TECHNICAL NOTE

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Screening of Amphetamine/Methamphetamine and Their Derivatives in Urine Using FPIA and Triage™₈ and the Scope and Limits of a Subsequent Identification by Means of the REMEDi™ HS System

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ABSTRACT: This study describes screening and identifying amphetamines, methamphetamines, and their derivatives in urine using immunochemical (Triage™₈, FPIA) and chromatographic techniques (REMEDi™ HS). Amphetamines, methamphetamines, MDMA (3,4-methylenedioxymethamphetamine), MDA (3,4-methylenedioxyamphetamine), MDE (3,4-methylenedioxyethylamphetamine), MBDB (N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine), BDB (3,4-(methylenedioxyphenyl)-2-butanamine), PMA (4-methoxyamphetamine), DOM (2,5-dimethyloxy-4-methylamphetamine), DOB (4-bromo-2,5-dimethyloxyamphetamine), amphetaminil, pholedrine, fenfluramine, and amfepramone were subjected to a comparative study. For this, the substances were analyzed to determine their specific threshold concentration for a positive detection in the Triage test and their limit of detection and positive threshold concentration for the FPIA test and the results compared.

Furthermore, the capabilities of a more detailed analysis with the REMEDi system were studied. This HPLC system was able to produce information on the single drugs and main metabolites found in the sample with the danger of false-positive or false-negative screening results greatly minimized.

KEYWORDS: forensic science, forensic toxicology, Triage, FPIA, REMEDi HS, urine, emergency medicine, amphetamines/methamphetamines, designer drugs

The consumption of amphetamine, methamphetamine, and their derivatives, the so-called designer drugs, has increased continuously in recent years. Designer drugs of the methylenedioxyamphetamine type such as MDMA (3,4-methylenedioxymethamphetamine), MDA (3,4-methylenedioxyamphetamine), MDE (3,4-methylenedioxyethylamphetamine), and others, and the butyl homologues BDB (3,4-(methylenedioxyphenyl)-2-butanamine) and MBDB (N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine) obtained by side-chain extension are because of their main effect referred to as entactogenes. Methoxyamphetamines such as DOM (2,5-dimethoxy-4-methylamphetamine), DOB (4-bromo-2,5-dimethoxyamphetamine), PMA (4-methoxyamphetamine), TMA (3,4,5-trimethoxyamphetamine), and DMA (2,5-dimethoxyamphetamine) preferably produce a hallucinogenic effect. More and more publications (1–5) on their abuse appear in literature and cases of intoxication are growing in number.

Therefore, the development of toxicological methods for their identification is of great clinical and forensic relevance.

The first step for the identification of drugs in urine is typically immunological screening (6–10). The rapid Triage™₈ immunoassay is a suitable tool for the simultaneous qualitative identification of the main metabolites of amphetamine/methamphetamine and designer drugs, tetrahydrocannabinol, opiates, cocaine, barbiturates, and tricyclic antidepressants within 15 min. The test is evaluated by visual inspection. In the case of a positive result with respect to the amphetamines/methamphetamines of interest here, that is, if the threshold concentration (1000 ng D-amphetamine or D-methamphetamine equivalents/mL urine) is exceeded, a colored bar will appear in the respective specific drug detection zone of the test cassette.

The fluorescence polarization immunoassay (FPIA) for the detection of amphetamines/methamphetamines II in urine is a fully automatic test which in comparison with the Triage allows even smaller concentrations to be detected. A result is usually considered to be positive if the urine sample contains a drug in a concentration which causes an immunological response corresponding to or exceeding the threshold concentration for that drug (e.g., 300 ng/mL D-amphetamine). The numerical data obtained also make it possible to interpret the results in quantitative terms.

The REMEDi™ HS system is an automatic HPLC setup for the detection, identification, and semiquantitative analysis of drugs in urine and serum. It consists of a combination of several HPLC columns performing the adsorption, concentration, and separation of neutral, acidic, and basic substances and a fast-scanning multiwave UV detector.

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One advantage of a chromatographic such as the REMEDi system is that the identification is for specific drugs and not for the compound class (11–13).

Materials and Methods

MBDB hydrochloride and BDB hydrochloride were purchased from Promochem GmbH (Wesel, Germany). All other drugs were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Stock solutions of each drug (1 mg/mL) were prepared with HPLC grade ethanol and stored at 4°C. We prepared a blank negative control from a urine pool that had been analyzed and found to be drug free.

Aliquots of the stock solutions were added to the negative urine pool to give final concentrations of 100 to 100 000 ng/mL.

