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Sensitive immunoassay for detection and quantification of the neurotoxin, tetramethylenedisulfotetramine (TETS)

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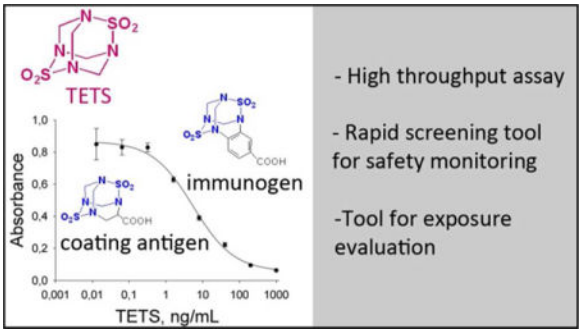
Abstract

Tetramethylenedisulfotetramine (TETS, tetramine) is a formerly used and highly neurotoxic rodenticide. Its lethality, recent history of intentional use for mass poisoning and the absence of a known antidote raises public health concerns. Therefore, rapid, high throughput and sensitive methods for detection and quantification of TETS are critical. Instrumental analysis method such as GC/MS is sensitive but not rapid or high-throughput. Therefore, an immunoassay selective to TETS was developed. The assay shows an IC_{50} of 4.5±1.2 ng/mL, with a limit of detection of 0.2 ng/mL, comparable to GC/MS. Performance of the immunoassay was demonstrated by a recovery study using known concentrations of TETS spiked into buffer, human and mouse serum matrices giving recoveries in the range of 80-120%. The assay demonstrated good correlation in TETS recovery with established GC/MS analysis. The immunoassay was then used to quantify TETS concentration in the serum of mice exposed to 2× LD_{50} dose of TETS and to monitor kinetics of TETS clearance from blood over a short period of time. TETS concentration in the serum reached 150 ng/mL without significant change over 4h post treatment. Results obtained with the immunoassay had good correlation with GC/MS analysis. Overall this immunoassay is an important tool to rapidly detect and quantify levels of TETS from biological samples with high sensitivity. The assay can be adapted to multiple formats including field or hospital use.

TOC image



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SUPPORTING INFORMATION

Additional information including text, four tables and seven figures are available in the supporting information.

Keywords

rodenticide; threat agent; antibody; mice; CounterAct; countermeasure

Introduction

Tetramethylenedisulfotetramine (TETS, tetramine) is an organic compound, initially developed as a rodenticide in the 1950s but was subsequently banned worldwide due to high neurotoxicity in all mammals. The compound is a noncompetitive blocker of chloride channels on the γ-aminobutyric acid (GABA) receptor of the neuronal cell membrane, leading to lack of inhibitory currents and excessive excitation in the central nervous system (CNS). Symptoms of poisoning with TETS are dose dependent, including seizures, status epilepticus and coma at high levels of exposure. The LD_{50} in mice is 0.1 mg/kg, and 5-10 mg is considered to be a lethal dose for humans[1]. Tetramine is simple to synthesize and handle. It is prepared from readily available starting materials. As predicted from its structure it neither absorbs ultraviolet light nor is it fluorescent and it ionizes poorly on LC-MS. It is a white odorless and tasteless powder with significant solubility in water. In addition, it is also extremely stable in the environment.[1] Despite being banned globally in 1984 it is still illegally available on the rural black market in China. As a result, TETS has been implicated in numerous cases of mass poisoning.[2,3] In the period of 2000-2012, at least 148 cases were reported in China; over 3526 people were reported to be exposed to TETS, resulting in 225 deaths.[2] Although, most of the known cases of poisoning with TETS occurred in China, one infant was poisoned in New York City, USA.[4] Six months after poisoning the infant remained severely developmentally delayed. Poisoning with TETS has long lasting effects. Even after patients are treated for seizures, there is evidence that numerous neurological lesions occur.[5,6] Most victims who have no seizures initially after exposure, develop them later on. On average recurrent seizures stop after 2.3 years with anti-seizure treatment, although cases that continued for 9 years have been reported.[5] Thus, poisoning with TETS leads to seizure-induced brain damage even in patients that survive acute intoxication. Poisoning with TETS may be fatal within an hour. There are no generally recognized antidotes for it and victims only receive supportive therapy and symptomatic treatment, including gastric lavage, dialysis, blood perfusion, and administration of drugs controlling seizures and acting on GABA receptors.[7,8] The high toxicity, unique physical properties, absence of antidote, and history of use as an agent of intentional mass poisoning make TETS a serious public health concern because it may pose a serious threat to the population.

