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CASE REPORT

False negative result for amphetamines on the Triage® Drug of Abuse panel?

The cause of the unusual phenomenon with experimental analyses

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Abstract On-site drug screening devices are widely used today for their simple test procedures and instantaneous results. Among other devices, a Triage® Drug of Abuse panel is considered to be highly reliable for its high specificity and sensitivity of abused drugs. Although it is known that a false positive amphetamine (AMP) result may be obtained from the urine samples containing putrefactive amines or ephedrine-related compounds, no clinical false negative methamphetamine results have been reported to date. However, a false negative Triage® result was obtained from the urine of a fatal methamphetamine poisoning victim taking Vegetamine® tablets. Further experimental analyses revealed that the cross-reactivity of methamphetamine and chlorpromazine metabolites, including nor-2-chlorpromazine sulfoxide, was the cause for a false negative Triage® reaction for AMP. Forensic scientists and clinicians must be aware of the limitations of on-site drug testing devices and the need for the confirmatory laboratory tests for the precise identification and quantification of drugs in suspicious intoxication cases, as also recommended by the manufacturers.

Keywords Immunoassay · Triage® Drug of Abuse panel · Methamphetamine

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Introduction

On-site drug screening tests are widely used today for their simple test procedures and instantaneous results. Forensic scientists, emergency department physicians, and laboratory technicians depend on the results for the rapid detection of abused drugs. Several laboratory and clinical studies have been carried out to assess the sensitivity and specificity of various drug testing devices [1–7]. The Triage® Drugs of Abuse (DOA) Panel (Biosite Diagnostics, San Diego, CA, USA) is one of the on-site drug testing devices most widely used for the detection of eight classes of abused drugs, namely amphetamines (AMP), cocaine metabolites, opiates, phencyclidine, tetrahydrocannabinol, barbiturates (BAR), benzodiazepines, and tricyclic antidepressants. Among various other devices, the Triage® DOA panel is considered to be highly reliable for its high sensitivity and specificity of drugs [5–7]. Although it is known that a false positive Triage® result for amphetamines may be obtained from a sample containing putrefactive amines or ephedrine-related compounds [8], no false negative Triage® results from clinical urine samples have been previously reported for amphetamines. In this study, the cause of a false negative Triage® result has been discussed with thorough experimental analyses, as the unusual phenomenon was observed in a clinical sample containing a significant amount of methamphetamine.

Materials and methods

Report of case

A woman in her thirties with a medical history of psychosis was found dead alone at home and empty packages of the

prescribed Vegetamine® A, with tablets containing phenobarbital (40 mg), promethazine (12.5 mg), and chlorpromazine (25 mg), were found nearby. The urine sample collected by a police investigator tested positive for BAR using the Triage® DOA panel. The body had been preserved at 4°C overnight and a forensic autopsy was carried out 2 days after the estimated death. A trained laboratory technician performed another Triage® test with clean supernatant of the urine sample collected during the autopsy and obtained the same result as the police investigator; positive only for BAR, with a clearly visible band for the positive control and no band for the negative control. The decedent, 155-cm tall and weight 55 kg, had no particular pathological findings except for the mild pericarditis and endocarditis. Although there were no identifiable tablet residues in the mouth nor stomach, pulmonary congestion and edema implicated the possibility of intoxication. Drug screening was carried out by gas chromatography–mass spectrometry (GC-MS) with a slight modification to our previously published method for the identification of any suspicious drugs [9] and methamphetamine, phenobarbital, promethazine, chlorpromazine, and their metabolites were detected in blood and urine samples, as shown in Fig. 1. The alcohol concentration in blood and urine was below the detectable level. The drug screening results of GC-MS implicated the possibility that the initial Triage® tests gave a false negative for amphetamines. Each detected drug was quantified to clarify the cause of death of the victim and investigations were made on a false negative Triage® result.

Quantification of drugs in the case

Phenobarbital and promethazine were purchased from Wako Pure Chemical Industries (Osaka, Japan), chlorpromazine was provided by Yoshitomi Pharmaceutical (Osaka, Japan) and methamphetamine hydrochloride was purchased from Dainippon Pharmaceutical (Osaka, Japan). Methamphetamine-*d*4 and amphetamine were generous gifts from the Department of Forensic Medicine, Fukuoka University School of Medicine (Fukuoka, Japan). Medazepam was obtained from Shionogi (Osaka, Japan). Pentobarbital and diazepam-*d*5 were purchased from Tokyo Chemical Industries (Tokyo, Japan) and Cerilliant (Round Rock, USA), respectively.