The respective reference substances were mixed thoroughly (vortex).

Triage™8

In order to determine the sensitivity with respect to amphetamine/methamphetamine and designer drugs, drug-free pool urine was prepared with 100 to 100 000 ng/mL single substance. This test differentiates between positive and negative specimens at the designated cut-off concentrations (Table 1).

FPIA

FPIA analysis was performed with the Amphetamine/Methamphetamine II Test according to manufacturer's recommendations using an Abbott TD_x/FL_x analyzer.

REMEDi™ HS

All reagents used for REMEDi HS were purchased from Bio-Rad Laboratories (Munich, Germany).

Samples for the limit of detection (LOD) were prepared in the concentration range 100 to 1000 (to 100 000) ng/mL urine. Identification of analytes in the LOD study required a peak height (Peak HT) of greater than 15 000 microabsorbance units (μAU). The chromatography report will automatically relate a so-called similarity factor (SF) to each peak to provide a measure of the quality of an identification. The SF will indicate the similarity between the spectrum of a peak and the existing entries in the library. The smaller this value the closer will the similarity be. An SF of ≤ 0.02 will relate to a candidate qualified for the identification of a substance, while values up to ≤ 0.06 are still usable for identification in general.

Results and Discussion

The results of the analysis of the several substances at different concentrations with the Triage™8 and FPIA amphetamine/methamphetamine II reagents are shown in Table 1. The threshold concentrations and limits of detection were determined in this work with the amphetamine/methamphetamine II test (status: May 1996, 1998 charge). The threshold concentration of a positive result in the Triage test is higher for all mentioned amphetamines/methamphetamines and their derivatives by the factor 3 to 6 as against the FPIA cut-off values for the respective substances. An exception can be observed for L-amphetamine in which the Triage test will respond at the 25-fold concentration of the cut-off value of L-amphetamine only.

The methylenedioxyamphetamines such as MDA, MDMA, and MDE and the butyl homologue BDB can be detected with both Triage and FPIA due to their adequate immunological response. This will require, however, that the available concentration is up to three times higher than the cut-off value of the calibrator D-amphetamine or than the threshold concentration of the Triage test. In the case of the butyl homologue MBDB a positive screening result will in both tests require a ten times higher concentration in the urine than would be necessary for the respective calibrators.

A positive result will not be obtained with the Triage test for the methoxyamphetamines such as DOM and DOB and, according to Ref 14 for TMA and DMA, even if a concentration 100 times

TABLE 1—*Results Triage and FPIA.*

	Triage™8	FPIA		
Test Compound	Threshold Concentration, ng/mL	Threshold Concentration, ng/mL	LOD, ng/mL	Concentration Added, ng/mL
D-Amphetamine	1,000	300	100	Calibrator
L-Amphetamine	25,000*	1,000	300	300–1,000
D,L-Amphetamine	2,000	300	150	150–300
D-Methamphetamine	1,000	300	150	150–300
D,L-Methamphetamine	2,000	500	150	150–500
MDMA	2,000*	600	300	300–1,000
MDA	1,500*	500	300	300–1,000
MDE	2,500*	1,000	300	300–1,000
MBDB	10,000	3,000	1,000	1,000
BDB	2,500	1,000	300	300–1,000
PMA	3,000	3,000	1,000	1,000–10,000
DOM	100,000†*	20,000	5,000	5,000–50,000
DOB	100,000†*	20,000	5,000	5,000–50,000
Amphetaminil	2,500	400	150	150–400
Pholedrine	1,000	200	100	100–1,000
Fenfluramine	4,000*	2,000	500	1,000–3,000
Amfepramone	100,000†	…	100,000	100,000

* Results are in agreement with Ref 14.
† Negative results.

higher than the threshold concentration (1000 ng/mL) is encountered. On the other hand, PMA will show a sufficient immunological response at 3000 ng/mL. The limit of detection of the FPIA will be exceeded for DOM, DOB, and PMA at concentrations in the range 1000 to 5000 ng/mL already so that the probability of false-negative results is clearly lower than in the Triage test though comparatively high concentrations are found.

The danger of false-positive screening results in the amphetamine/methamphetamine tests exists especially in the case of amphetaminil, pholedrine, and fenfluramine, which are chemically related to this group. Pholedrine, for example, is identified in both tests with the same cross-reactivity as D-amphetamine, while amphetaminil and fenfluramine will show positive results in higher concentrations only. Amfepramone will even at 100 μg/mL urine not give a positive response. But it is reported in literature (15) that the urine from persons taking amfepramone may contain metabolites which are characterized by a high cross-reactivity.

