Screening method for stimulants in urine by UHPLC-MS/MS. Identification of isomeric compounds

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ABSTRACT

A fast screening method for the detection of more than 60 stimulants in urine was developed. The method consisted of a dilution of the urine (1:5 v/v) and analysis by ultraperformance liquid chromatography coupled to tandem mass spectrometry, using a C18 column (1.7 μm particle size), a mobile phase containing deionized water and acetonitrile with formic acid, and gradient elution. The chromatographic run time was 5 min. The detection was performed in positive mode electrospray ionization, monitoring one or two specific ion transitions for each analyte. Appropriate repeatability was obtained, with relative standard deviation (RSD) values below 25% for most of the analytes. Regarding intermediate precision, estimated during routine work, higher RSDs were obtained, probably due to between day differences in the status of the mass spectrometer and in the chromatographic system. Matrix effect ranged from 60 to 255% with RSD lower than 35% for the majority of compounds. Despite the matrix effect observed, the signal/noise ratio of the analytes spiked at 50 ng/mL was greater than three in all tested samples, allowing a correct detection of all substances at the MRPL required by WADA and demonstrating the suitability of the method. The method was tested in administration study samples and satisfactorily in operation for more than one year with routine doping samples. The presence of isomeric stimulants with closely similar chromatographic and/or mass spectrometric properties did not allow the unequivocal identification of these compounds after the first analysis. Different possibilities for separation and identification of isomeric compounds are presented.

INTRODUCTION

The list of prohibited substances and methods of the World Antidoping Agency (WADA) includes more than two hundred pharmacologically and chemically different substances and prohibited methods[1]. Doping control laboratories need to monitor the prohibited substances and/or their metabolites mainly in urine samples. In case of positive results during the initial testing procedure (screening), laboratories apply a second analysis for confirmation purposes. Screening and confirmation analyses have to be performed in a short period of time according to the International Standard for Laboratories[2], specially important during major sports events where 24 h reporting times are needed. Therefore, laboratories are forced to develop high throughput screening and confirmation methods. The use of fast methods has been also a demand in clinical testing for a long time; diagnostic is facilitated by fast analysis, which is important in some emergency cases.

Doping control laboratories use multi-analyte methods to screen for groups of prohibited substances[3]. For long time, screening and confirmation methods were based on gas chromatography coupled to mass spectrometry (GC-MS)[4]. However, at present, liquid chromatography coupled to mass spectrometry (LC-MS) is currently the technique of choice for the screening and confirmation of most of the groups of substances of doping interest[5-24].

Different strategies have been described in order to reduce the complexity of the doping control analyses and the total analysis time. LC-MS, either tandem MS or high resolution MS, allow the comprehensive screening of different groups of substances with a simple sample preparation without the need of specific derivatizations as it was needed for GC-MS based methods[4]. In recent years, the availability of robust, sensitive and reliable MS detectors allows the development of methods where sample preparation and/or purification steps are not needed (direct injection or “dilute and shoot” methods)[18,19,22,24-26]. The direct analysis of conjugated metabolites by LC-MS has also been described to avoid the hydrolysis step during sample preparation[27]. “Dilute and shoot” methods are also especially interesting for compounds with high volatility (e.g.

some compounds of the group of stimulants) that may be lost during the evaporation step of the organic solvent used in liquid-liquid or solid-phase extractions[28].

Another strategy to reduce the analysis time is the use of ultraperformance liquid chromatography (UHPLC) that enables the work with columns packed with small particles (< 2µm). UHPLC is able to produced high resolution by using high linear velocities which results in a reduction of the analysis time. Methods using UHPLC coupled to tandem MS or to high resolution MS have already been described for the screening and confirmation of some doping agents[15,18-20,23,26], demonstrating the reliability of that approach for screening purposes.

Stimulants are a group of substances nowadays frequently analyzed in doping control and analytical toxicology by “dilute and shoot” techniques[18,19,22,24]. They contain an easily-ionizable nitrogen atom and, therefore, the ionization efficiency using electrospray ionization is high. Moreover, although they are metabolized, most of them are excreted in urine in unchanged form in considerable amounts and the detection level required is high[29]. All these factors make “dilute-and-shoot” techniques a good choice for screening analysis of stimulants.

The objective of the work was to develop a fast method to screen for stimulants. A “dilute and shoot” method able to detect more than 60 stimulants by UHPLC-MS/MS is described. Due to isomeric nature of most stimulants, the possibilities for unequivocal identification of the sets of various isomers are also presented.

EXPERIMENTAL

Chemicals and reagents

Standards used were: Levmetamphetamine (Alltech, Barcelona, Spain); nikethamide (Basf, Ludwigshafen, Germany), prolintane (Boehringer Ingelheim, Ingelheim, Germany); strychnine (Bristol-Myers Company, Nova York, USA); methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), methylenedioxymethamphetamine (MDMA) and methylenedioxymethamphetamine d_5 (MDMA d_5) (Cerilliant, Texas, USA); furfenorex, ethylamphetamine and chlorphentermine (Doping Analytical Section, Rigshospitalet, Copenhague); dobutamine (European Pharmacopeia, Strasbourg, France); norephedrine (Impex-quimica, Barcelona, Spain); propylhexedrine (Knoll, Ludwigshafen, Germany); clobenzorex (Laboratorio Llorente, Madrid, Spain); amiphenazole (LGC Standards, Luckenwalde; Germany); norpseudoephedrine (Mack GmbH , Illertissen, Germany); fencamfamine (Merck, Darmstadt, Germany); etaphedrine (Merrel Dow, Horgen, Switzerland); amphetamine (Ministerio de Sanidad y Consumo, Madrid, Spain); crotetamide and carphedon (National Analytical Refrence Laboratory, Pymble, Australia); p-hydroxy-amphetamine, dimethylamphetamine, fencamine, p-methylamphetamine, ortetamine, isometheptene, cyclazodone, cropropamide, famprofazone and fenetylline (NMI Australian Government, Sidney, Australia); selegiline (Orion-Farmos Pharmaceuticals, Espoo, Finland); phentermine (Pfizer, Milan, Italy); mefenorex (Roche, Monza, Italy); benzylpiperazine, pseudoephedrine, tuaminoheptane, methylephedrine, methamphetamine(d-), phendimetrazine, methylhexaneamine, mephentermine, norfenfluramine, fenfluramine, benzphetamine, benfluorex, diphenylamine used as internal standard (ISTD), chloramphetamine, heptaminol, ephedrine, caffeine, fenproporex, pentetrazole and methoxyphenamine (Sigma-Aldrich, St Loiuse, MO, USA); p-hydroxy-metamphetamine, phenmetrazine and pipradol (Toronto Research Chemicals, Toronto, Canada); amfepramone (Uriach, Barcelona, Spain); phenpromethamine, sibutramine, prenylamine, mephedrone, 1,4-dimethylpenthylamine, 2-amino-N-ethylphenylamine and fenbutrazate (World Association of Anti-Doping Scientists).

Acetonitrile (LC gradient grade) was purchased from Merck (Darmstadt, Germany), formic acid (LC/MS grade) and MBTFA (N-methyl-bis-trifluoroacetamide) were purchased from Sigma-Aldrich (St Loiuse, MO, USA). All other solvents and reagents were of analytical grade. Ultrapure water was obteined using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

Standard solutions

Stock standard solutions of 1 mg/mL of the compounds in study were prepared in methanol. Working solutions of 100 and 10 µg/mL were prepared as 1:10 and 1:100 dilutions of the stock solution. All solutions were stored at −20 ºC.