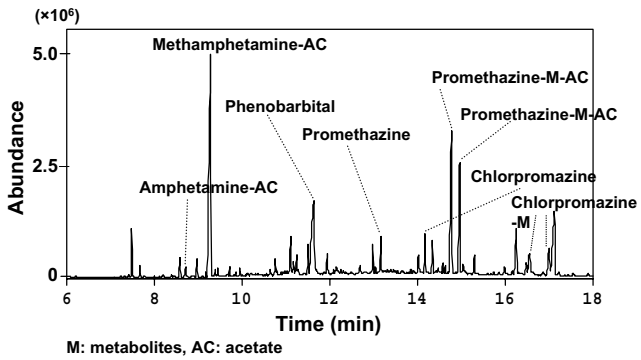

Fig. 1 A total ion chromatogram of the derivatized extract from the urine of the cadaver

Concentrations of methamphetamine and its metabolite amphetamine in whole blood and urine samples were determined by GC-MS using a Focus™ column (Varian, Lake Forest, CA, USA) and acetylation with our previously published method [10]. Methamphetamine-*d4* was used as an internal standard.

Promethazine and chlorpromazine in whole blood and urine samples were extracted as described for methamphetamine analysis and submitted to GC-MS without acetylation. Medazepam was used as an internal standard.

Phenobarbital in whole blood and urine samples was extracted by Bond Elut Certify™ columns and analyzed by high performance liquid chromatography (HPLC). Pentobarbital was used as an internal standard.

Triage® tests with methamphetamine and the parent compounds of Vegetamine®

The detected amounts of methamphetamine, phenobarbital, promethazine, and chlorpromazine in the urine of the cadaver were added to the control urine samples and the prepared urine samples were submitted to Triage® analyses to identify the substance responsible for a false negative reaction. Methamphetamine was also added to urine samples from previous autopsy cases in which the components of Vegetamine® were detected by GC/MS screening.

Animal experiments

This experiment was reviewed by the Committee for Ethics regarding Animal Experiments in the School of Medicine, Kyushu University and it was carried out under the control of Guidelines for Animal Experiments in the School of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government of Japan. Moreover, this study followed the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

A total of ten Wistar rats (6 weeks old, 200 g, Kyudo, Kumamoto, Japan) were divided into five groups. The first group was a control. Phenobarbital, promethazine, chlorpromazine, and the mixture of three drugs were injected transesophageally into the second, the third, the fourth, and the fifth group of rats respectively, after being anesthetized

with diethyl ether. Urine (24 h) was collected from each rat and 14.9 μg/ml of methamphetamine was added to each urine sample for the Triage® tests.

The Triage® tests with methamphetamine and chlorpromazine metabolites

All 15 chlorpromazine metabolites used in this study were generous gifts of Dr. Manian at the National Institute of Mental Health (Washington, DC).

Urine samples containing each of 15 major chlorpromazine metabolites and methamphetamine (14.9 μg/ml) were prepared and submitted to Triage® analyses to identify the substances responsible for a false negative reaction. Following the result, further Triage® tests were performed by changing the concentrations of methamphetamine and nor-2-chlorpromazine sulfoxide in the urine samples.

Quantification of nor-2-chlorpromazine sulfoxide in urine sample of the case

The concentration of nor-2-chlorpromazine sulfoxide in the urine sample of the cadaver was determined by liquid chromatography–mass spectrometry (LC-MS) as follows; 1 ml of urine sample was mixed with 1 ml of distilled water and 10 μl of IS solution (1,000 ng diazepam-*d5* in methanol) in a 10 ml centrifuge tube and sonicated for 5 min. The preparation was centrifuged at 850×*g* for 15 min. The supernatant was then transferred to the Focus™ column sequentially conditioned with 1 ml of methanol and 1 ml of distilled water. The column was rinsed sequentially with 1 ml of distilled water and 1 ml of 10% acetonitrile. The analytes were eluted with 0.75 ml of 0.1% TFA in acetonitrile and 0.75 ml of 0.2% ammonia in acetonitrile. The eluate was evaporated to dryness under the stream of nitrogen, the residue was dissolved in the 100 μl of HPLC mobile phase (25% acetonitrile in 0.05% formic acid) and 5 μl of this solution was submitted to LC-MS. The determination of nor-2-chlorpromazine sulfoxide was carried out using a Waters UPLC-MS system (Milford, MA, USA) with ZQ 2000 mass detector controlled by Masslynx ver. 4.1. Chromatographic separations were performed on a Waters Acquity UPLC BEH C18 column (100×2.1 mm; particle size 1.7 μm). The mobile phase was composed of a mixture of 0.05% formic acid in water (A) and 0.05% formic acid in acetonitrile (B). The gradient was delivered at 0.4 ml/min as follows: 0–0.5 min, 25% solvent B; 4–6 min, 30% solvent B; 9.5 min, 40.5% solvent B; 11–13 min, 90% solvent B; 13.1–17 min, 25% solvent B; and loop time was 17 min. The column was maintained at 40°C. The mass spectrometer was operated with a positive electrospray source. The ionization source conditions were as follows: capillary voltage of 3.0 kV, source temperature of 120°C, desolvation temperature of 350°C, and cone voltage of 30 V. The cone and desolvation gas flows were 50 and 600 l/h, respectively. With these UPLC-MS conditions, the compounds were analyzed by scan mode with a scan time of 0.4 s. The $[M+H]^+$ ions, m/z 307 and m/z 290, were used for nor-2-chlorpromazine sulfoxide and IS, respectively.