Thus, the methoxy derivatives of the drug class of amphetamines/methamphetamines cannot always be reliably detected with the FPIA or particularly with the Triage test. The concentrations of one or several substances found in the urine are often not sufficient to produce a positive screening result. Besides, even a positive screening result will not yield any information of greater detail about the single substances contained. It will also not be possible to differentiate between amphetamines/methamphetamines and their derivatives and chemically related substances such as pholedrine and fenfluramine. There is the danger of getting false-negative or false-positive results.

A tool offered for a more detailed analysis is the REMEDi system which in the ideal case can identify single substances in the urine automatically within 30 min without an extractive preparation. The information produced is sufficient for clinical emergency cases and can be used readily in a more detailed GC-MS confirmative analysis if needed for forensic purposes.

The results of the REMEDi tests made (software version 5.32.11) are listed in Table 2 detailed according to retention times, relative retention times to the internal standards IS1 (N-ethyl-nordiacepam) and IS2 (chlorpheniramine), peak height, limit of detection (ng/mL), and a so-called similarity factor.

REMEDi™ HS is capable of identifying all mentioned substances out of the amphetamine/methamphetamine group and their derivatives as well as amphetaminil, pholedrine, fenfluramine, and amfepramone if contained as single substances within the specified limits of detection of 100 to 300 ng/mL urine. The only exception is D,L-methamphetamine with a sensitivity of 500 ng/mL urine.

If several substances are contained in the same sample, higher requirements will be made on the identification depending on the agent combination encountered, e.g., adequate differences with respect to retention times and UV spectra and the availability of appropriate concentrations of the single components. Furthermore, it can be seen from Table 2 that the results of the identification obtained with the single injection method were good (SF 0.001 $\leq$ 0.020) or still usable (SF 0.027 to 0.060).

Based on the retention values the single substances may be subdivided into three groups so that also in the case of an incomplete chromatographic resolution it will be possible to produce detailed information of the single substances expected to be encountered.

- 4.13 to 4.31 min: amphetamines, amphetaminil (decomposes into amphetamine), MDA, PMA, DOB, DOM, BDB
- 4.53 to 4.85 min: pholedrine, fenfluramine
- 5.05 to 5.35 min: MDE, MDMA, MBDB, methamphetamine

Amfepramone with its retention time of 6.95 min cannot be classified into any of above groups.

Table 3 lists the results of urine analyses for drug abuse from spiked samples.

TABLE 3—*Results of urine analyses from spiked samples.*

Sample	Triage™$_8$	FPIA, μg/mL	REMEDi™HS
Urine 1	Negative	Negative [0,02]	MBDB* and BDB*/MDA
Urine 2	Positive	Positive [3,80]	Methamphetamin* and MDA*

* Identified by GC-MS analysis.

TABLE 2—*Results REMEDi.*

	REMEDi™HS					
Test Compound	RT, min	RRT 1	RRT 2	Peak-HT by Sensitivity, μAU	SF	LOD ng/mL
D-Amphetamine	4.31	1.408	0.451	50,000	0.015	100
L-Amphetamine	4.31	1.407	0.457	20,000	0.018	100
D,L-Amphetamine	4.31	1.416	0.460	35,000	0.006	100
D-Methamphetamine	5.35	1.710	0.578	17,000	0.019	100
D,L-Methamphetamine	5.35	1.721	0.575	15,000	0.042	500
MDMA	5.09	1.658	0.539	42,000	0.050	300
MDA	4.13	1.345	0.438	52,000	0.053	100
MDE	5.05	1.645	0.536	49,000	0.035	100
MBDB	5.15	1.661	0.531	47,000	0.049	100
BDB	4.31	1.360	0.443	64,000	0.028	100
PMA	4.20	1.355	0.446	30,000	0.005	300
DOM	4.27	1.386	0.456	57,000	0.010	100
DOB	4.18	1.357	0.445	47,000	0.001	100
Amphetaminil	4.31	1.418	0.475	15,000	0.001	100
Pholedrine	4.53	1.495	0.468	25,000	0.050	100
Fenfluramine	4.85	1.569	0.517	45,000	0.005	200
Amfepramone	6.95	2.213	0.733	17,000	0.003	100