Sample preparation procedures

Preparation for LC-MS/MS

Urine sample (0.5 mL) was centrifuged at 3500 rpm for 5 min. An aliquot of 300 µL was transfered to vial and diluted 1:5 with a mixture of ultrapure water:acetonitrile (99:1, v/v) containing formic acid (1%) and diphenylamine (1 µg/mL) as internal standard (ISTD).

Preparation for GC/MS (underivatized extracts)

A previously described procedure was used[30]. Urine samples (5 mL) were spiked with 25 µL of the ISTD solution (diphenylamine, 1 µg/mL) and were made alkaline with 0.5 mL of 5M KOH. Samples were extracted with tert-butylmethyl eter (2 mL) using salting-out (anhydrous sodium sulphate, 3 g). After agitation (40 mov/min, 20 min) and centrifugation, the organic phase was evaporated to dryness under nitrogen stream in a water bath at 25ºC. The residue was reconstituted with methanol (100 µL) and analyzed by GC/MS.

Preparation for GC/MS (derivatized extracts)

Samples were extracted as indicated above using MDMA D_5, as ISTD (25 µL of a 100 µg/mL solution). The organic phase was evaporated to dryness, reconstituted with 100 µL of MBTFA and derivatized at 60ºC for 30 min in a dry bath. Extracts were analyzed by GC/MS.

UHPLC-MS/MS instrumental conditions

LC-MS/MS analyses were carried out using a triple quadrupole mass spectrometer (Xevo TQ MS) provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters Associates, Milford, MA, USA) coupled to an ultraperformance liquid chromatographic (UPLC) system (Acquity, Waters Associates) for the chromatographic separation. Cone gas as well as desolvation gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. The nitrogen desolvation temperature was set to 450ºC and the source temperature to 150ºC. Argon was used as collision gas. Electrospray in positive ionization mode was used and the capillary voltages was set at 3.5 kV.

Detection was performed in multiple reaction monitoring mode (MRM). Electrospray ionization (ESI) working parameters (cone voltage, collision energy) were optimized for each analyte by direct infusion of individual standard solutions (10 µg/mL) into the mass spectrometer at a flow rate of 10 µL/min with mobile phase (50:50,v:v, water:acetonitrile with 0.01% formic acid) at 200 µL/min**.** Cone voltage, ion transitions and collision energies used for each analyte are listed in Table 1. Interscan times of 3 ms, interchannel delays of 3 ms and dwells time between 5 to 160 ms were applied. Acquisition was optimized to 9-12 data points per peak. MassLynx 4.1 software was used to acquire and process data (Waters Corporation, Milford, MA).

The chromatographic separation was carried out on a Waters Acquity UPLC™ system using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm particle size) (Waters Corporation, Milford, MA). The column temperature was set to 45 ºC. The mobile phase consisted of deionized water with 0.01% formic acid (A) and acetonitrile with 0.01% formic acid (B). Separation was performed

at a flow-rate of 0.6 mL/min with the following gradient conditions: from 0 to 1.2 min, 1% B, increase to 90% B in 2.6 min, 90% B during 0.2 min, decrease to 1% B in 0.1 min and equilibration to initial conditions for 0.90 min. The total run time was 5 min and the sample volume injected was 10 µL.

For the separation of isomeric compounds (Groups A-F, Table 3), four different optimized gradients were used:
Gradient A/B: 0 min 1% B, increase to 3% B in 4.2 min, increase to 90% B in 3.8 min, 90% B for 0.5 min, decrease to 1% B in 0.1 min, and equilibration for 1.4 min.
Gradient C: 1% B for 1min, increase to 15% B in 7 min, increase to 90% B in 1 min, 90% B for 1 min, decrease to 1% B in 0.1 min, and equilibration for 1.9 min.
Gradient D: 5% B for 1.2 min, increase to 90% B in 16.8 min, 90% B for 0.7 min, decrease to 5% B in 0.1 min, and equilibration for 1.2 min.
Gradient E/F: 1% B for 1.2 min, increase to 90% B in 6.8 min, 90%B for 0.7 min, decrease to 1%B in 0.1 min, and equilibration for 1.2 min.

GC/MS instrumental conditions

GC/MS analysis were conducted using a 5890 GC gas chromatograph coupled with a 5973 MS mass spectrometer (Agilent Technologies, California, USA). The instrument was equipped with an autosampler 7673 (Agilent Technologies). The capillary column used was a 5% phenylmethyl silicone (12m length, 0.2mm internal diameter, 0.33µm film thickness) (Agilent Technologies). The carrier gas was helium (flow rate 0.9-1.1 mL/min). The injector and the transfer line temperatures were 280°C. The volume of sample injected was 3 µL in split mode (1:10-1:15).

Different temperature programs were applied to achieve the separation of the isomeric compounds:
Ramp 1: initial temperature was kept at 110°C during 14 min, increasing to 280ºC at 20°C/min and held at 280ºC for 4 min.

Ramp 2: initial temperature was set at 90°C, increasing to 300ºC at 20°C/min and held at 300ºC for 4 min.
Ramp 3: initial temperature was set at 90°C, increasing to 290ºC at 20°C/min and held at 290ºC for 5 min.
The analyses were performed in scan mode (m/z 50 to 600 and 40-400) and the characteristic ions for each compounds are listed in Table 3.

Validation study

The method was validated for qualitative purposes according to a previously described protocol[31].

Selectivity and specificity were studied by the analysis of 50 different blank urine samples. The absence of any interfering substance at the retention time of the compounds of interest and the internal standard was verified.

The detectability of the analytes was evaluated by analysis of six replicates of blank urine samples spiked with the analytes at 50 ng/mL, corresponding to 50% of the minimum required performance limits (MRPL) defined by WADA[29].

Intra-assay precision was assessed by analysis of six replicates of blank urine samples spiked at two different concentrations 50 and 100 ng/mL (low and high quality control samples, respectively) on the same day. Intermediate precision was estimated by analysis of one replicate of a quality control sample spiked with a selection of compounds, on six different days. The precision was expressed as the relative standard deviation (RSD) of the ratio between the areas of the compound and the ISTD.

Matrix effect was evaluated by the analysis of six different urines spiked at a concentration of 50 ng/mL of each compound. The areas of the analytes in these samples were compared with the area obtained after the analysis of water spiked at the same concentration.

The carry over between samples was assessed by analysis of a blank sample just after analysis of a sample spiked with the analytes at high concentration.

Analysis of authentic urine samples

The screening method was applied to urine samples obtained in excretion studies involving the administration of single oral doses of the compounds to healthy volunteers. The clinical protocol was approved by the local Ethical Committee (CEIC-IMAS, Barcelona, Spain). Urine samples were collected before administration and up to 24 h after administration at different collection periods. The following compounds were studied: amphetamine, benfluorex, clobenzorex, ephedrine, etaphedrine, fencanfamine, fenfluramine, fenproporex, heptaminol, isometheptene, MDMA, mephedrone, mefenorex, methamphetamine, methylhexaneamine, methoxyphenamine, niketamide, phentermine, pipradol, prolintane and pseudoephedrine.