Currently, gas chromatography (GC) with different detection systems (MS[9–13], MS/MS[9,11], nitrogen/phosphorus detection[14], flame thermionic detection[15]), is the main analytical technique used for TETS detection and quantification. This is due in part to poor ionization on LC-MS. Quantification protocols with good sensitivity were developed for tetramine detection in blood (8 - 500 ng/mL and LOD 1 ng/mL),[14] urine (LOD around 3 ng/mL), [9,10,15] food (down to 0.2 ng/g)[12] and beverages (0.5 - 100 ng/mL)[11,13] Based on clinical reports, depending on the severity of poisoning, the concentration of tetramine in the blood of victims varies widely from low ng/mL up to Gg/mL.[7,16,17] In urine, the concentration of

TETS is typically similar or slightly higher than in blood.[10,17] The low detection limits, achieved with GC methods require laborious sample preparation in order to reduce matrix interference with analysis. In addition, instrumental methods are expensive, slow, require advanced infrastructure and are inappropriate as a high throughput technique, as well as requiring specific expertise and a well-trained operator. On the other hand, immunoassays are widely used for detection of small molecules in environmental[18–20] and biological[18,20,21] samples, as well as in food[18,22,23] and beverages.[24,25] They usually have high selectivity toward their corresponding analytes with sensitivity greater or comparable to instrumental methods. Furthermore, they provide the advantage of minimal or no sample preparation, thus dramatically decreasing the labor involved in analysis. These attributes allow high throughput screening and quantification. An additional benefit of immunoassay is its potential to be packaged in a field portable platform for on-site detection.[26–28]

The tetramine structure appears to be an ideal candidate for an immunoassay. It is sterically constrained, lacking freedom of the rotation about its bonds and has several heteroatoms to provide recognition points for the antibody. Surprisingly, to the best of our knowledge, there is no antibody selective to TETS reported in the literature. We suppose this deficiency may be due to the challenging properties of TETS that make it inert and difficult to modify as well as potentially dangerous in order to produce appropriate haptens for immunization. We developed a simple and general chemical approach for the synthesis of TETS-like compounds and created a library of TETS analogs several of which were suitable haptens for immunoassay development.[29]

In this work we used the haptens and analogs of TETS to develop the first quantitative immunoassay selective to tetramine. The sensitivity of the developed immunoassay was compared to a GC/MS method, performed in conditions similar to methods published in the literature.[9,10,14,15] Its performance was evaluated in spike-and-recovery studies performed with buffer, mouse and human serum. The resulting data were compared to the data obtained with GC/MS. The developed assay was used to monitor kinetics of TETS in the blood of mice treated with tetramine.

MATERIAL AND METHODS

Information concerning chemicals and instruments, immunization and antiserum preparation, extraction procedure and the GC/MS method is detailed in the Supporting Information (SI).

Preparation of immunogens and coating antigens

Synthesis of TETS analogs and corresponding haptens was reported by Barnych et al.[29]. For immunoassay development, haptens with a reactive carboxylic acid group were conjugated to proteins by a N-hydroxysuccinimide (NHS) (Haptens 2d, 2j, 2c in Table 1) method. Haptens having an aromatic amine group ($-NH_2$) were conjugated to the protein by the diazotization method for coating antigens and through glutaraldehyde chemistry for use as immunogens. Haptens 6a and 6b were conjugated to the protein via a succinate linker or via a click chemistry approach.[30] All haptens (Table 1, Table S1) were conjugated to thyroglobulin (Thy) for immunogen preparation and to bovine serum albumin (BSA) and

conalbumin (CON) for coating antigen screening. The conjugation protocols are detailed in the SI.

Reagent optimization

Each serum was screened against 1 homologous and 5 heterologous haptens using a three point competitive ELISA at 0, 50 and 5000 Gg/L TETS concentration. Selected coating antigens were used to obtain 8-point full competition curves to determine and compare assay sensitivities (Table S2). In further assay development, the dilution of serum from rabbit #3442 was optimized in checkerboard titration with coating antigen 2c-CON.

Optimization of the assay conditions

The sensitivity of the ELISA was optimized through the following parameters. **(a) Protein in blocking buffer.** The effect of different proteins on assay sensitivity was assessed on plates blocked with 1% solution of BSA, skim milk or ovalbumin in PBST. **(b) Protein in antibody buffer.** The effect of protein presence in the buffer was evaluated with BSA tested at 0, 0.5 and 1% concentration prior to addition to analyte on the plate. **(c) Co-solvent in analyte buffer.** The effect of organic solvent on assay sensitivity was studied by analyzing standard solutions of TETS prepared in PBS containing 10, 20 or 40% of methanol and DMSO (prior to addition of antibody in the well). **(d) Ionic strength.** The effect of ionic strength on assay sensitivity was evaluated from the assays performed in 5 mM, 10 mM, 20 mM and 30 mM PBS (pH 7.5).

Indirect competitive ELISA

Plates were coated with 0.5 Gg/mL of antigen (Hapten 2c/CON) in coating buffer (100 μL/well). After incubation for 1 h at room temperature (RT), the solution was replaced with blocking buffer (200 μL/well) and plates were incubated over night at 4°C or for 1-4 h at RT. Plates were washed with washing buffer (phosphate buffered saline (PBS) containing 0.05% Tween 20, PBST) 3 times prior to sample loading. TETS solutions (calibration curve or sample) in assay buffer were loaded on the coated plate (in duplicates or triplicates) at 50 μL/well. An equal volume of anti-TETS antiserum diluted in PBS containing 1% BSA was added. The plate was incubated for 1 h at RT and then washed 5 times with wash buffer. Goat anti-rabbit IgG-HRP conjugate was added at 100 μL/well in a 1:10000 dilution as instructed by the manufacturer. The plate was incubated for 1 h at RT and washed 5 times. Substrate solution was added (100 μL/well) and left to develop color for about 10 min. The reaction was stopped by addition of 2 M H_2SO_4 (50 μL/well) and absorbance was read at 450 nm. SigmaPlot 11.0 software was used for curve fitting and data analysis.

