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Rapid Detection of Irreversible Acetylcholineasterase Inhibitor by Mass Spectrometry Assay

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Here we developed a rapid method to detect acetylcholinesterase (AChE) activity by matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) for screening irreversible AChE inhibitors. Due to its good salt-tolerance and low sample consumption, MALDI-FTMS could facilitate rapid detection, especially detection in real application. AChE activity was determined through calculating abundance of substrate and product in mass spectrometry. By this approach, we investigated the relation of organophosphorous (OP) concentrations and AChE inhibition. Shown in different inhibition curves from different OP pesticides, enzyme inhibitions still kept good correlation with concentration of OPs. Finally, this AChE-inhibited method was applied to screen whole bloods of four decedents and discuss their death reason. In contrast to healthy persons, three of decedents showed low AChE activity, and probably died for irreversible AChE inhibitors. Through the following detecting in GC-MS/MS, the possible death reason of these three decedents was confirmed, and another decedent actually died for sumicidin, a non-AChE inhibitor. It demonstrated that screening irreversible AChE inhibitors by detecting enzyme activity in MALDI-FTMS provided fast and accurate analysis results and excluded another toxicants not functioning on AChE. This method offered alternative choices for indicating the existence of enzyme inhibitors.

Keywords acetylcholinesterase, irreversible inhibitor, organophosphorous pesticide, mass spectrometry, rapid detection

Introduction

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the neurotransmitter acetylcholine, producing choline and an acetate group. It is mainly found at neuromuscular junctions and cholinergic nervous system. AChE has a very high catalytic activity and plays an important role in mammalian physiological process.[1]

AChE activity would be inhibited by various compounds reversibly or irreversibly. The irreversible inhibition takes place via the covalent reaction of the serine hydroxyl (OH) in the enzyme active site.[2-4] Recently, owing to the extensive use of pesticides, those usual irreversible AChE inhibitor including organophosphorous (OP) and carbamate pesticides exist anywhere in our life, which threaten human health indiscernibly and seriously. Therefore, it is necessary to develop fast and sensitive methods for detecting the irreversible AChE inhibitors.

In order to screen AChE inhibitors, the most commonly used methods have been developed about measuring AChE activity. It is reported that many assays can be used to indicate the existence of AChE inhibitors via detecting enzyme activity, such as thin layer chromatography,[5,6] fluorometric detection,[7,8] chemometrics assays,[9,10] capillary electrophoresis[11] and biosensors.[12,13] Through detecting the decrease amount of substrate acetylthiocholine (ATCh) and the increase of product thiocholine (TCh) from enzymatic reaction, AChE activity is determined. Up to the present time, mass spectrometry with various ionization modes and detector techniques has been applied to the identification of enzyme inhibitors.[14-20] The known OP compounds, carbamate pesticides and some alkaloids can be

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separated and quantitatively analyzed directly, while the unknown inhibitors become aware via detecting the decrease of enzymatic hydrolysis product.[21-25] In high performance liquid chromatography (HPLC), the novel enzymatic microreactor is involved, which realizes the online detection and fast analysis.[26,27] However, the complex matrixes such as whole blood are apt to perplex the detection results. Besides, most of methods need tedious pretreatment in order to maintain the normal work of instruments.

Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) has also attracted great attention in studying enzyme activity and enzyme inhibitor.[28-30] Due to its salt-tolerance and low consumption, MALDI-FTMS has advantages in dealing with complex matrix and achieving high-throughput screening. In this work, we applied MALDI-FTMS to detect the whole blood from four poisoning decedents and healthy persons. It turned out that whole blood of three decedents with low AChE activity showed these persons died for irreversible AChE inhibitors such as OP pesticides and another decedent whole blood show equal AChE activity with healthy persons. In further investigation by GC-MS/MS method, three OP pesticides were detected in three decedents with low enzyme activity and sumicidin (not AChE inhibitor) existed in the decedent with normal enzyme activity. These results confirmed the judgment of enzymatic method. This enzyme-inhibited method based on MALDI-FTMS shows new perspectives for rapid and accurate response to AChE inhibitor.