The method was also applied to routine anti-doping control for a period of one year with analysis of more than 3000 samples

RESULTS AND DISCUSSION

Method development

The compounds studied are nitrogen containing compounds and most of them bear a phenylalkylamine structure (**Table 1**). For that reason, they were easily ionized in positive mode ESI, specially using the mobile phase of water and acetonitrile slightly acidified with formic acid. Most of the compounds formed the protonated molecule $[M+H]^+$. In source fragmentation was observed for pipradol, where the ion resulting from dehydratation of the protonated molecule, $[M+H-H_2O]^+$, was the most abundant specie (**Table 1**).

ESI working parameters are listed in **Table 1**. In general, low cone voltages and collision energies were used due to the molecular size of the compounds and the stability of the product ions formed. Due to similarities in structures among the compounds of the group, similar fragmentation routes and product ions were also obtained. Different isomeric groups are observed, and they are indicated in **Table 1**. The product ion mass spectra of the compounds included in each isomeric group are presented in **Figure 1**.

Data acquisition for analysis of urine samples was performed in MRM mode**.** For screening purposes, one ion transition was used to monitor most of the compounds. However, for some compounds two ion transitions were measured to increase specificity. A total of 72 ion transitions were measured with time windows of 0.3-0.6 min around the expected retention time of the compound **(Table 1)**. The dwell times were automatically adjusted by the instrument software to obtain between 9 and 12 data points per peak, allowing an adequate definition of the chromatographic peaks.

Regarding chromatographic conditions, apart from formic acid, no other mobile phase additives were needed either to promote ionization or to improve chromatographic behavior of the studied compounds. The gradient was optimized to provide a reasonable short chromatographic time allowing for the analysis 63 compounds in only 5 min (**Table 1**). Taking into account the sample preparation used, the chromatographic gradient was optimized to elute polar

matrix compounds (e.g. salts) before elution of the analytes in order to avoid problems due to matrix effects. For that reason, the initial percentage of organic solvent was very low, 1%. For the same reason, although not needed for chromatographic separation, the final content of the organic solvent was increased to 90% in order to have a good clean-up of the chromatographic column after each sample analysis.

Due to the high ionization efficiency obtained for the majority of compounds, a simple dilution of the urine was studied as a sample preparation. Due to the complexity of the urine matrix, it is expected that urine dilution results in significant matrix effects produced by urinary interfering compounds that co-elute with the analytes and interfere with ionization. A dilution factor of 5 was selected as a compromise between sensitivity and matrix effects. The composition of the solvent used for dilution was optimized to obtain adequate signal intensities and chromatographic peak shapes for all compounds. Splitted peaks were obtained for some analytes due to the pH environment and the fact that the amines are in equilibrium with their respective conjugated acids. In order to avoid them, the diluting solution was acidified to favor one of the species. Trifluoroacetic acid (TFA) and formic acid were evaluated, and results obtained were similar for both modifiers. Taking into account that TFA may produce problems of contamination in the instrument, formic acid was selected. Different percentages of formic acid in the dilution solvent were studied (0.1%, 1% and 5%). Improvement of peak shape was observed as the percentage of acid increased, especially for some analytes. However, high percentages of acid may also result in contamination problems in the instrument. Therefore, 1% formic acid was selected as a compromise. Finally, the percentage of organic modifier in the dilution solvent was also studied. A mixture containing the same proportion of water and acetonitrile as the initial composition of the mobile phase (99:1,v/v) was selected, that offered optima signals and peak shapes, especially for some compounds (e.g., p-hydroxy-amphetamine).

Validation of the screening method

The screening method was validated for qualitative purposes for the majority of compounds. A summary of the validation results is presented in **Table 2**.

Selectivity and specificity were evaluated by analysis of fifty blank urine samples. The method showed good selectivity and specificity with no interfering peaks. Some interfering peaks were observed close to the expected retention time of some analytes, and two characteristic ion transitions were monitored to improve selectivity in these cases (see **Table 1**). Some isomeric compounds were not chromatographically separated (**Table 1**, **Figure 1**) and unequivocal identification was not possible in the screening step. The identification of isomeric compounds is described in the specific chapter.

The fulfillment of the WADA criteria regarding the detectability of the analytes was verified by analysis of samples spiked at 50% of the MRPL[29]. For all analytes, a signal/noise ratio greater than 3 was obtained, indicating that the limit of detection is lower than 50 ng/mL for all analytes. .

Moreover, the detectability was evaluated using six different urine samples to study the matrix effect. Matrix effect was observed for most of the compounds, either decreasing or increasing the response, and ranged from 60 to 255% with RSD lower than 35% for the majority of compounds, indicating reproducibility between urine samples (**Table 2**). The presence of matrix effect was expected, taking into consideration the simple sample preparation and the fast chromatographic analysis. It is accepted that the matrix effect in qualitative analysis is less critical mainly for analytes showing high ionization efficiency, as it is the case of stimulants, and the matrix effect is evaluated to ensure that ion suppression does not make the analyte undetectable at the required concentration level in a specific urine [29]. Despite the matrix effect observed in our method, the signal/noise ratio of the analytes spiked at 50 ng/mL was in all samples tested greater than 3, allowing a correct detection of all substances at the MRPL required by WADA and demonstrating the suitability of the method.

Intra-assay precision (n=6) was calculated by analysis of quality control samples at two concentrations levels, 50 and 100 ng/mL (**Table 2**). Appropriate

repeatability was obtained, with RSD values within the range 0.7-27.1% and 0.3-23.1% for the low and high quality control samples, respectively. Regarding intermediate precision, estimated for a selection of analytes during routine work, higher RSDs were obtained. These high RSDs obtained for some analytes are probably due to differences between days in the daily status of the mass spectrometer and of the chromatographic system. Small differences in chromatographic analysis can produce distinct elution of matrix interferences and, in consequence, in matrix effects on the analytes. So, taking into account that the methodology proposed is used as screening tool and all the analytes are detected at the level required the high values of intermediate precision are not considered a drawback.

Carry over between samples was also studied. No signal was observed in blank samples analyzed after samples spiked with high concentrations of the compounds.

As a final validation, administration study samples were analysed. The method was able to detect the administration of amphetamine, benfluorex, clobenzorex, etaphedrine, fencanfamine, fenfluramine, fenproporex, heptaminol, isometheptene, MDMA, mephedrone, mefenorex, methamphetamine, methylhexaneamine, methoxyphenamine, niketamide, phentermine, pipradol, prolintane, ephedrine and pseudoephedrine in samples collected up to 24h after intake of a single oral dose. As examples in **Figure 2**, chromatograms of blank urine samples are compared with those obtained for samples collected after administration of clobenzorex, fenfluramine, heptaminol, niketamide and benfluorex. For some of the compounds, the monitoring of metabolites in addition to the unchanged drugs helps in the detection of positive urines. As can be seen in **Figure 2**, after administration of clobenzorex, peaks of the parent drug and the metabolites, p-hydroxy-amphetamine and amphetamine, are observed in urine. For fenfluramine, the metabolite norfenfluramine is also detected. In these regards, the future incorporation of phase II metabolites (glucuronides or sulphates), not limited by the sample preparation step, may considerable improve the detection of some prohibited stimulants[27,32]

In order to evaluate the reliability for routine work, the described method was applied to the analysis of authentic doping control routine samples received in the laboratory for more than one year. In our hands, the system demonstrated to be stable and reliable with simple maintenance operations. Moreover, the method was subjected to the external quality control of WADA for a period of one year with successful results in the identification of positive samples to prolintane, isometheptene, cyclazodone, norpseudoephedrine, ephedrine and pseudoephedrine.