Matrix effect

The effect of matrix on the assay performance was evaluated using mouse serum, human serum and human urine spiked with TETS. Spiked matrix was allowed to stand for at least 1 h. Competition curves were obtained by a serial dilution of the analyte in the assay buffer that is PBS containing either 20% or 40% methanol. The assay buffer also contained 0%, 20%, 40% or 100% of a neat matrix (matrix content is indicated as a concentration prior to addition of the antibody in the well).

Immunoassay validation

Validation studies were performed by 1) evaluating the recovery from buffer, mouse and human serum fortified with varying TETS concentrations measured by immunoassay and GC/MS using both neat and extracted samples; 2) applying immunoassay to analyze serum samples obtained from mice treated with TETS. Sample preparation for validation studies was done in a blind fashion by another operator and analysis of samples from exposure studies are detailed in the SI.

RESULTS AND DISCUSSION

1. Hapten design and immune response in rabbits

The design and synthesis of haptens is a critical step in immunoassay development. The literature data show that the most sensitive competitive immunoassays are those developed when the coating antigen is different from the immunizing antigen (heterologous assay).[31–34] Not only the carrier protein should be different but also at least the handle and preferably the chemical structure of the hapten should be modified. The smaller the hapten the more important hapten design becomes. Therefore, the affinity of the antibody should be strong enough to bind to the coating antigen but remain lower than the affinity towards the target analyte.

The first series of haptens (Table 1, haptens 6a and 6b) preserved most of the TETS structure except one nitrogen and one $–SO_2$ group, which were replaced by carbon atoms. On the side of the molecule missing those functional determinants, an amine ($-NH_2$) or a hydroxy (−OH) functional group was added. Each hapten was then conjugated via different chemistries to create heterology in a linker arm. The first approach included reaction of haptens with succinic anhydride, followed by conjugation of the resulting hemisuccinates to the protein via standard EDC/NHS chemistry. The second approach took advantage of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, probably the most widely known example of click chemistry (Table S1). Click chemistry has not been widely used in immunoassay coupling, but this study illustrates its applicability. Haptens 6a and 6b were first transformed into an amide and an ester of the azidoacetic acid respectively which then were reacted with BSA-pentynoic acid conjugates under reported optimized conditions.[30] Haptens conjugated to Thy (Table S1) were used for immunization and the titer was assessed in a homologous assay with BSA as the carrier protein. Immunogens 6bS-Thy and 6aS-Thy (Table S1) raised a strong response in rabbits giving very high titers even when tested at 0.01 Gg/mL of coating antigen and 1:25 000 dilution of the serum after the first immunization. This high titer remained unchanged following boosting injections. We hypothesize that the polyclonal serum was highly reactive toward the triazole moiety present on the linker arm. In turn, immunogens 6bCC-Thy and 6aCC-Thy resulted in very low titers that slowly increased over the immunization period of 3 months. However, none of the obtained sera had sufficient affinity to TETS. It is possible that elimination of the nitrogen and $-SO_2$ functionalities resulted in haptens differing from TETS with enough significance to decrease antibody affinity to TETS relating to the coating antigen, making the assay insensitive to the analyte.

In order to better mimic the TETS structure we developed a second series of haptens using a novel approach to the synthesis of TETS analogs recently reported by our group.[29] This group of haptens (Table 1) has a structure that preserves all the heteroatoms of TETS and differs only in one of the methylene bridges that was replaced by an ethylene bridge carrying a side chain with functional group. Heterology is achieved with different linker arms including a short arm (hapten 2c) or a flexible long arm (hapten 2d), rigid arm (hapten 2j) or a long arm with a bulky aromatic ring (hapten 2k). All haptens were conjugated to Thy for immunization, while BSA and CON were used for protein heterology in coating antigens. Immunizations were performed with haptens 2j, 2k and 2d.

2. Identification of sensitive pairs of coating hapten and antibody

Sera from 6 rabbits were screened in a four-point (5000, 500, 50, 0 ng/mL TETS) competitive format against 5 coating antigens. Figure 1 shows the percentage of inhibition of antibody binding to the plate in the presence of TETS at 50 ng/mL (one point concentration shown). A colored bar shows the percentage of inhibition for selected sera tested on one of 6 coating antigens, including homologous (same haptens as immunogen) and heterologous (different hapten for immunization). Absence of the bar means that selected sera did not recognize corresponding coating antigen. Among tested combinations the following ones seemed to be the most promising for further exploration because the inhibition was above 20 % at 50 ng/mL TETS: 2165/2c, 2165/2k, 2165/6bCC, 2168/2c, 2168/2k, 2168/6bCC, 3442/2c, 3442/2k, 3443/2c, 3443/2k. These serum/coating antigen pairs were tested in an 8 point competitive format (Table S2). The combination of serum 3442 with coating antigen 2c/CON was chosen for the following studies because it had the highest sensitivity, high slope of 0.75 (target slope is 1.0 or more) and high signal-to-noise ratio.

