, 200.1-502.6 ng mL^{-1}). The IC_{15} was assigned as the developed method's LOD. Sensitivity and specificity of our developed method were calculated comparing to the standard chromatographic method, as shown in the Table 1. The sensitivity and specificity of methomyl detection were 56.52% (95% CI, 41.11-71.07%) and 100% (95% CI, 63.56-100%), respectively. For chlorpyrifos, sensitivity was 100% (95% CI, 87.23-100%) and specificity was 96.30% (95% CI, 87.03-99.91%).

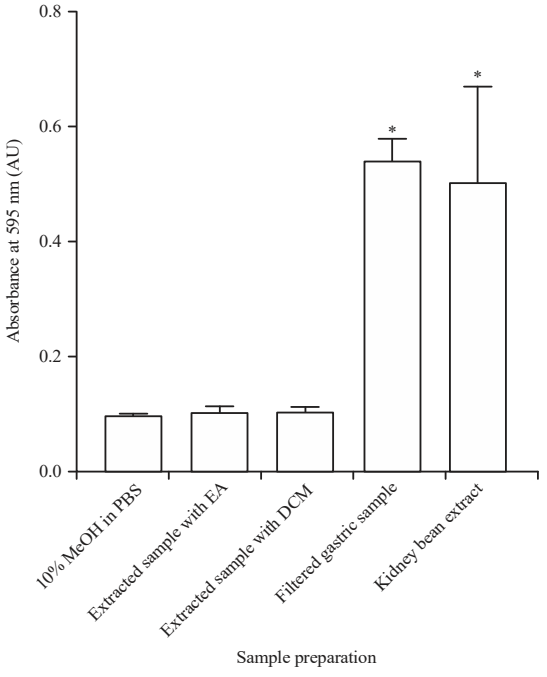

Fig. 1: Esterase activity of different preparation samples
Esterase activity was determined by the absorbance at 595 nm, *p<0.01 compared to 10% MeOH in PBS, EA: Ethyl acetate, DCM: Dichloromethane

Table 1: Sensitivity, specificity, accuracy ANA-phytoesterase enzyme inhibition method to detect methomyl and chlorpyrifos

ANA-phytoesterase method (n = 54)	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)
Methomyl detection compared to LC-MS/MS	56.52 (41.11-71.07%)	100% (63.56-100%)	62.93% (48.74-75.71%)
Chlorpyrifos detection compared to GC-FPD	100% (87.23-100%)	96.30% (87.03-99.91%)	98.15% (90.11-99.95%)

LC-MS/MS: Liquid chromatography-mass spectrometry, GC-FPD: Gas chromatography with flame photometric detector, CI: Confidence interval

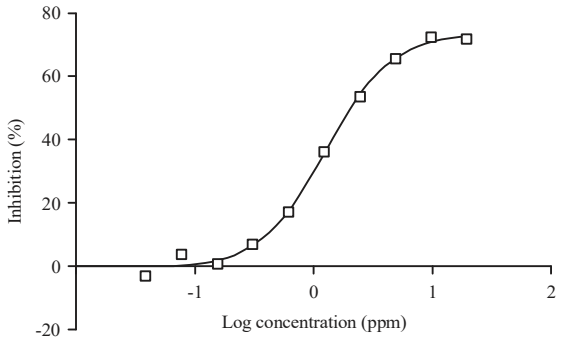

Fig. 2: Standard inhibition curve for methomyl
IC_{50}: 1.288 ng mL^{-1}, IC_{15}: 422.6 ng mL^{-1}, ppm: 10^3 ng mL^{-1}

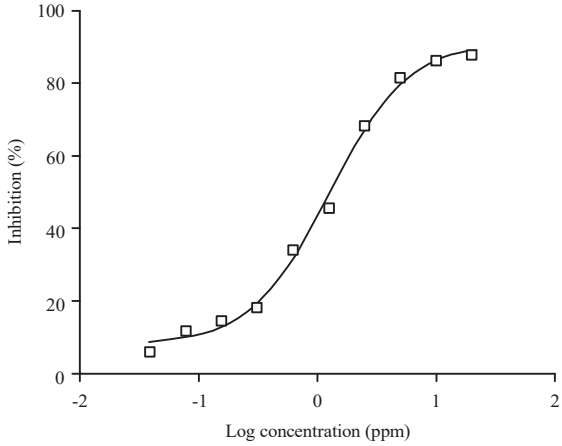

Fig. 3: Standard inhibition curve for chlorpyrifos
IC_{50}: 1.265 ng mL^{-1}, IC_{15}: 339.8 ng mL^{-1}, ppm: 10^3 ng mL^{-1}