Identification of isomeric stimulants

As mentioned before, there were some isomeric groups including compounds with the same ion transitions and very close retention time in the conditions of the screening analysis (**Table 1**). These isomeric groups are indicated as A, B, C, D, E and F in **Table 1**, and their structures and product ion mass spectra can be seen in **Figure 1**. Alternative approaches have to be used for identification of a presumptive analytical finding. The procedures available to identify isomeric compounds are listed in **Table 3**.

The first possibility is the reanalysis of the samples using LC specific gradient for the separation of each isomeric group, and specific MS/MS methods including characteristic ion transitions (**Table 3**). In this study, compounds within all isomeric groups were separated using improved chromatographic gradients, and the separations achieved are shown in **Figure 3**. As an example, for the identification of dimethylamphetamine, ethylamphetamine and mephentermine (group F), in addition to the use of a specific gradient elution (gradient F), the presence of the product ion at m/z 133 in the mass spectra of the precursor m/z 163 helps in the identification of the compound as mephentermine, as the product ion is not present in the mass spectra of the other two compounds (see **Figures 1 and 3**).

Another possibility to identify isomeric compounds is the use of GC-MS analysis, without or with derivatization (**Table 3**). In that case, the urine sample

has to be subjected to an extraction procedure before the chromatographic analysis (see experimental section). Using GC-MS without derivatization, identification of different isomeric groups can be easily achieved because of the different retention times and also different electron ionization mass spectra (see groups C and E). The separation of the diastereomeric pairs norephedrine/norpseudoephedrine (group A) and ephedrine/pseudoephedrine (group B) can be accomplished by GC/MS using a very slow temperature program (ramp 1).

Using GC-MS as TFA-derivatives, some isomeric compounds can be also distinguished because of the different retention times and electron ionization mass spectra (see groups C, E and F)[33].

The method developed, not being a chiral oriented analysis, does not separate the d- and l-enantiomers of amphetamine and metamphetamine. For these enantiomeric compounds (group C), a chiral separation, such as the diastereomeric derivatization with l-N- trifluoroacetyl-1-prolyl chloride[34] should be carried out.

CONCLUSIONS

A fast screening method based on UHPLC-MS/MS analysis was developed and validated for the simultaneous detection of 63 stimulants in urine at the concentration levels required by WADA. Neither extraction nor evaporation is required for processing the urine sample, which minimizes the loss of volatile analytes during the sample preparation.

The existence of isomeric stimulants with close retention times and/or similar ion transitions did not allow the unequivocal identification of these compounds in the initial testing procedure. Different possibilities for separation and identification of isomeric compounds were proposed, including the use of improved chromatographic gradients or the analysis by GC-MS without or with derivatization.

As a result of simple sample preparation and short LC-MS/MS analysis, a fast turn-around time is achieved. These features make the developed method highly interesting for routine anti-doping purposes.

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Table 1. Compounds included in the screening procedure: monoisotopic mass (MM), retention times (RT), relative retention times (RRT), precursor ion (PI), product ion (DI), cone voltage (CV) and collision energy (CE). Isomeric group indicates those compounds with similar retention time and product ion mass spectra.

Analyte	MM (Da)	RT (min)	RRT	PI (m/z)	DI (m/z)	CV (V)	CE (eV)	Isomeric group
p-hydroxy-amphetamine	151.10	1.82	0.49	152	135	15	10	-
p-hydroxy-metamphetamine	165.12	1.94	0.53	166	135/107	20	10/15	-
Heptaminol	145.15	1.99	0.54	146	128	15	10	-
Benzylpiperazine	176.13	2.03	0.55	177	91	25	20	-
Norpseudoephedrine	151.11	2.04	0.55	152	134	10	5	A
Norephedrine	151.11	2.04	0.55	152	134	10	5	A
Amiphenazole	191.05	2.10	0.57	192	117/106	30	25	-
Ephedrine	165.12	2.14	0.58	166	148	10	10	B
Pseudoephedrine	165.12	2.15	0.58	166	148	10	10	B
Methylephedrine	179.13	2.19	0.59	180	162	30	10	-
Metamphetamine (d/l)	149.12	2.21	0.60	150	119/91	20	10/15	C
Phenprometamine	149.12	2.21	0.60	150	119/91	20	10/15	C
Phenmetrazine	177.12	2.22	0.60	178	115	30	35	-
Amphetamine	135.10	2.23	0.60	136	119/91	15	10/15	-
Etaphedrine	193.15	2.23	0.60	194	176/60	25	15/20	-
MDA	179.09	2.24	0.61	180	163	15	10	-
Phendimetrazine	191.13	2.25	0.61	192	146/117	32	25	-
Caffeine	194.08	2.27	0.62	195	138	35	25	-
Amfepramone	205.15	2.28	0.62	206	105	30	20	-
Methylhexaneamine	115.14	2.29	0.62	116	57	15	10	D
Phentermine	149.12	2.29	0.62	150	133/105	15	10/15	E
MDMA	193.11	2.29	0.62	194	163	20	15	-
Dobutamine	301.17	2.30	0.62	302	107	30	20	-
Fenproporex	188.13	2.31	0.63	189	119	15	10	-
Strychnine	334.17	2.31	0.63	335	184	35	40	-
Mephentermine	163.14	2.32	0.63	164	133/91	22	15	F
Dimethylamphetamine	163.14	2.32	0.63	164	119/91	22	15	F
Tuaminoheptane	115.14	2.34	0.63	116	57	15	10	D
Pentetrazole	138.09	2.35	0.64	139	96/69	25	15	-
Ethylamphetamine	163.14	2.36	0.64	164	119/91	20	15	F
Methoxyphenamine	179.13	2.36	0.64	180	149	20	15	-
MDEA	207.13	2.36	0.64	208	163	20	15	-
Fencamine	384.23	2.36	0.64	385	236	35	20	-
p-methylamphetamine	149.12	2.37	0.64	150	133/105	15	10	E
Ortetamine	149.12	2.39	0.65	150	133/105	10	10/15	E
1,4-dimethylpenthylamine	115.13	2.39	0.65	116	57	15	10	D
Mephedrone	177.11	2.39	0.65	178	145	25	20	-
Isometheptene	141.15	2.41	0.65	142	69	20	15	-
Nikethamide	178.11	2.42	0.66	179	108	20	20	-
Selegiline	187.14	2.43	0.66	188	91	20	15	-
Fenetylline	341.19	2.43	0.66	342	207	30	25	-
2-amino-N-ethylphenylamine	177.15	2.43	0.66	178	133	20	15	-
Chloramphetamine	169.07	2.47	0.67	170	153	15	10	-

Norfenfluramine	203.09	2.52	0.68	204	187	20	10	-
Furfenorex	229.16	2.53	0.69	230	148/81	20	15	-
Chlorphentermine	183.08	2.54	0.69	184	167/125	15	10/15	-
Carphedon	218.11	2.54	0.69	219	202/174	15	10/15	-
Mefenorex	211.11	2.56	0.69	212	119	22	15	-
Propylhexedrine	155.17	2.59	0.70	156	69	20	15	-
Pipradol*	267.16	2.61	0.71	250	130	40	30	-
Fencamfamine	215.17	2.65	0.72	216	171	20	15	-
Benzphetamine	239.17	2.66	0.72	240	119/91	25	15/20	-
Prolintane	217.18	2.68	0.73	218	105/91	30	20	-
Fenfluramine	231.12	2.68	0.73	232	159	25	20	-
Cyclazodone	216.09	2.72	0.74	217	146	20	15	-
Clobenzorex	259.11	2.72	0.74	260	91	25	15	-
Crotetamide	226.17	2.74	0.74	227	182/86	5	5	-
Cropropamide	240.18	2.95	0.80	241	196	10	5	-
Sibutramine	279.18	2.96	0.80	280	139/125	25	15	-
Benfluorex	351.14	2.97	0.80	352	230	25	20	-
Prenylamine	329.21	3.04	0.82	330	212	30	20	-
Famprofazone	377.25	3.07	0.83	378	229	30	20	-
Fenbutrazate	367.21	3.08	0.83	368	204	30	20	-
ISTD	169.09	3.69	1.00	170	93	35	25	-

*The precursor ion for pipradol is $[M+H-H2O]^+$.