3. Assay optimization

We then performed a series of optimization experiments to attempt to achieve better sensitivity and to characterize the assay for further applications. *a. The effect of blocking agent* on assay characteristics was evaluated first. The most common proteins of BSA, skimmed milk and ovalbumin (OVA) were used as 1% solutions in PBST. Blocking with skimmed milk resulted in significantly stronger reduction of the overall read-out signal compared to other proteins tested (Figure S1). Skim milk also gave the highest sensitivity with the IC_{50} value being 8 ng/mL vs 20 and 26 ng/mL obtained with OVA and BSA respectively. One probable reason for this observation is that casein, the major protein in skim milk, is smaller in molecular mass (~ 20 kDa) and thus blocks the surface of the plate more efficiently than BSA or OVA (66 kDa and 45 kDa). Better blocking then results in decreased non-specific binding of antibodies and improves assay sensitivity.

Addition of the protein to the antibody assay buffer also had a significant effect on assay sensitivity. Addition of 1% BSA to the antibody solution (or 0.5% BSA on the plate at final dilution) resulted in a 2.4× increase in sensitivity (IC_{50} 8 ng/mL) compared to the assay with no protein added to the antibody solution (IC_{50} 19 ng/mL). This effect is indeed protein dependent because addition of a smaller amount of BSA (0.5 %) also improved the IC_{50} value (IC_{50} 11 ng/mL, Figure S2). Interestingly, the overall signal of the assay also increased when some protein was added to antibody solution.

b. Co-solvent selection. Organic co-solvents are often added to the immunoassay to improve analyte solubility or as a part of the sample preparation procedure. Methanol and DMSO were evaluated in the assay at final concentrations of 5, 10 and 20 % (Figure S3). The signal intensity did not change greatly when methanol was used at different concentrations. Similarly, sensitivity varied slightly giving IC_{50} values ranging between 3.8 ng/mL (20% methanol), 7.7 ng/mL (10% methanol), and 10 ng/mL (5 % methanol). However, DMSO had a dramatic effect on signal intensity but almost no effect on sensitivity. The signal suppression was concentration dependent, causing a larger decrease with higher concentrations of the solvent. These data suggest that methanol is a better co-solvent for the TETS ELISA in the current format, and it can be used at a wide range of concentrations in the sample with minimal effect on assay performance.

Ionic strength of the buffer did not have a significant influence on assay sensitivity, but the maximum absorbance was negatively affected (Fig. S4). This suggests that the binding interaction between antibody and coating antigen is gradually suppressed in solutions with higher ionic strength. Therefore, the following experiments were performed in 10 mM PBS.

The optimized ELISA included coating antigen-antibody pair 2c-CON/3442 with coating antigen concentration of 0.5 Gg/mL. The coated plate was blocked with 1% skim milk. The analyte was loaded in assay buffer containing 20% methanol in PBS, pH 7.5. Serum dilution was 1:3000 in PBS containing 1% BSA, before addition to the plate. The heterologous assay had a linear range ($IC_{20\text{-}80}$) of 0.7-30 ng/mL of TETS in assay buffer and IC_{50} value of 4.5±1.2 ng/mL (concentration reported as final in the well or 0.45 ng of TETS in the well, tested in triplicate for 9 days) (Fig. 2). The LOD in the buffer was determined as the IC_{10} value, and estimated to be 0.2 ng/mL (0.02 ng TETS in the well). To the best of our knowledge, this is the first immunoassay sensitive to TETS reported in the literature. The sensitivity of the assay is comparable to the sensitivity of reported GC/MS methods (in house and reported LOD ranging from 1 to 8 ng/mL following clean-up).[9–11,14,15]

4. Cross-reactivity (CR)

As evidenced from the literature and based on multiple physiological and toxicological criteria, tetramine is a noncompetitive blocker of the GABA type A receptor ($GABA_AR$).[35–37] TETS competes with ethynylbicycloorthobenzoate (EBOB), a standard ligand of $GABA_AR$, for binding to the receptor. A number of other organic small molecules have also been shown to have good affinity toward GABA receptors.[38] For instance, at 1 μM TETS inhibits 57% of EBOB binding, while fipronil and picrotoxinin give 69% and 51% inhibition, lindane and the cyclodiene α-endosulfan give 66% and 103% inhibition, respectively.[38] Therefore, these compounds might share certain structural features that enable their binding to the same target site on the protein, and thus may also bind to the antibody developed to TETS. Figure S5 shows inhibition curves obtained with 5 tested molecules. Polychlorinated cage compounds like the cyclodiene heptachlor and the halogenated terpene toxaphene showed some inhibition in the assay, although with IC_{50} values were over 1000 ng/mL. This observation correlates with findings of Esser, et al.[39] who reported a monoclonal antibody (mAb), that was developed to cyclodienes to be sensitive to TETS with CR of 17% compared to α-endosulfans, or 2% compared to

toxaphene. The authors supposed that the sulfamide moiety of TETS is comparable with the planar Cl-C-CCl_2-C-Cl group of the cyclodienes, thus leading to ligand recognition.[39] Other compounds (fipronil, picrotoxin and pentylenetetrazol) tested in the assay did not show any significant inhibition. Indeed CR was not expected since these compounds bind to a series of diverse sites on the GABA-gated chloride channels.