Experimental

Reagents and materials

Acetylcholinesterase (AChE) from electrophorus electricus (Type VI-S, EC 3.1.1.7, 426 U•mg^{-1}), acetylthiocholine iodide, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma Chemical Co. (St Louis, MO). All the organophosphorus pesticides were from Shanghai Institute for Food and Drug Control (Shanghai, China). The ultrapure water was purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Methanol and acetonitrile were of HPLC-grade quality from Merk (Darmstadt, Germany). Polyethylene glycol-200 (PEG-200), PEG-400, hydrochloric acid (HCl) and trifluoroacetic acid (CF_3COOH), were from Shanghai Chemical Reagent Corporation (Shanghai, China).

Materials preparation

AChE solutions were prepared and diluted by 50 mmol•L^{-1} Tris-HCl buffer (pH 8.0) to 5 U•mL^{-1}, and were stored at −20 ℃ in dark. The substrate acetylthiocholine (ATCh) solutions (5 mmol•L^{-1}) were prepared with Tris-HCl buffer freshly. All of the OP pesticides were made by fresh ultrapure water and stored at −20 ℃. The matrix solution was prepared fresh daily by adding 150 mg of DHB to 1 mL of ethanol/water solution (V ∶ V = 50 ∶ 50), and 1% CF_3COOH was added.

Whole blood samples preparation

Five whole blood samples were collected from Shanghai Institute of Forensic Science. Taking 2—3 mL of each blood sample in a 10 mL glass tube and adding 5 mL $CHCl_3/CH_2Cl_2$ (V ∶ V = 1 ∶ 1) solution, the mixture was agitated and extracted with a blender for 1 min. Then extracted solutions (5 mL) were dried via air blow-down techniques, and 500 μL of each sample was centrifuged for 5 min at 10000 r/min. The supernatants (100 μL) were diluted by 10 times since AChE activity in human whole blood was too high to detect in a short time. The whole blood dilutions were collected as acetylcholinesterase source.

Measurement of AChE and reaction conditions

Enzyme reactions were carried out in eppendorf plastic (EP) tubes. AChE solution (10 μL) and 10 μL of inhibitors (different pesticides solutions) were added and incubated for 30 min at 37 ℃ with 10 μL of ultrapure water as control group. Then, the substrate (ATCh, 5 mmol•L^{-1}, 10 μL) was added in the reaction for 20 min at 37 ℃ until acetonitrile (30 μL) was added to enzyme system and quenched the reaction.

Each whole blood dilution (10 μL) and substrate solution (10 μL) were incubated in an EP tube for 10 min at 37 ℃ until acetonitrile (30 μL) was added and quenched the reaction. The relative blank group was performed as whole blood (10 μL) being quenched by acetonitrile (30 μL) firstly, then the substrate ATCh solution was added.

MALDI-FTMS conditions and analysis

Experiments were conducted using an Ionspec 4.7 T HisRes MALDI-FTMS (Ionspec, Irvine, CA, USA). The external Ionspec MALDI ion source used an air-cooled Nd:YAG laser (355 nm, New Wave Research, Fremont, CA) with a gradient filter for adjusting the UV-laser power. Ions, generated from a MALDI source, were transferred via a quadruple ion guide to the capacitive coupled closed cylindrical cell. The intensity of MALDI-laser irradiation was varied between 35% and 45% as needed. The laser irradiation pulse time was set at 50 ms. For low-mass region, the quadruple guide had an applied voltage of 30 V (base to peak) at a frequency of 725 kHz. The mass spectrometry was calibrated with PEG-200 or PEG-400 for each test. The acquisition mass-to-charge (m/z) range was 80—200.

