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Comparison and Development Suggestions about Three Detection Methods for Tetrodotoxin

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Abstract. Tetrodotoxin poisoning cases are widely, frequently occurred by consuming noncompliant seafood such as puffer fish. Through extensively compare the existing detection method, 3 representative ways of detection method are elected. Mouse bioassay and ELISA belong to biological detection method, mass spectrometer and nuclear magnetic resonance, which belongs to Physical and chemical assay. We discuss the shortage of those method, compare the pros and cons with other method, and carry out some possible solutions to the current problems intended to provide reference for studies of the relevant area.

Keywords: Tetrodotoxin, detection method, bioassay, nuclear magnetic resonance, mass spectrometer

1. Introduction

Puffer fish is a long historical delicacy in Japan, although it leads to a large number of poisoning and a high mortality rate in Japan [1]. In modern times, Japanese scientist Yoshizumi Tahara have first discovered tetrodotoxin (TTX) in the body of puffer fish in 1909. Hirata Yoshimasa developed a preparation method of crystal toxin in 1950[2]. TTX is a potent low molecular weight neurotoxin that inhibits the sodium channel on the nerve membrane; TTX is more than a thousand times more toxic to humans than cyanide; there is no known antidote for TTX [3]. Tetrodotoxin is a name that is easy to be misunderstood, the public thinks TTX is only found in pufferfish, but actually, TTX exists in a large number of marines and some terrestrial, including, but not limited to, pufferfish, xanthid crabs, starfishfrogs of the genus Atelopus, and California newts [4]. Wrong cognition of TTX increases the possibility of ingestion. TTX has the characteristic of being both heat stable and water-soluble, so improper cooking will not eliminate the toxicity, instead, it will increase the toxicity [3]. Selective consumption of the muscle tissues carrying TTX can effectively avoid TTX poisoning, but it increases

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the complexity of the preparation of related dishes [4]. Detection of TTX can be used to determine the lethal dose for humans and animals; find out the amount of TTX that exists in the organism or the food to prevent accidental poisoning and death. Also, it can be used to help the improvement of the pharmacology of TTX, thus providing technical support for the research and development of relevant detoxification agents.

The detection of TTX becomes critical. The common TTX detection approaches are divided into two categories, biological detection method, and physical and chemical testing method. Biological detection methods contain mouse bioassay, immunoassay, SPR sensor, and aptamer detection technology. The physical and chemical testing method includes fluorescence spectrophotometry, chromatographic detection method, mass spectrometric detection, nuclear magnetic resonance (NMR), and infrared spectroscopy method. Pretreatment is highly related to the detection effect. With the maturing of detection technology, the time used for detection is cut down, but the pretreatment still last long. The current pretreatment is Liquid phase extraction and solid phase extraction, supplemented by ultrasonic, heating and other means, so as to realize the extraction, purification and enrichment of TTX, such as Liquid chromatography-mass spectrometry(LC-MS)[5]. Varies of methods can be applied to different situations. Different methods have different advantages, such as immunoassay having relatively high sensitivity; LC-MS is sensitive, simple, and efficient. The progress and development of the detection methods of TTX lead to higher efficiency and precision of data found and analysis [5].

While various detection methods are held in high regard, they are still far from practical. Bioassays encompass receptor binding assays [6], immunological methods [7], and mouse bioassays, which have a limited TTX identical range[2]. An improved competitive inhibition enzymatic immunoassay method has been proposed, which has greatly improved the precision and repeatability of bioassays. However, because of the expensive nature of the antibody used in this method, making this method could not be applied widely [8]. Further, as for the chemical and physical assays. The difficulties from nuclear magnetic resonance (NMR) that in genuine examples Intense interference from matrix components may degrade spectral quality [9]. Chromatography was held as improvement, due to the structural analysis problem of TTX and its derivatives can be resolved because mass spectrometry detects low limits and has high sensitivity and can also provide rich structure information. The results of the qualitative analysis are trustworthy, the sample size is small, and the scope of the analysis is broad, moreover nearly all TTX derivatives can be found. Gas chromatography Mass spectrometry was thought to be a solution to the NMR issue that derivatization necessitates a large amount of sample while also being time-consuming and having poor reproducibility due to the evaporation characteristics of TTX [10]. As compare, LC-MS is considered as the best choice of determination of TTX [11].