When a series of analyte analogs are examined, their CR data may give some information about the binding preferences of the antibody. Figure S6 shows the CR pattern for serum 3442. A rabbit was immunized with hapten 2j having a benzene ring directly attached to the TETS cage. Accordingly, the serum showed a high CR toward analogs with highly similar structures (hapten 2j and analog 2i, Fig. S6). Lack of the phenyl ring (2a) significantly decreased the strength of the interaction between the antibody and TETS analog, however it was almost completely restored by introducing a substituent on the ethylene bridge (2c, 2b), suggesting that the nature of the side group is not important for binding efficiency. On the other hand, the bulky substituent in 2g led to less efficient interaction with the antibody and thus lower CR. Significantly lower CR to 4a and 2e compared to 2a is probably due to structural changes that resulted in a different relative position of the two sulfamide groups. In addition, the absence of CR toward 6bCC and 6aCC probably indicates that the presence of both sulfamide groups is important for antibody recognition. Finally, it is interesting to note that the selectivity of antibody to 2a and TETS is almost equal, suggesting that for antibody recognition the structure of the bridge, either methylene or ethylene does not have any influence.

5. Matrix effect on assay performance

Complex samples are challenging for accurate analysis and quantification because of the interfering components present in the sample matrix. For analytical instrumental methods like GC- or LC/MS sample clean-up is an inevitable step that is laborious and time consuming. On the other hand, even though the immunoassay performance may also be affected by the matrix, there are a number of simple methods to account for it including addition of the blank matrix to the calibration curve. Simple dilution of the sample also significantly decreases interference. In this study, commercial blank mouse and human serum, as well as human urine from healthy volunteers were tested. Mouse serum had a strong effect on assay performance. Figure S7 A shows significant decrease of the signal in the assay with 10% mouse serum. Interestingly, further increase in mouse serum content in the buffer did not give significantly higher signal suppression. It is worth noticing that as little as 1% mouse serum caused a pronounced decrease in the signal (data not shown).

Compared to mouse serum, human serum had only a minor effect on the inhibition curve (Fig. S7C). This is a big advantage compared to instrumental methods since the analysis can be performed without any sample treatment. It is of particular importance when urgent evaluation must be performed after exposure to unknown chemicals.

Interestingly, even though a urine matrix is supposed to be less complex compared to serum, it caused significant suppression of the immunoassay signal (Fig. S7E). However, case studies reported by Chau, et al.[40] and Lu, Wang, Yan, Xiao and Stephani[6] showed that concentration of TETS in human blood, urine and other biofluids is high, at least 50 ng/mL

in mild poisoning cases. Accordingly, a 10-20× dilution will be necessary to adjust the concentration of TETS in samples to be within the analytical linear response range of the assay. Therefore, despite the interference of the matrix on assay performance, sample dilution significantly improves assay performance (Fig. S7).

Another series of tests were performed to evaluate if adding higher concentration of methanol to the assay buffer may precipitate protein and/or salts from the samples, thus improving the assay signal. We tested two conditions where the assay buffer contained 10% methanol (Figures S7 A, C, E) or 20% methanol (Figures S7 B, D, F). Indeed, some differences were observed, in particular for the urine samples. When these samples were diluted 5-10 times (10-20% of matrix) in the buffer with 20% methanol, the signal did not differ significantly from the control, where no matrix was added. This is a significant improvement since urine samples could be diluted and tested in the assay without further sample treatment. However, this effect was not consistent with matrix. For human serum, better results were obtained with buffer containing 10% methanol, while for mouse serum the amount of co-solvent did not significantly influence the assay performance. Therefore, further experiments were conducted in assay buffer containing 10% methanol as the final concentration. These data also suggest that blank matrix should be added into the calibration curve samples to account for signal suppression for analysis of serum samples obtained from mice, but not for human serum (urine samples were not further analyzed). It should be noted that prior to the immunoassay analysis all biosamples should be adjusted to optimized assay conditions (pH and co-solvent). Therefore, while the assay LOD in the buffer is 0.2 ng/mL, the LOD of the method differs for different matrices and varies in the range of 0.4-0.8 ng/mL.

Extraction procedure and efficiency—For analysis of samples from asymptomatic victims that may have experienced exposure to a very small amount of tetramine, such a high dilution of the sample is not applicable. Therefore, extraction of the analyte from the matrix is an inevitable sample preparation procedure. To evaluate the efficiency of extraction, we used ^{14}C-labeled TETS previously synthesized by our group.[38] Use of radioactive material for the development and validation of an extraction procedure is relatively little used and rarely appreciated in analytical chemistry. However, it is a very attractive method for extraction optimization since it allows fast and easy tracking of the distribution of spiked radioactive material within the sample and subsequent extraction fractions, by counting the number of decay events. This in turn allows more rapid optimization of the extraction conditions to achieve higher recoveries and cleaner extracted material. For TETS we used simple liquid-liquid extraction with ethyl acetate (EtOAc). Blank EtOAc, human urine, mouse and human serum were spiked with an aliquot of ^{14}C-TETS with theoretical radioactivity of 1200 disintegrations per minute (dpm). Each matrix was spiked with TETS and extracted 3 times followed by liquid scintillation counting. To account for possible quenching due to the matrix, the same matrix was extracted first and then spiked with ^{14}C-TETS. Activity of ^{14}C-TETS was determined first in spiked EtOAc and gave 1029±36 counts per minute (cpm) (n=5, Table S3). There was no significant quenching observed in any of the tested matrixes, as can be seen from Table S3. Radioactivity measurements in extracted samples revealed that liquid-liquid extraction with

EtOAc was very efficient for TETS, as evidenced by high recovery values for mouse and human serum at 93 and 90% respectively, and a 95% recovery from human urine.