The matrix solution was deposited on the stainless steel target to produce a microcrystalline layer of matrix, followed by sample deposition on the preformed matrix layer. These wet sample spots were getting dried slowly to produce a microcrystalline layer which was homogeneous crystals of matrix and samples, and was analyzed

by MALDI-FTMS.

The abundances of peaks reflected the content of compounds. The conversion (C) was calculated as follows:

$$C=[[\mathrm{TCh}]/([\mathrm{ATCh}]+[\mathrm{TCh}])]\times 100\%$$

where [TCh] is replaced by the aboundance of TCh. [ATCh] is replaced by the aboundance of remaining ATCh as well as the fragment of ATCh. The inhibition (I) was calculated as follows:

$$I=[(C_0-C_\mathrm{i})/C_0]\times 100\%$$

where C_0 is the conversion rate of ATCh to TCh with ultrapure water as control group. C_i is the conversion rate of ATCh to TCh inhibited by OP pesticide.

GC-MS/MS analysis

GC-MS/MS analysis was performed on an Agilent 5975 tandem quadrupole mass spectrometer coupled with the Agilent 6890 gas-chromatograph (Agilent Inc. USA). The analytical column was an HP-5MS fused silica capillary column (30 m×0.25 mm ×0.25 μm) (Varian Inc. USA).

Samples were analyzed in the electron impact ionization (EI) mode with the ionization energy of 70 eV. The compounds were separated on an HP-5MS and eluted with helium gas (99.999%) at a constant flow rate of 1.0 mL•min^{-1}, and employed split injection mode at a split ratio of 1∶20. The initial oven temperature was maintained at 100 ℃ for 1 min, and then progressively heated up to 280 ℃ at a ratio of 20 ℃•min^{-1}, finally kept for 15 min. The injection port temperature and interface temperature were both 260 ℃. Ionization source temperature, quadrupole temperature and transfer line temperature were kept at 230, 150 and 280 ℃ respectively. Full scan mass spectra were acquired in the mass range within 50—500 amu. Data acquisition was performed in full scan mode and screened by selected ion monitor (SIM).

Results and Discussion

MALDI-FTMS performance for low weight molecule

MALDI-FTMS has superiority in determining compounds. Figure 1 depicts a typical mass spectrum from AChE catalyzed ATCh to TCh system based on high resolution MALDI-FTMS. The signals of substrate acetylthiocholine $[\mathrm{ATCh}]^+$ (m/z 162.1) and the product thiocholine $[\mathrm{TCh}]^+$ (m/z 120.1) are recognized clearly. The ion signal of m/z 103.0 is from the fragment of acetylthiocholine (m/z 162.1), which had been discussed in our previous study.[28] Previously, it was reported that the relative change of abundances of peaks could reflect the change of contents of compounds in our laboratory. Therefore, the abundances of substrate and product could be used for calculating enzyme activity (expressed as conversion of substrate), which make it possible to assess following enzyme inhibition. In order to obtain reliable results about enzyme activity, the data reproducibility of substrate conversion had been investigated. The percentage relative standard deviation (RSD) values in dot, dot-to-dot and sample-to-sample were evaluated and all of RSD values were within 6%, which confirmed the accuracy of quantitative analysis of enzyme activity.

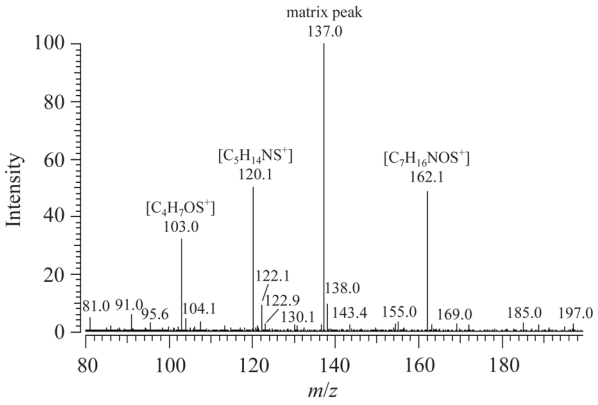

Figure 1 The MALDI-FTMS spectrum obtained from AChE catalyzed ATCh to TCh system.