Table 2. Validation results: intraday and intermediate precisions, and matrix effect.

Analyte	Intraday precisions (n= 6)		Intermediate precision (n=6)		Matrix effect	
	Conc (50 ng/mL)	Conc (100 ng/mL)				
	RSD (%)	RSD (%)	Conc (ng/mL)	RSD (%)	mean (%)	RSD(%)
Amfepramone	4.0	5.6	100	36.8	-	-
Amiphenazole	6.0	2.9	100	31.8	71.3	56.7
Amphetamine	5.6	3.7	100	53.5	-	-
Benfluorex	0.7	0.3	100	10.2	122.2	28.8
Benzphetamine	2.5	0.5	100	6.5	106.7	29.3
Benzylpiperazine	3.5	4.8	100	24.0	-	-
Caffeine	1.4	11.7	100	11.3	-	-
Carphedon	26.0	8.7	100	34.3	254.9	26.4
Chloramphetamine	10.3	9.7	-	-	-	-
Chlorphentermine	9.7	21.9	-	-	110.9	30.4
Clobenzorex	5.4	5.8	100	49.2	107.1	31.4
Cropropamide	6.1	10.2	100	48.6	107.2	24.0
Crotetamide	2.9	4.7	100	31.8	112.9	6.8
Cyclazodone	25.0	1.9	100	9.5	181.7	48.3
Dimethylamphetamine	13.4	6.6	-	-	-	-
Dobutamine	16.1	7.6	100	39.0	111.0	31.7
Ephedrine	14.0	8.0	1000	31.7	99.9	20.7
Etaphedrine	6.1	2.3	100	69.4	97.3	26.0
Ethylamphetamine	12.2	11.6	100	8.3	126.5	32.9
Famprofazone	3.0	8.5	-	-	99.8	9.5
Fenbutrazate	5.1	4.2	100	12.1	119.3	38.3
Fencamfamin	10.6	4.5	100	12.1	-	-
Fencamine	15.6	22.6	100	78.5	87.6	28.3
Fenetylline	12.8	10.9	100	49.8	-	-
Fenfluramine	11.8	4.4	100	16.4	118.4	8.4
Fenproporex	13.6	7.4	100	59.6	100.1	35.6
Furfenorex	22.7	7.6	100	64.1	118.7	40.2
Heptaminol	13.1	7.7	-	-	-	-
Isometheptene	4.6	1.2	100	10.7	97.7	12.0
MDA	1.0	5.2	-	-	-	-
MDEA	20.7	4.3	100	45.8	102.3	17.4
MDMA	14.4	7.2	100	38.5	119.6	17.4
Mefenorex	17.2	3.8	100	21.6	117.3	18.9
Mephentermine	10.4	6.2	100	11.1	-	-
Methamphetamine(d-)	3.7	3.5	100	42.0	94.1	13.1
Methamphetamine(l-)	13.6	7.1	-	-	-	-
Methoxyphenamine	7.0	6.3	100	55.0	-	-
Methylephedrine	8.8	7.1	1000	58.5	118.6	22.9
Methylhexaneamine	7.8	6.2	100	40.0	128.2	22.5
Nikethamide	17.7	2.6	100	21.0	90.0	17.4
Norfenfluramine	10.3	6.2	100	66.1	72.2	33.0
Norpseudoephedrine	2.2	5.5	1000	59.0	127.6	21.4
Norephedrine	9.2	19.6	-	-	-	-
Pentetrazole	1.3	1.3	-	-	-	-
Phendimetrazine	4.2	6.0	-	-	-	-
Phenmetrazine	10.8	9.9	100	9.2	99.1	12.7
Phenpromethamine	2.9	6.3	-	-	-	-
Ortetamine	6.0	4.0	-	-	-	-
Phentermine	13.9	6.1	100	61.2	59.5	44.6
Pipradol	4.0	6.1	100	23.8	-	-
p-Methylamphetamine	18.0	15.6	100	51.8	-	-
p-hydroxy-amphetamine	27.1	6.3	100	68.8	82.5	19.8
p-hydroxy-metamphetamine	16.6	3.8	100	-	149.0	13.9
Prenylamine	2.1	1.0	100	17.6	185.9	21.9
Prolintane	13.3	5.6	100	10.4	109.6	6.5
Propylhexedrine	3.9	4.6	100	52.4	89.8	25.2
Pseudoephedrine	4.7	7.2	-	-	-	-
Selegiline	3.0	4.0	-	-	-	-
Sibutramine	5.1	3.4	100	7.5	-	-
Strychnine	13.8	22.1	100	15.2	-	-
Tuaminoheptane	6.4	15.4	100	32.3	78.3	39.2
1,4-dimethylpenthylamine	17.7	23.1	-	-	-	-

Table 3. Separation conditions by LC-MS/MS and GC-MS for the identification of the compounds of the diferent isomeric groups. For each condition, retention time (RT) and characteristic ion transitions or m/z of the analytes.

Group	Identification of isomers	Compound	LC-MS/MS		GC/MS underivatized		GC/MS N-TFAderivatives	
			RT (min)	Transitions	RT (min)	m/z	RT (min)	m/z
A/B	LC-MS/MS (Gradient A/B) GC-MS underivatized (Ramp 1)	Norpseudoephedrine	2.57	152>134;117	3.52	44,77,105	-	-
		Norephedrine	2.24	152>134;117	3.59	44,77,105	-	-
		Ephedrine	3.30	166>148;133	4.56	58,77,105	-	-
		Pseudoephedrine	3.70	166>148;133	4.66	58,77,105	-	-
C	LC-MS/MS (Gradient C) GC-MS underivatized (Ramp 2) GC-MS-N-TFA derivatives (Ramp 3) GC-MS chiral separation[a]	Metamphetamine(d)	4.56	150>119;91	1.82	58,91,134	2.98	91,110,118,154
		Metamphetamine (l)	4.56	150>119;91	1.82	58,91,134	2.97	91,110,118,154
		Phenprometamine	4.56	150>119;91	1.86	44,77,91	2.91	105,118,140
D	LC-MS/MS (Gradient D)	Methylhexaneamine	2.76	116>57;43	n.a[b]	n.a	n.a	n.a
		Tuaminoheptane	3.14	116>57;43	n.a.	n.a.	n.a.	n.a
		1,4-dimethylpenthylamine	2.94	116>57;43	n.a.	n.a.	n.a	n.a
E	LC-MS/MS (Gradient E) GC-MS underivatized (Ramp 2) GC-MS N-TFA derivatives (Ramp 3)	Phentermine	2.86	150>133;105	1.72	58,91,134	2.42	91,114,132,154
		p-methylamphetamine	3.09	150>133;105	2.08	44,77,91,105	2.85	105,132,140
		Ortetamine	3.00	150>133;105	2.12	44,77,91,105	2.88	105,132,140
F	LC-MS/MS (Gradient F) GC-MS underivatized (Ramp 2) GC-MS N-TFA derivatives (Ramp 3)	Dimethylamphetamine	2.80	164>119;91	2.19	72,91,148	2.30[c]	72,91
		Ethylamphetamine	2.92	164>119;91	2.11	44,72,91,148	3.22	91,118,140,168
		Mephentermine	2.96	164>133;91	2.25	72,91,148	3.15[d]	91,110,168
ISTD		Diphenylamine (Gradient A/B)	7.38	170>93	-	-	-	-
		Diphenylamine (Gradient C)	9.31	170>93	-	-	-	-
		Diphenylamine (Gradient D)	10.11	170>93	-	-	-	-
		Diphenylamine (Gradient E/F)	5.99	170>93	-	-	-	-
		Diphenylamine (Ramp 1)	-	-	15.80	169	-	-
		Diphenylamine (Ramp 2)	-	-	4.46	169	-	-