Immunoassay evaluation with spiked samples

To characterize the immunoassay developed to quantitatively detect TETS in biofluids, we performed a recovery evaluation from spiked matrices, including buffer, mouse and human serum all with and without an extraction step. Since we did not expect to obtain urine samples from exposed humans (not available) or mice (very low volumes), urine matrix was not included in spike-and-recovery studies. Buffer and control sera samples were spiked with TETS ranging from 2-5000 ng/mL and then quantified in a blind fashion (by a different operator). Good recoveries were obtained with the ELISA generally ranging from 80 to 120% in buffer (Table 2, part 1). Recoveries from mouse serum were slightly lower for samples spiked with higher concentrations. This is an expected observation. Samples with high concentration spikes had to be largely diluted to obtain a signal in the linear range while the calibration curve was built with a blank serum added to account for matrix effect. This dilution resulted in decreased matrix effect and slight underestimation of TETS concentration. Recoveries from human serum were consistent with theoretical spike concentrations with some insignificant overestimation. It should be noted that the calibration curve was built in the assay buffer containing no human serum. Thus, the slight overestimation might be related to a minor matrix effect of the human serum. Nevertheless, the overall recovery results suggest that the ELISA is an excellent tool for rapid, cheap, high throughput and very sensitive analysis of a variety of samples that does not require special sample treatment, clean-up or pre- concentration. For comparison purposes, we also performed analysis of a separate series of spiked samples, where TETS was extracted and then analyzed with the immunoassay. As expected, cleaned up samples had improved recovery values making them more consistent with theoretical values and decreased recovery variation (Table 2, part 2).

Similar experiments were conducted with an instrumental analytical method- GC/MS run under conditions similar to the EPA approved method[41] (Table 2). Consistent correlation with theoretical values and ELISA results was observed. However, despite addition of isotope labeled ^{13}C-TETS as an internal standard and clean-up with solid-phase microextraction (SPME), GC/MS data were variable and generally provided overestimated results showing a strong effect of the matrix on the instrumental analysis (Table 2, part 1). Recoveries from serum samples were particularly affected by the matrix. Therefore, for the mouse serum series we also tried LLE as an alternative to SPME. Simple LLE improved recovery values, but produced some underestimated values (Table S4). Table 2 part 2 shows that LLE efficiently removed TETS from the matrix and provided clean extracts for ELISA resulting in excellent recoveries mostly in the range of 80-120%. However, this clean- up procedure was not sufficient for GC/MS, resulting in overestimation of 40-160%. Both methods gave highly variable results for the low range of spiked concentrations 2-5 ng/mL, indicating this concentration to be near the limit of quantification of the methods.

The results obtained are in good agreement with published data. They show that indeed, compared to LLE, more sophisticated methods for sample extraction and clean-up are

needed to provide more accurate results with the instrumental methods. However, reported modern techniques for sample preparation, including SPME, stir bar sorptive extraction or membrane assisted solvent extraction remain time consuming and may require sophisticated techniques to perform high throughput samples preparation and analysis.[9,11,12,14,15] Therefore, the immunoassay developed in this work provides an advantageous alternative to instrumental methods. It is a fast, cheap and high throughput method with adequate accuracy, while not requiring any sample pre-treatment.

Quantification of TETS in the serum of exposed mice

We sought to evaluate the ELISA in real samples from exposed subjects. Since human samples are unavailable, we generated in-house samples by exposing mice to TETS at 2 × LD_{50} dose (0.2 mg/kg, intraperitoneal route). Animals were protected from lethality by pre-treating them with 10 mg/kg riluzole, a sodium channel blocker. The immunoassay was used to quantify the TETS concentration in the serum and to monitor the kinetics of TETS clearing from blood over a short period of time. Table 3 gives TETS concentrations detected in groups of animals bled at 30 min, 60 min, 120 min and 240 min after treatment. Over the 240 min period, TETS remained in the blood at 150 ng/mL with only slight differences between different time points and different animals. This concentration corresponds to about 10% of the injected dose. These data were confirmed with GC/MS analysis that resulted in similar TETS concentrations in corresponding samples. The rest of the injected TETS might have distributed in other organs and tissues, with the major part being excreted with feces as reported by Radwan et al.[42] Even though limited data are available on the biology of TETS and there are no published data on TETS pharmacokinetics when administrated through injection, it is known that tetramine is slowly eliminated from the body.[40] For example, from case studies, Chau et al.[40] reported that TETS was identified in vomitus and blood even 1 week after poisoning. They also noted that TETS has a high volume of distribution and a slow metabolism causing it to stay in the body for up to 6 months after exposure. In a separate study[42], where tetramine was orally administrated to mice, TETS appeared in the blood within 10 min after dosing and its concentration remained constant for 3 days. These few published data support our findings in this work. They also support the hypothesis that the high toxicity of TETS seen with only moderate binding to the GABA A chloride channel is explained in part by slow clearance. They also caution that effective therapies need to be long lasting.