Relation between AChE inhibition and inhibitor concentration

We investigated the relation between AChE inhibitions and inhibtior concentrations, in order to examine the reliability of enzyme-inhibited method to screen AChE inhibitors based on MALDI-FTMS. Three OP pesticides were used as AChE inhibitors, and they were diluted to different concentration range based on their different toxicity.[31] As shown in Figure 2, it illuminated that AChE inhibitions caused by dichlorovos (DDVP) in the range between 2 and 500 μg•L^{-1} increased along with the incremental DDVP concentration. Similarly, Figures 3 and 4 displayed the corresponding inhibition curves of malathion (2—100 μg•L^{-1}) and

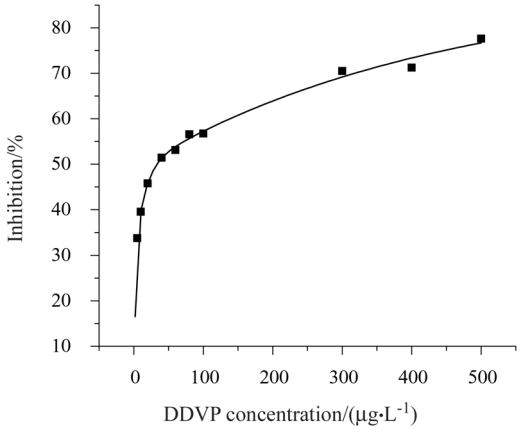

Figure 2 AChE inhibition caused by DDVP (2—500 μg•L^{-1}).

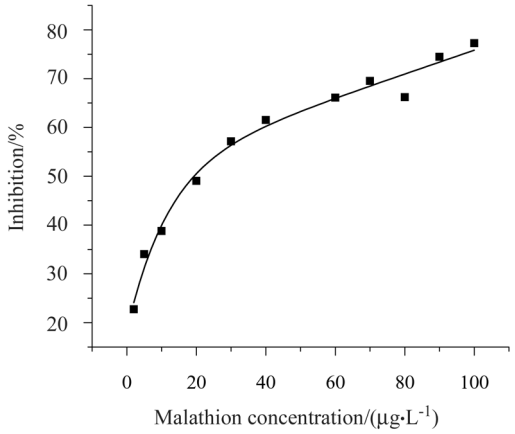

Figure 3 AChE inhibition caused by malathion (2—100 μg•L^{-1}).

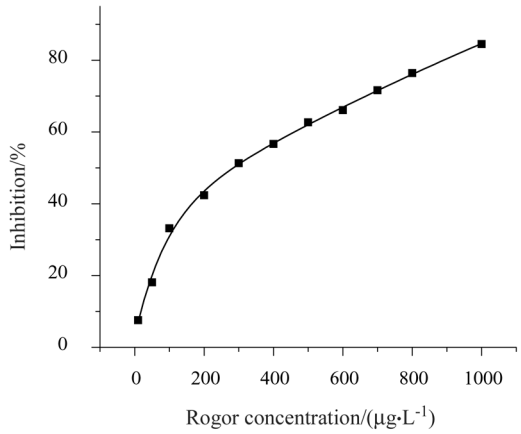

Figure 4 AChE inhibition caused by rogor (10—1000 μg•L^{-1}).

rogor (10 and 1000 μg•L^{-1}) respectively. It demonstrated that MALDI-FTMS was a reliable technique to detect AChE activity and inhibition, which laid the foundation for studying AChE inhibitors.

Besides, the limits of detection about these AChE inhibitors had been studied by detecting AChE activity. When the percentage of inhibition was more than 20%, the sample was designated of causing significant inhibiting action. OP pesticides solutions were diluted continuously to obtain the reasonable LODs. The detection limits of malathion obtained from our method was as low as 0.035 μg•L^{-1}, which was much lower than the result from the literature (1 μg•L^{-1}),[13] which showed advantages for assaying AChE inhibitors in real samples and demonstrated the good sensitivity of our proposed method.