		MDMA D_5 (Ramp 3)	-	-	-	-	4.95	158

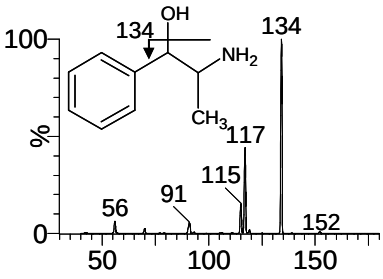
[a]Chiral separation of d- and l-metamphetamine (see reference 34), [b] not analyzed, [c] underivatized, [d]partially derivatized. Most abundant ions are underlined.

Figure 1. Chemical structures, product ion mass spectra and fragmentation pattern of isomeric estimulants.

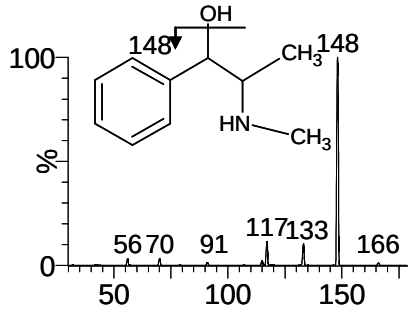
Figure 2. LC-MS/MS results of urine samples obtained after administration of clobenzorex, fenfluramine, heptaminol, nikethamide and benfluorex. Chomatograms of the characteristic ion trasitions of the analytes obtained after analysis of a blank sample (up), and after analysis of a post administration sample (down). Results of the different metabolites detected are shown for clobenzorex and fenfluramine (A, B).

Figure 3. Chromatograms using specific chromatographic gradients for the separation of isomeric compounds.

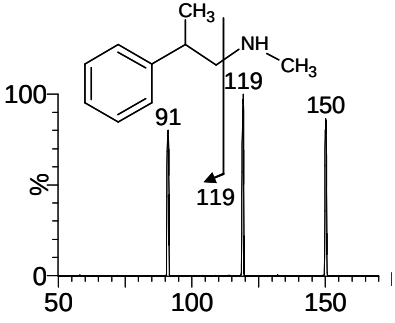
A. Norephedrine
Norpseudoephedrine



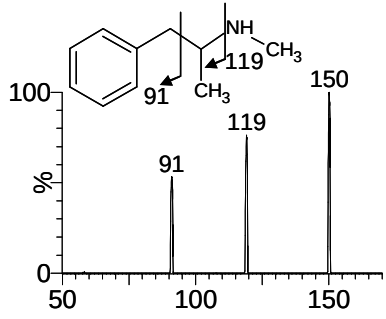
B. Ephedrine
Pseudoephedrine



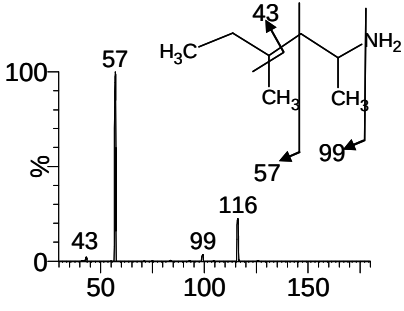
C. Phenprometamine



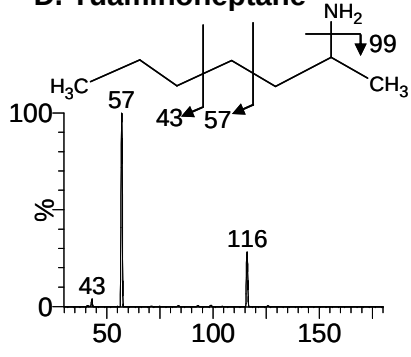
C. Metamphetamine (d/l)