Table 1 The concentration of drugs detected in the cadaver's whole blood and urine

Substance	Concentration (μg/ml)		
	Right heart blood	Left heart blood	Urine
Methamphetamine	9.9	11.4	14.9
Amphetamine	0.91	0.92	1.41
Phenobarbital	22.0	23.9	25.9
Promethazine	0.48	0.45	1.01
Chlorpromazine	0.29	0.27	0.51

Results

The concentrations of methamphetamine, amphetamine, phenobarbital, promethazine, and chlorpromazine in the whole blood and urine samples from the cadaver are shown in Table 1. Among the laboratory and animal experiments, a false negative Triage® result for amphetamines was obtained from the urine samples produced after metabolizing Vegetamine® as shown in Tables 2 and 3. Out of the 15

Table 2 The Triage® analyses with methamphetamine and the components of Vegetamine®

Urine number	Type of urine	Drug/s added	Triage® results	
			AMP	BAR
1	Control	Methamphetamine, phenobarbital, promethazine, chlorpromazine	+	+
2	Control	Methamphetamine, phenobarbital	+	+
3	Control	Methamphetamine, promethazine	+	−
4	Control	Methamphetamine, chlorpromazine	+	−
5	Urine samples from previous autopsies with Vegetamine® detected	Methamphetamine	−	+
6			−	+
7			−	+

Amount of drugs added: methamphetamine, 14.9 μg/ml; Phenobarbital, 25.9 μg/ml; promethazine, 1.01 μg/ml; chlorpromazine, 0.51 μg/ml

Table 3 The Triage® analyses with rat urine samples

Rat number	Drug/s administered	Drug added to the collected urine	Triage® result	
			AMP	BAR
1	None (control)	Methamphetamine 14.9 μg/ml	+	−
2			+	−
3	Phenobarbital		+	+
4			+	+
5	Promethazine		+	−
6			+	−
7	Chlorpromazine		−	−
8			−	−
9	Phenobarbital, promethazine, chlorpromazine		−	+
10			−	+

Amount of drugs administered: Phenobarbital, 10 mg/kg; promethazine, 5 mg/kg; chlorpromazine, 5 mg/kg

chlorpromazine metabolites tested, a false negative Triage® result was obtained only when nor-2-chlorpromazine sulfoxide and methamphetamine were added to urine, as shown in Table 4. Via the analyses shown in Table 5, it was proven that by adding a two times higher concentration of nor-2-chlorpromazine sulfoxide than methamphetamine to a urine sample, a false negative Triage® result could be obtained. The concentration of nor-2-chlorpromazine sulfoxide in the urine of the cadaver presented was measured as 2.29 μg/ml.

Table 4 The Triage® analyses with chlorpromazine metabolites

Name of substance	Drug added	Triage AMP result
7-Hydroxychlorpromazine	Methamphetamine 14.9 μg/ml	+
7-Hydroxychlorpromazine sulfoxide		+
7-Hydroxydidesmethyl chlorpromazine		+
7,8-Dihydroxychlorpromazine		+
Nor-2-chlorpromazine sulfoxide		−
7-Hydroxydesmethyl chlorpromazine		+
Chlorpromazine sulfone		+
Nor-1-chlorpromazine sulfoxide		+
Chlorpromazine 5-n-dioxide		+
Nor-1-chlorpromazine		+
Chlorpromazine 5-oxide		+
Nor-2-chlorpromazine		+
7,8-Dioxochlorpromazine		+
Chlorpromazine n-oxide dehydrate		+
7,8-Dihydroxychlorpromazine		+