Detection of AChE activity of five whole blood samples

Finally, we applied this mass spectrometry based method to detect AChE activity of whole blood of four decedents with unknown death reasons and healthy persons. The whole blood samples of healthy persons were from persons who all died in car accidents. The whole bloods from these persons were collected and mixed to obtain the final healthy samples which was used for detecting AChE activity and served as control group. These decedents were characterized as poisoning symptoms, which had been caused by unknown compounds. There were so many candidate toxicants that it had difficulties in looking for the criminal poison. In this case, whole blood samples from these decedents were extracted and prepared for detecting AChE activity in order to judge whether these persons died for irreversible AChE inhibitors. All of the whole bloods were diluted by 10 times since AChE in healthy persons with high catalytic efficiency was not easily operational and practicable. When enzymatic reactions in the whole bloods were quenched by ACN, lots of proteins in whole bloods would deposit. And the following centrifugation would remove the deposited proteins and exclude the disturbance from macromolecules in matrix. Meanwhile, the concentration of enzyme substrate ATCh used here for AChE activity was much higher than low molecular weight compounds in blood samples and would exclude the disturbance of these compounds. Plus, ATCh used in our work was not a native substrate of AChE and other substrates of AChE such as ACh in matrix would not disturb the targeted compound analysis.

As shown in Table 1, in comparison with AChE activity of healthy people, three decedents (marked as 1390, 1477 and 1462) had obvious lower enzyme activity, while another decedent (marked as 1420) had normal even higher enzyme activity. These results indicated that it was irreversible AChE inhibitors that caused those three decedents' death probably. Meanwhile, the decedent with a 1420 symbol probably died for some other toxicants. Although numerous AChE inhibitors existed in our life, taking enzyme activity as the single criterion for responding the enzyme inhibitor was convenient for fast screening, and helpful for excluding non-AChE inhibitors poisoning.

Table 1 AChE activity of five whole bloods from decedents and healthy person

Whole blood	Conversion/%
#1390	0
#1477	0
#1462	0
#1420	100
Blank	70.8

Detection of AChE inhibitor via GC-MS/MS

In order to testify whether there had AChE inhibitors in those whole bloods with inactive AChE and find out what these inhibitors were, we introduced GC-MS/MS to detect the whole blood samples directly. The results were as follows: high concentration dichlorphos and dipterex were detected in sample marked as 1390; low concentration dichlorphos was detected in sample marked as 1477; rogor and omethoate were detected in

sample marked as 1462; sumicidin was detected in sample marked as 1420. Among these compounds, dichlorphos, dipterex, rogor and omethoate were all OP pesticides, which were definite AChE inhibitors. Meanwhile, sumicidin was not designed as AChE inhibitor, thus, the whole blood containing sumicidin had equal AChE activity with whole blood from healthy person. Therefore, through detecting AChE activity, it was convenient and fast to identify the existence of AChE inhibitors. Coupling with MALDI-FTMS, high throughput detection had been realized easily, and this method was suitable for detecting real samples.

Conclusions

A simple and convenient approach about screening irreversible AChE inhibitors by MALDI-FTMS is developed in this work. It is helpful for excluding non-AChE inhibitor toxicant quickly and judging the existence of AChE inhibitors via detecting AChE activity. In comparison with other detection method, mass spectrometry provides accurate analysis results about enzyme activity. Furthermore, MALDI-FTMS shows advantages to analyze real samples, due to its salt tolerance and high-throughput. The method used in this paper identified that three of decedents had died for irreversible AChE inhibitors, whose whole bloods were figure out containing OP pesticides in deem by the following GC-MS/MS analysis. The application of MALDI-FTMS detecting AChE activity and screening irreversible AChE inhibitors, offers an alternative choice for rapid screening enzyme inhibitors.

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