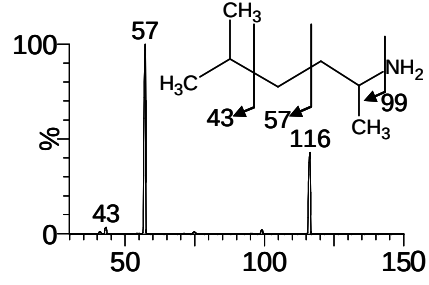
D.Methylhexaneamine



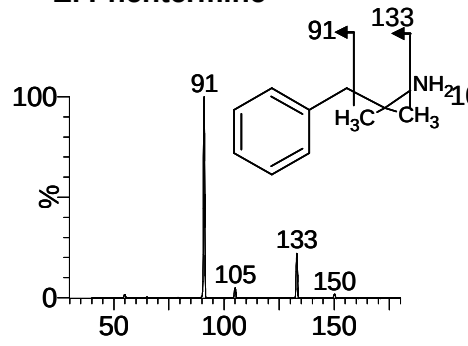
D. Tuaminoheptane



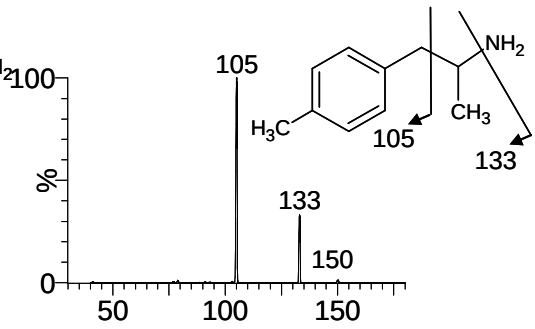
D. 1,4-dimethylpenthylamine



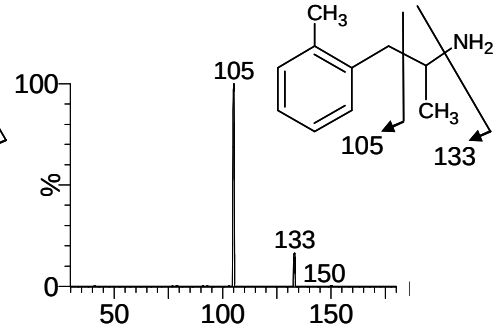
E. Phentermine



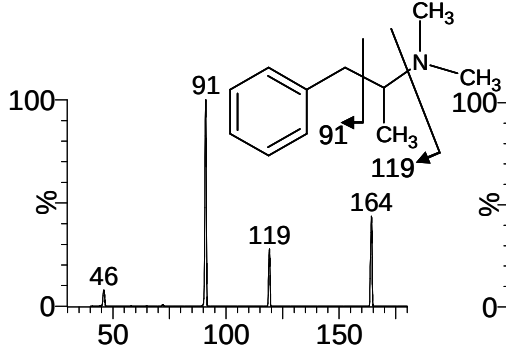
E. P-methylamphetamine



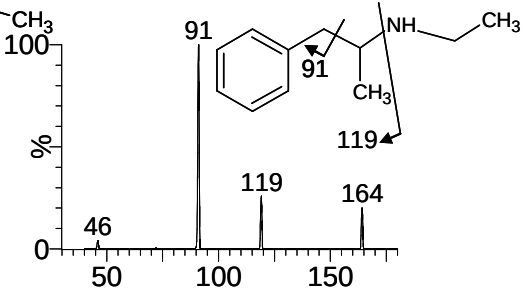
E. Ortetamine



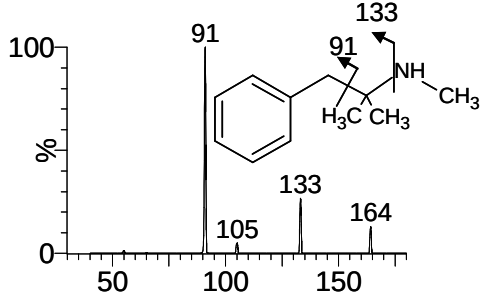
F. Dimethylamphetamine

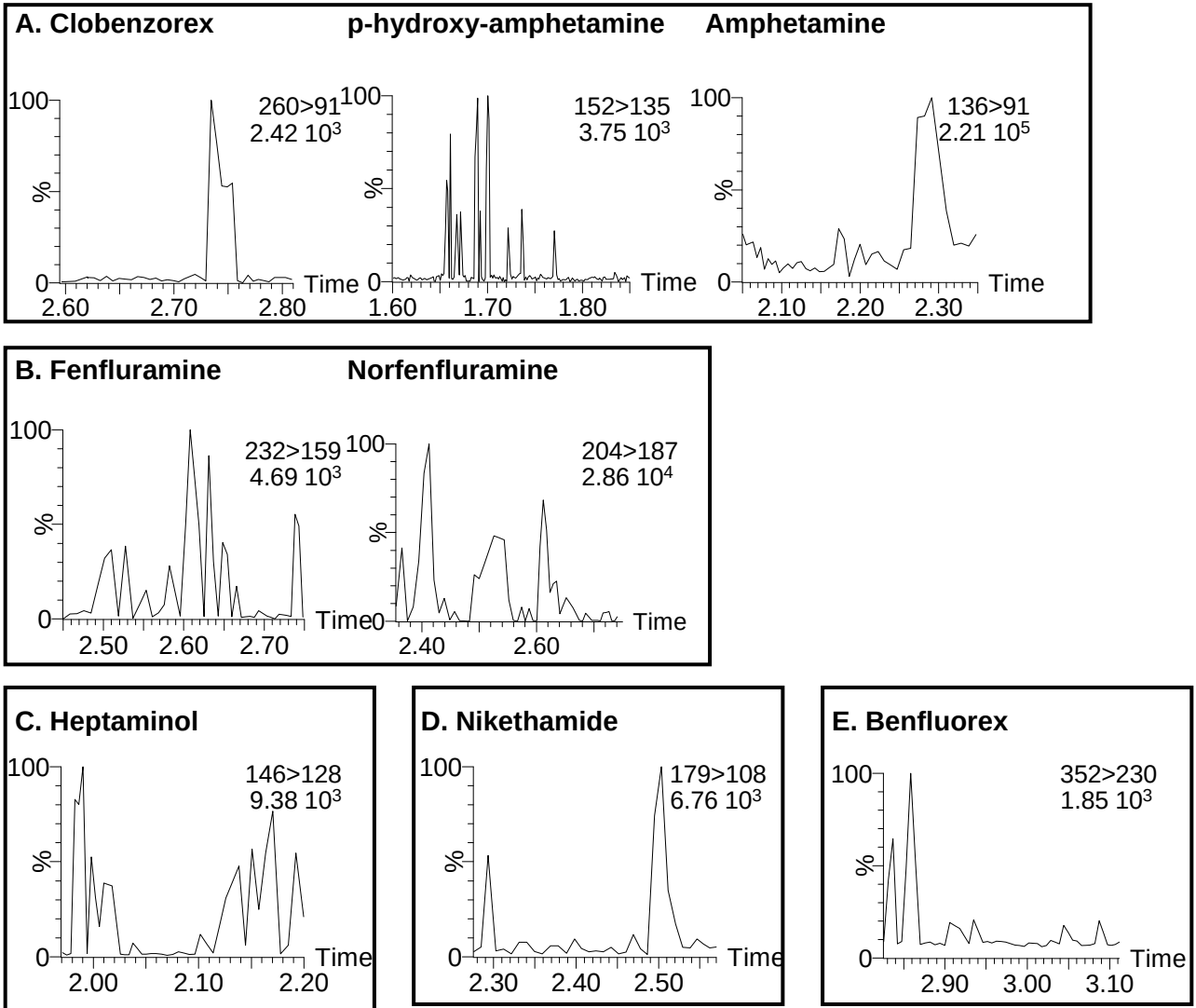


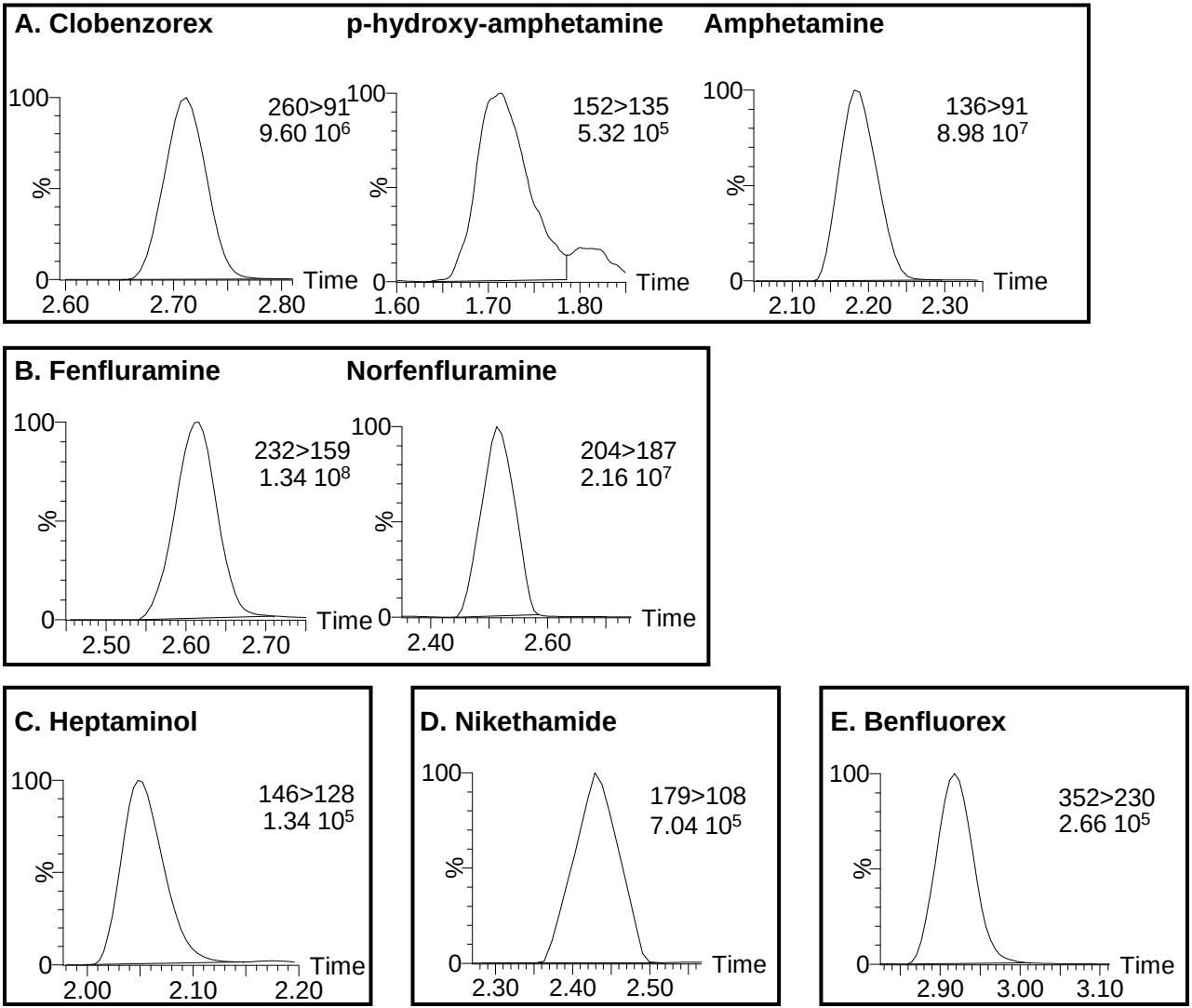
F. Ethylamphetamine

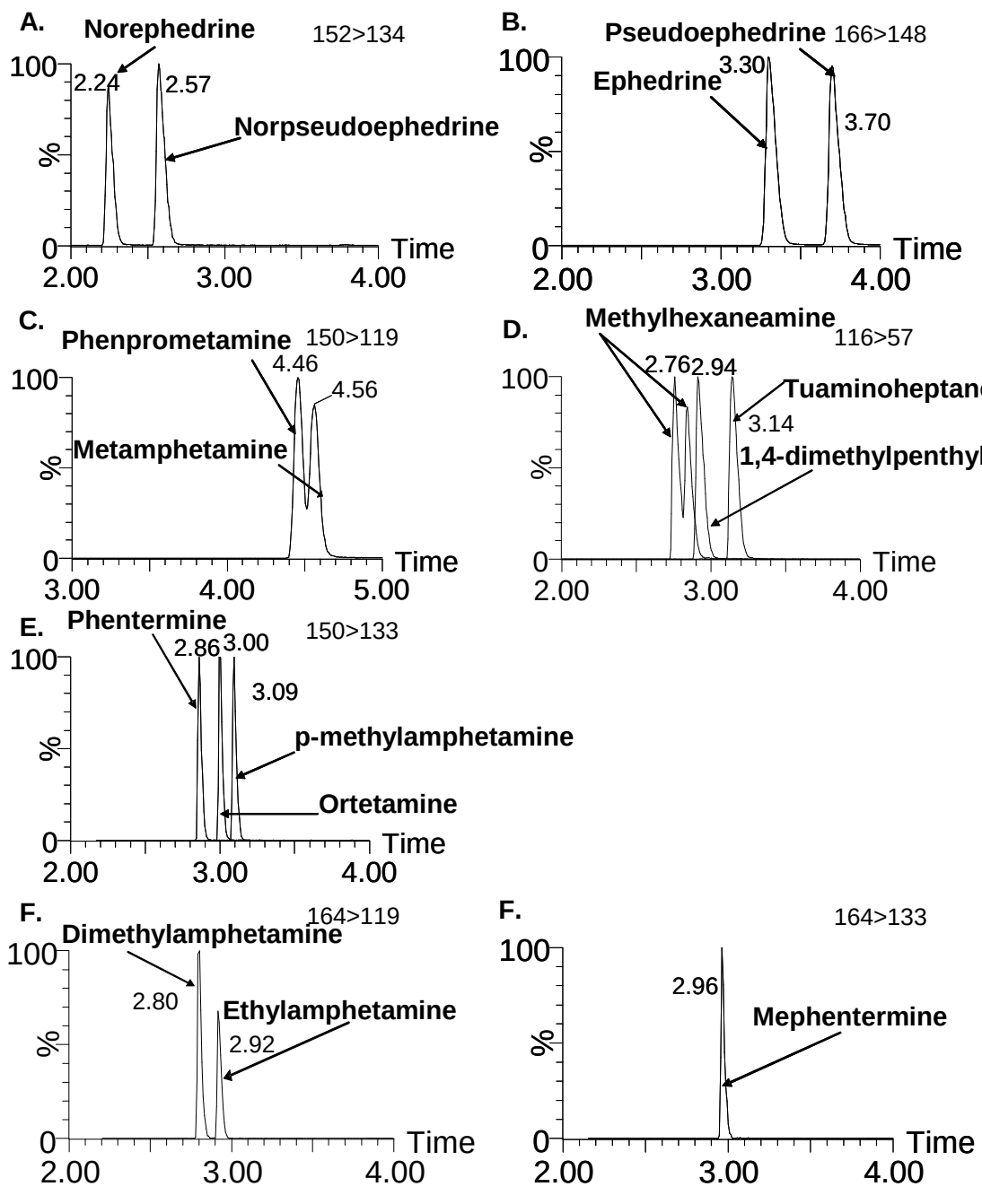


F. Mephentermine




A. Clobenzorex
p-hydroxy-amphetamine
Amphetamine
100
%
0
260>91
2.42 10^3
Time
2.60
2.70
2.80
152>135
3.75 10^3
1.60
1.70
1.80
136>91
2.21 10^5
2.10
2.20
2.30
B. Fenfluramine
Norfenfluramine