The amount of chlorpromazine metabolite: 100 μg/ml

Table 5 The Triage® analyses with various concentrations of nor-2-chlorpromazine sulfoxide and methamphetamine

Concentration (μg/ml)		Triage® AMP
Nor-2-chlorpromazine sulfoxide	Methamphetamine	
0	14.9	+
10	0	−
5	14.9	+
10	14.9	+/−
25	14.9	−
50	14.9	−
75	14.9	−
100	14.9	−
2	1	−
10	5	−
20	10	−
40	20	−

Discussion

According to the drug concentrations determined, the right heart blood of the cadaver contained a therapeutic level of phenobarbital (22.0 μg/ml), promethazine (0.48 μg/ml), and chlorpromazine (0.29 μg/ml) but almost a lethal level of methamphetamine (9.91 μg/ml), based on the report by Schulz et al. [11]. The results were in accordance with methamphetamine intoxication, which suggested that the results of the initial Triage® tests were false negative for amphetamines. It might have been impossible to identify the accurate cause of the death of the woman without the detailed analyses by GC-MS, especially because there was no typical anatomical evidence of methamphetamine use such as cardiomyopathy nor injection marks in the presented case [12–15].

The laboratory Triage® test results suggested the metabolites of phenobarbital, promethazine, or chlorpromazine, not the parent compounds, were responsible for the false negative result for amphetamines, as a false negative result was obtained only from the urine samples with Vegetamine® components and their metabolites as shown in Table 2. Furthermore, from the results of animal experiment, it was indicated that chlorpromazine metabolites were the cause for the false urine negative results obtained with a Triage® panel. More than 160 possible metabolites have been postulated for chlorpromazine but only about 20 have been isolated to date [16]. However, out of the 15 major chlorpromazine metabolites tested in this study, it was proven that nor-2-chlorpromazine sulfoxide interferes with the correct Triage® reaction.

The Triage® DOA panel uses a competitive binding immunoassay for the detection of abused drugs. The test involves a soluble phase reaction, a solid phase reaction and

integrated controls. The panel consists of a reaction cup containing three beads for the soluble phase reaction. The purple bead is the conjugate bead containing colloidal particles to which the drug and its metabolites are attached. The white bead is the antibody bead containing monoclonal antibodies specific for the drugs and its metabolites detected. The remaining yellow bead is the buffer. The solid phase reaction area is a membrane with immobilized antibodies in eight different detection zones. The testing procedures are simple. An operator is requested to add a sample to the reaction cup, transfer the mixture to the membrane after 10 min of incubation, then add wash solution to the area. If the drug is present in the sample, it will bind to the antibodies in the white bead as insufficient antibodies are left to bind all the conjugated drug of the purple bead. When the mixture is transferred to the membrane, some or all the conjugated drug is free to bind to the fixed antibodies. The colloidal dye produces a line on the membrane of the drug detection zone for a positive result. If drug is absent or below the cut-off concentration, the antibodies in the white bead will bind with the conjugated drug in the purple bead. There is no free conjugated drug to bind to the antibodies immobilized on the membrane. The drug–dye conjugate is washed away by the wash solution, so no line appears on the membrane.

By changing the concentration ratio of methamphetamine and nor-2-chlorpromazine sulfoxide in a urine sample, it was proven that a false negative Triage® result will be obtained when the concentration of nor-2-chlorpromazine sulfoxide in the sample is almost twice as high or higher, than that of methamphetamine (Table 5). The molecular weight of nor-2-chlorpromazine sulfoxide is 306.81, whereas that of methamphetamine is 149.24. This means that an equal mole of nor-2-chlorpromazine sulfoxide and methamphetamine, or higher moles of nor-2-chlorpromazine sulfoxide than methamphetamine, cross-react with each other and lead to a false negative Triage® result. A possible cause for this phenomenon is that the nor-2-chlorpromazine sulfoxide interferes with the binding of methamphetamine to the antibodies in the white bead by forming a complex with an equal mole of methamphetamine. As a result, the complex with no affinity to the antibodies in the white bead allows the conjugated drug in the purple bead to bind to the antibodies instead, producing a negative result for amphetamines in the detection zone (Fig. 2).

The concentration of nor-2-chlorpromazine sulfoxide in the urine of the cadaver was 2.29 μg/mL. This is much lower than the concentration required to obtain a false negative Triage® reaction in a sample containing 14.9 μg/ml of methamphetamine. It is therefore expected that nor-2-chlorpromazine sulfoxide is not the sole responsible substance, but chlorpromazine metabolites other than the 15 substances tested in this study may have also contributed to the initial false negative Triage® results in the presented case.