There is limited knowledge on TETS distribution in the human body and more research is required to understand its pharmacokinetics. The antibodies against TETS and corresponding immunoassay provide very useful tools and are clearly a significant progress in the analysis field. Immunoassay provides a convenient way for high throughput screening of samples with high sensitivity and good accuracy. The immunoassay can be employed for monitoring TETS distribution and rates of its elimination in treated animals. The antibodies also can be used to show that ^{14}C in tissue following ^{14}C TETS administration is in fact TETS. In view of poisoning cases reported in China, the developed immunoassay may be formatted into a portable biosensor device to provide a rapid screening tool with simple yes/no readout when rapid identification of unknown material is needed, for instance in subway stations, airports, or governmental institutions and may aid in therapy.

Recently, there have been reports of intentional poisoning of children and adults at public organizations like daycares and grocery stores in China.[43–45] Reports indicate that 30 kindergarten children were poisoned and two victims died.[44] Therefore, rapid and cheap immunoassay based screening tools may allow establishment of a security routine and control in public organizations involving food consumption. Additionally, formatted as a more sophisticated biosensor it can be used for evaluation of the degree of exposure or poisoning in victims.

In conclusion, this is the first report in the literature describing development of a sensitive immunoassay selective to TETS. The assay showed a detection limit around 0.2 ng/mL that is lower or in the range of the GC/MS limit of detection. As an advantage, the immunoassay does not require sample pre-treatment, clean-up or pre-concentration prior to analysis. The immunoassay has the potential for being adapted into a sensor for rapid in-field screening and monitoring purposes. The advantage of the high throughout and cheap analysis may help for in-time detection of contamination with TETS or early detection of the toxin in the blood of the victims. Timely, selective and quantitative detection of the poisoning with TETS may allow fast and appropriate treatment of the victims resulting in better clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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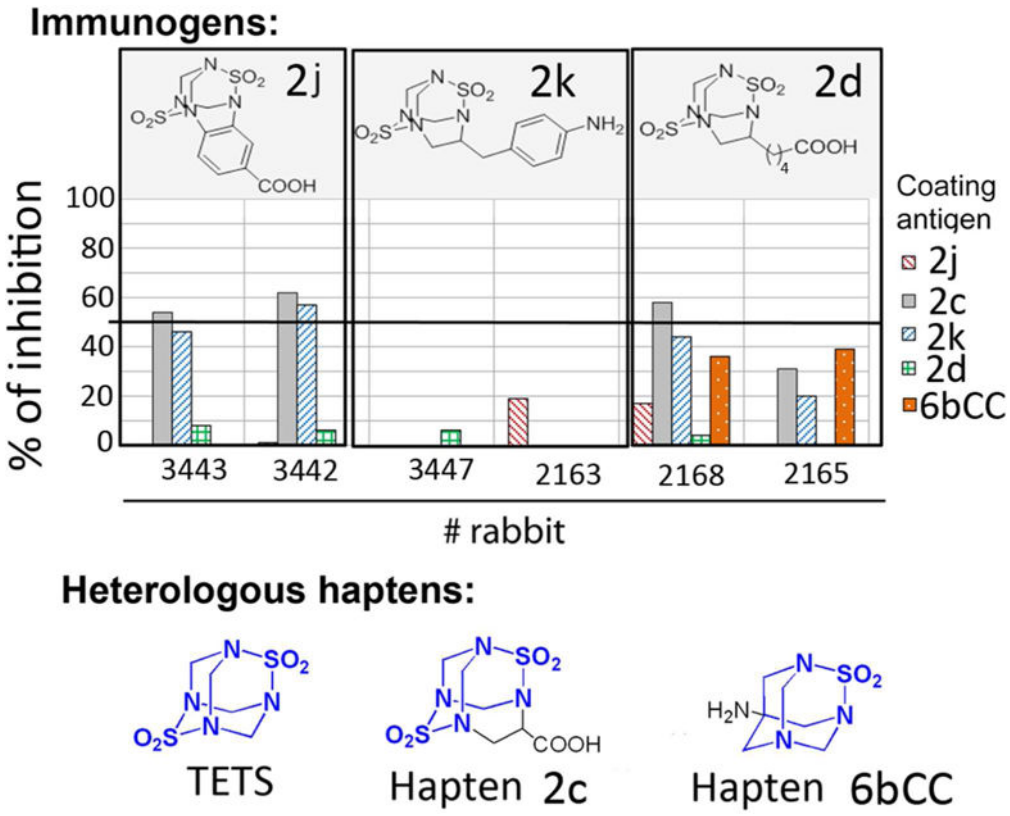


Figure 1.

Screening for successful pairs of coating antigen/serum. Criteria of success is 50% of inhibition and over, at 50 ng/mL TETS. Absence of the bar indicates that selected sera did not recognize corresponding coating antigen. Conalbumin was used as a carrier protein.