2. Detection method

2.1 General introduction of the current detection method

By further digging into the three most used and representative detection methods. The bioassay, Mass spectrometry, and Nuclear magnetic resonance are considered as detecting the TTX from bioassay, and physical and chemical assay respectively.

2.2 Bioassay

Biological approaches are widely used in the detection of tetrodotoxin. The mouse bioassay is the earliest way of detection (MBA). Healthy male mice of body weight between 20-22g are used during the experiment, different concentrations of TTX were injected intraperitoneally, then record the time costed for mice to die. A linear relationship is discovered between the inverse of time to death and the dose of TTX, in order to quantified TTX [12]. This is shown in figure 1.

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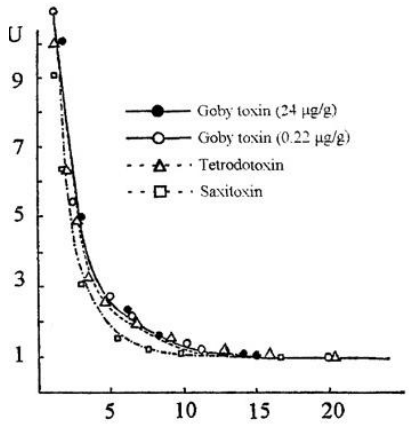

Figure 1. Dose-death time curve for goby toxin, TTX and saxitoxin (STX) with mouse bioassay technique [13].

This bioassay has several advantages, low cost, only require easy operation, the experimental phenomenon is obvious and the results can be collected simply. However, it also has significant shortages. For instance, it is susceptible to interference, having bad measurement repeatability, the pretreatment is sophisticated, the detection limits are high [14]. The ethical issues related to mouse bioassay also have some problems. Since animals are also living things on the earth, their lives also should be respected. But the destiny for the experimental mice is death. Although there are currently many approaches for mice to die without painfulness, mercy killing, death by CO2, or killed immediately after anaesthesia. But the moral principles for doing experiment on mice is still a globally appearing issue, therefore this method is not suggested. Moreover, MBA cannot distinguish TTX analogous from TTX since those toxin types induce the same symptomology, such as mice may response similarly to STX (saxitoxin) [14]. Those problems are commonly faced in biological detection and cannot be avoided. The individual differences between mice can only be reduced, but it is impossible to exterminate. Various growth factors, such as insulin-like growth factor, can regulate the mice body size to be consistent, but each mouse metabolization during the experiment can various from each individual, causing experimental errors. Those problems are irreversible in mouse bioassay, but another biological detection method, ELISA, can solve some of them. ELISA belongs to immunoassay, an analytical method for detection of target substances based on the principle of specific binding of antigen and antibody. ELISA detection method is mainly established by antigen antibody reaction, adsorption of ultra-small amount of antigen antibody on solid phase support through enzyme catalysis to maximize the immune reaction to achieve the purpose of qualitative or quantitative detection - a detection technology. It has the characteristics of high sensitivity, strong specificity and easy operation[15]. Yet, ELISA still has some limitations, such as the pretreatment is complex and the cost is high, but it has recovered some problems occurred in MBA. The development of Li yue etc. provide a visible limit of detection (vLOD) of 10 μg kg-1, which is much lower than the MBA [16]. The detection method is simple, rapid, sensitive, and can meet the demand of practical work. the limitation occurred in MBA can be solved in ELISA, Therefore, it provides a better alternative to the standard method for quantitative detection of TTX.

2.3 Mass spectrometry

Relying on the site of Mass spectrometry, by detecting qualitatively and quantitatively by their respective m/z and abundance[17]. Since the most basic Mass spectrometry is unavailable to use by detection of mixture, several further improvements was developed that thought as a solution for detection between mixtures, for example, Gas chromatography, Liquid chromatography, Paper chromatography, thin layer chromatography and Supercritical fluid chromatography. Taking perspectives from two most representative chromographs. Firstly, a detection technique, GC-MS, by

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combining their characteristics was developed, with showing characteristics of high resolution, avoids the peak of some unnecessary substances, high sensitivity, suitable for qualitative and accurate quantitative [11]. However, there is disadvantages that derivatization is a time-consuming process that requires a lot of samples and has poor reproducibility. Besides, TTX is a compound which is high volatilization, therefore it may cause error for the result. Further, as for the GC-MS to make the outcome reliable and validity, only applied to small range of compounds, which has low volatilization and thermal stable. For example, the compounds which are thermal unstable, such as ethyl diazoacetate, GC-MS showing poor stability and accuracy similar as detection of tetrodotoxin [18]. Thus, to improving this defect, the second one LC-MS, a more stable methods, has been developed.