DISCUSSION

Kidney bean esterase, identified as a member of carboxylesterase, was used as a novel alternative enzyme to detect OPs and carbamates detection in food and environment by inhibition essay[10]. The ANA can react with carboxylesterase and also can react with ChE[15] which not only can be found in blood, but also in the stomach content[14]. This is consistent with the finding of expression of esterase activity among simple filtrated gastric content samples in this study. It can be concluded that the gastric content, even though underwent the filtration procedure, could not be directly used for ChE inhibitor insecticides detection by ANA-phytoesterase method. Simple LLE method was selected to be the sample preparation method, due to its simplicity and efficiency[20]. This study showed both dichloromethane and ethyl acetate, as extraction solvent, could equally eliminate the matrix effect of intrinsic esterase enzyme from the gastric content samples. However, there are a few advantages of using dichloromethane as the extraction solvent instead of ethyl acetate, more rapid evaporation rate[21], nonflammable solvent[21,22] and its relative low toxicity [23]. For these reasons, dichloromethane seemed to be more suitable solvent for practical extraction procedure of the developed method. Generally, carbamate or OPs poisoning diagnostic test is based on the principle of ChE activity. Biggs' method is used to measure the ChE activity by detection the acidity of acetic acid, the product of ChE activity on acetylcholine, with bromothymol blue[24]. Later, this colorimetric method was developed into cholinesterase reactive paper, which is widely used for insecticide exposure risk screening among Thai agricultural workers[25]. However, there are some conditions effects on serum ChE. For example, people with type II Diabetes mellitus or Alzheimer disease tend to have elevated serum ChE level[26]. While, certain types of opioids-morphinans, phenanthrenes and morphones were reported to decrease the serum ChE[27]. High performance chromatography techniques, with high sensitivity and specificity, are the standard method for insecticide detection[7,20]. But this required expensive equipment and cost to analysis. Enzyme inhibition essay using Ellman reagent, as a less complicated method, still requires normal serum as a source of ChE and spectrophotometer[7]. In this study, the developed ANA-phytoesterase enzyme inhibition method could directly detected the esterase enzyme inhibitor as well and overcome the limitation of cholinesterase reactive paper. The kidney bean extract, which was used as alternative source of esterase enzyme to normal serum, is considered to be the advantage due to the low price and availability of kidney bean. Moreover, the developed method result is indicated by the visible color change, required no equipment to interpretation. Therefore, the ANA-phytoesterase method is a good method for ChE inhibitor insecticides in the practical aspect. The developed ANA-phytoesterase method had good specificity for both methomyl and chlorpyrifos detection in the gastric content and could effectively rule out those cases of non-methomyl/chlorpyrifos ingestion, avoiding unnecessary treatment. The result of poor sensitivity for methomyl

detection was not beyond expectation, because the LC-MS technique can detect methomyl at much lower concentration than the developed method. Despite of the poor sensitivity to detect insecticides, the developed method was sufficient to detect those cases of methomyl/chlorpyrifos self-ingestion. Because the reported toxic concentration of methomyl (2,100 ng mL^{-1}) by Kudo *et al.*[12] and reported concentration of chlorpyrifos from gastric content in self-poisoning case (9,400 ng mL^{-1}) by Martínez *et al.*[13] were both beyond the LOD of this developed colorimetric method. The extraction procedure using dichloromethane as a solvent may yield different result for AChE inhibitor insecticides other than methomyl and chlorpyrifos since different agents possess different solubility in dichloromethane. The further study on developing more rapid procedure, on-site application and also focusing on other cholinesterase inhibitor agents is suggested.

CONCLUSION

This study showed the developed method effectiveness to detect methomyl and chlorpyrifos in gastric content sample. Methomyl and chlorpyrifos could be satisfyingly detected at the level less than the reported toxic concentration. Moreover, this method requires neither expensive laboratory equipment nor expert analyst for interpretation. In conclusion, the developed method could be a good screening test for AChE inhibitor insecticides self-ingestion.

SIGNIFICANCE STATEMENT

The developed colorimetric method for detecting cholinesterase inhibitor can be used as alternative diagnostic test in case of self-ingestion for laboratory or hospital where neither complicated nor expensive equipment is available.

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