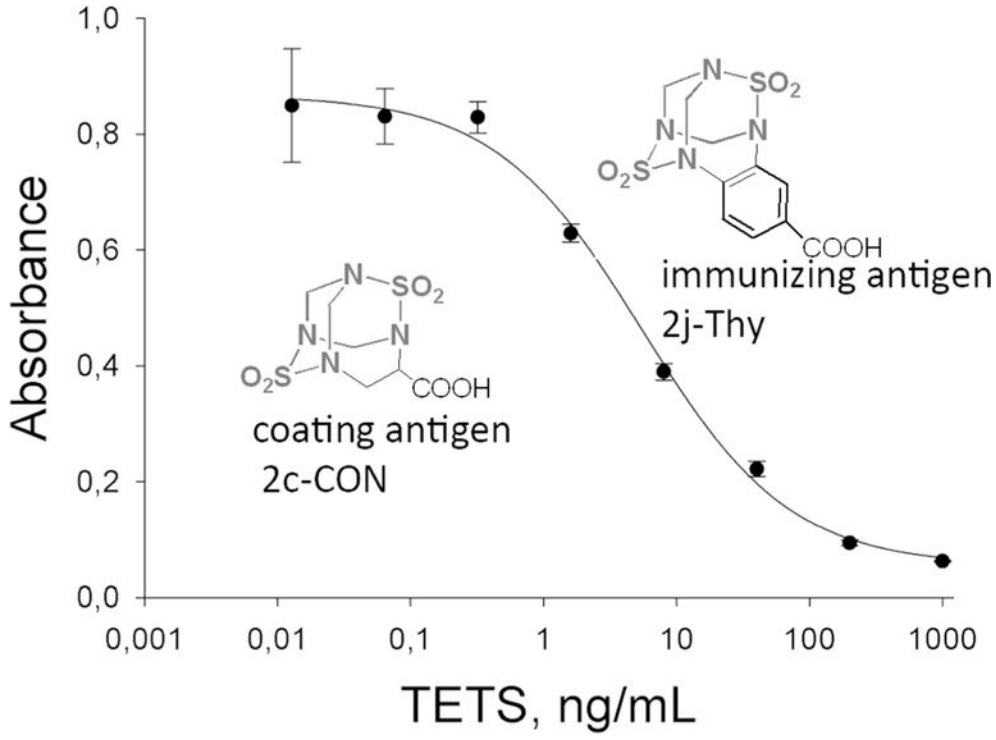


Figure 2.

TETS competitive curve with rabbit serum 3442 in assay buffer. Assay conditions: coating antigen 0.5 Gg/mL (2c-CON, on the left), anti-TETS serum in 1/6000 dilution; goat anti-rabbit IgG-HRP (1/10000). IC_{50} 4.5±1.2 ng/mL (n=9) (or 0.45±0.12 ng in the well).

Table 1

Selected haptens for immunoassay development.

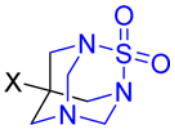
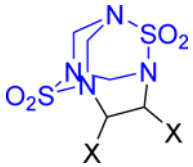
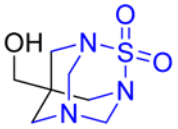
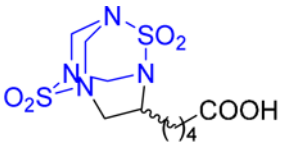
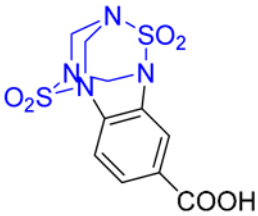
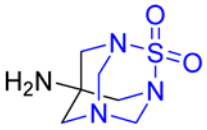
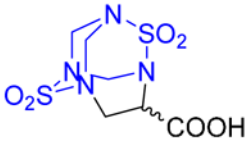
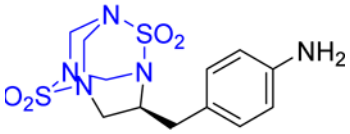
1ST SERIES	2ND SERIES
	
 Hapten 6a	 Hapten 2d
	 Hapten 2j
 Hapten 6b	 Hapten 7c
	 Hapten 2k
Resulting antibodies did not sufficiently recognize TETS	Resulted in antibodies selective to TETS

Table 2

Recovery of TETS from spiked samples

Spike, ng/mL	ELISA, ng/mL	%*	GC/MS, ng/mL	%*
1. Without extraction Buffer				
Buffer				
4	5±1.2	125	6.7	167
10	13.4±4.9	134	<LOD	N/A
50	55±14	110	58	115
500	400±68	80	371	74
2000	1930±330	96	1590	79
Mouse serum				
2	1.4±0.2	72	<LOD	N/A
8	7.6±2.6	95	56	697
40	26±7	64	59	147
300	161±38	54	228	76
4000	3820±1220	95	4360	109
Human serum				
3	4.3±2.4	143	7.9	264
20	15.5±3.6	77	36.8	184
40	56±16	141	59	148
200	202±36	101	274	137
3000	3610±350	120	4200	140
2. With extraction				
Mouse serum				
4.6	11.6±4.3	252	17.8	387
6.5	9.5±2.1	147	9.4	144
23.8	28±10	117	34.4	144
455	447±17	98	836	184
1456	1440±390	99	2324	160
Human serum				
3.7	3±0.1	81	0.7	19
12.2	11.1±1.6	91	13.0	106
45.5	49±7	108	65.6	144
238	218±30	91	383	161
2381	2250±370	95	3920	165

*% of recovery

Table 3

Quantification of TETS in the serum of exposed mice.

Mouse, number	min after injection	ELISA, ng/mL[#]	GC/MS ng/mL
1	30	174±40	150
2		149±32	143
3		122±18	113
1	60	101.3[*]	122
2		137±22	177
3		112±17	185
1	120	99±27	114
2		N/A	102
1	240	192±42	189
2		103±20	88
3		118±28	112
4		157±7	N/A

[#] data presented as mean ±SD, tested on three different days with a triplicate measurement for each day;

[*] tested one day in triplicate; N/A –not measured