Among various branches of mass spectrometry have been created to make the detection, focusing on Tetrodotoxin toxicology, with LC-MS being the most popular method. And the LC-MS is the Fundamental of MS using the separation process was carried out using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA, USA) connected to an API 3000 (Applied Biosystems, Warrington, UK) triple quadruple mass spectrometer (QqQMS) outfitted with a Turbo-assisted ion spray (ESI) ionization source (Sciex, Toronto, ON, Canada) [19]. The LC-MS carried characteristics of high sensitivity, low operating cost can analyze a variety of TTXs, suitable for qualitative and accurate quantitative detecting. [19] Although, the LC-MS is considered the best choice of determination of TTX for MS. There are still several difficulties, such as high requirements for equipment and personnel, organic solvents required and matrix effects occurred, needed to be overcome. From the example of protein precipitation of the bioassay, the matrix effect has a huge influence on the outcome produced by the LC-MS [20]. In addition, according to the measurement of leukocyte cystine, it has a similar issue to the matrix effect of LC-MS [21]. The matrix effect could be avoided. By adding known volumes of stable isotope-labeled standards to the sample being analyzed, LC-MS/MS analyses rely on stable isotope dilution assays to counteract matrix effects[22]. Further, because of the high requirement for the preparation of the LC-MS technique is still an irreversible defect for the time being at the present state. Thus, from the site of the mass spectrum, LC-MS is regarded as the highest efficiency one for detecting the tetrodotoxin toxicology due to its high stability and wide range of detecting compounds, instead of requiring to be capable with high-skills labor forces and equipment.

2.4 Nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) is a powerful means of detecting the structure of organic matter and is a non-destructive testing technique that doesn't destroy the sample. It is a technique that detects the local magnetic field around the nucleus of an atom. When a nucleus receives energy input from another source in an applied magnetic field, an electron energy level transition occurs. Energy level transitions are the basis for obtaining a nuclear magnetic resonance signal. The NMR spectrometers used for analysis are divided into two broad categories: high-resolution and wide-line NMR spectrometers. Among them, high-resolution NMR spectrometers are more widely used. Correlation spectroscopy (COSY), a kind of two-dimensional nuclear magnetic resonance (2D-NMR), is used in the detection of toxins, due to the ability to provide richer molecular information, it is conducive to the detection of molecular structures, especially complex molecules [3]. In the case of detecting toxins of brackish water puffer [23], the 500MHz analysis was carried out on a Varian Unity plus 500 spectrometers. The toxin was treated in D2O before putting in an NMR tube for further 1H-NMR analysis. After analysis, the toxin of brackish water puffer has been proven that is tetrodotoxin, due to the high degree of structure overlap. However, NMR lacks the ability to separate samples, and it is difficult to analyze multi-component mixtures. The information provided only at the atomic level, has low sensitivity and large errors, requiring multiple sets of samples [5]. Moreover, the precision of NMR mainly depends on the strength of the magnetic field, so there are higher requirements for equipment, and the price is relatively expensive. Under the current technology, it is still difficult to solve the above problems of NMR, but with the development of technology, the new NMR instrument may be able to provide solutions to the above problems.

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3. Conclusion

Three detection methods applied to tetrodotoxin detection, Bioassay, Mass spectrometry, and Nuclear magnetic resonance, still have certain defects and limitations, and there is no way to eliminate them perfectly under the current scientific and technological means. But nevertheless, they are all applicable and provide an effective, indelible effect for the detection of tetrodotoxin. For medical testing, in addition to detection technology, proper extraction is also an important way to improve the ability of testing. Therefore, the extraction of target poisons cannot be ignored, especially in highly toxic biotoxin poisoning accidents. For tetrodotoxin metabolism fast, not only need a precise, sensitive detection method, but also a high recovery, bottom matrix effect extraction method. From the perspective that in addition to exploring and improving existing technical defects, future research should continue to develop new and more complete detection methods and extraction methods, providing more convenient and beneficial tools for the detection of tetrodotoxin.

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