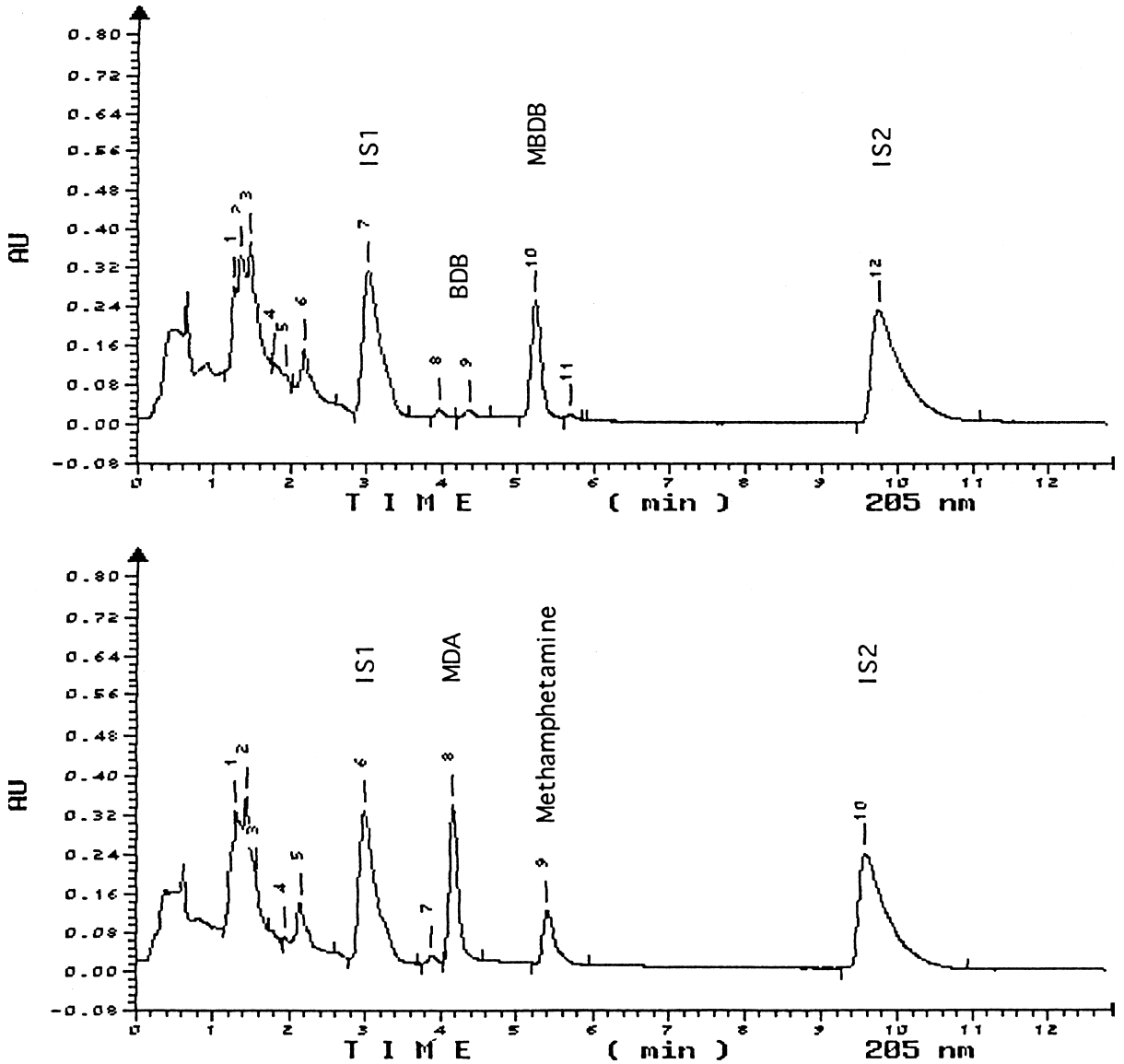

FIG. 1—*Chromatograms of spiked urine samples. Above: 3, caffeine, 6, endogenous peak, 1, 2, 4, 5, 8, 11, unknown. Below: 2, caffeine, 5, endogenous peak, 1, 3, 4, 7, unknown.*

The advantages of an additional analysis with the dem REMEDi™ HS system are shown in Fig 1. Significant information is obtained with respect to single substances, also for low sample concentrations. A specific confirmation procedure by GC-MS may follow depending on the definition of the problem.

Conclusion

Irrespective of whether the rapid Triage™₈ immunoassay or the FPIA for amphetamines/methamphetamines II can produce a positive screening result in this class of drugs, much more comprehensive information can be obtained with the REMEDi™ HS system. This HPLC system is a suitable tool to obtain data on the single drugs and main metabolites found in the sample with the danger of false-positive or false-negative results greatly minimized. It is essential for the treatment of clinical emergency cases and in preselecting possible candidates for a forensic confirmative analysis.

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