On the other hand, despite the urine amphetamine concentration higher than the cut-off value of 650 ng/ml, the Triage® result was false negative. It implies that amphetamine, as well as methamphetamine, is cross-reacting with one or more substances with the components of a Vegetamine® tablet. The identification of the cross-reacting substance should be included in the future studies.

There is a report on a false negative amphetamine Triage® reaction at extremely high concentrations (1,000–

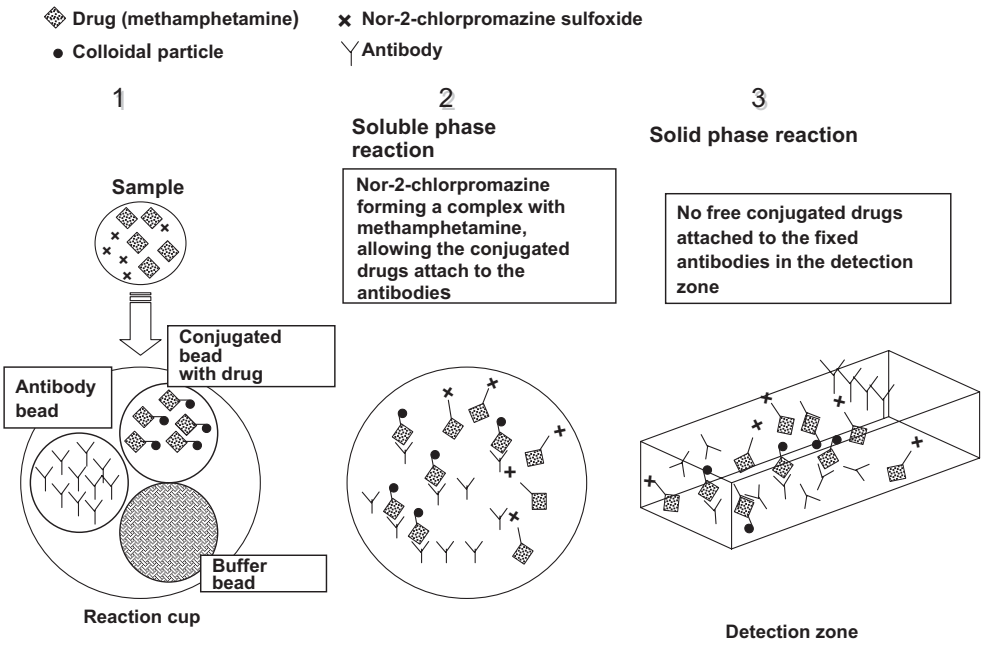

Fig. 2 A possible mechanism of a false negative result on a Triage® DOA panel

1,500 μg/ml) [4], but it is suspected that a false negative result in that report was produced by a different mechanism to the one discussed in our study, as the reported experiment was carried out by artificially adding an excessive amount of methamphetamine alone to a control urine sample.

There have been published reports on the cross-reactivity of chlorpromazine metabolites in the early version of the monoclonal enzyme multiplied immunoassay technique (EMIT), EMIT dau (Syva, Palo Alto, California) amphetamine assay [17, 18]. Another report informed the false positive results in EMIT II Plus monoclonal amphetamine/methamphetamine immunoassay caused by promethazine metabolite/s [19]. The limitation of on-site drug screening kits has always been brought to discussion [20, 21], but this is the first study to identify the responsible compound for a false negative amphetamine result in the Triage® DOA panel. Promethazine and chlorpromazine possess similar phenothiazine structures and it is possible that phenothiazine-associated compounds cross-react in the widely distributed immunoassays, causing a false result in some cases. Many antipsychotic drugs prescribed worldwide contain promethazine and/or chlorpromazine, the derivatives of phenothiazine. Forensic scientists must always be aware of the possibility of methamphetamine poisoning in cases of sudden death of patients taking phenothiazine-associated drugs and perform confirmatory laboratory tests in suspicious situations.

Conclusion

Drug abuse, including methamphetamines in Japan and other countries worldwide raises the need for fast and accurate screening tests. False negative test results are important because they represent patients or decedents who had significant concentrations of drug in the urine, but who would not have been identified as drug users. This study has proven that nor-2-chlorpromazine sulfoxide plays a significant role in obtaining a false negative methamphetamine result in the Triage® DOA panel. Forensic scientists and clinicians must be aware of the limitation of the on-site drug testing devices and the need for the identification and quantification of drugs by specific alternative methods in suspicious intoxication cases, as also recommended by the manufacturers.

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