232>159
4.69 10^3
2.50
2.60
2.70
204>187
2.86 10^4
2.40
C. Heptaminol
146>128
9.38 10^3
D. Nikethamide
179>108
6.76 10^3
E. Benfluorex
352>230
1.85 10^3
2.90
3.00
3.10


A. Clobenzorex
260>91
9.60 10^6
p-hydroxy-amphetamine
152>135
5.32 10^5
Amphetamine
136>91
8.98 10^7
B. Fenfluramine
232>159
1.34 10^8
Norfenfluramine
204>187
2.16 10^7
C. Heptaminol
146>128
1.34 10^5
D. Nikethamide
179>108
7.04 10^5
E. Benfluorex
352>230
2.66 10^5
100
%
0
Time


A.
Norephedrine
152>134
2.24
2.57
Norpseudoephedrine
B.
Pseudoephedrine
166>148
Ephedrine
3.30
3.70
C.
Phenprometamine
150>119
4.46
4.56
Metamphetamine
D.
Methylhexaneamine
116>57
2.76
2.94
Tuaminoheptane
3.14
1,4-dimethylpenthylamine
E.
Phentermine
150>133
2.86
3.00
3.09
p-methylamphetamine
Ortetamine
F.
Dimethylamphetamine
164>119
2.80
Ethylamphetamine
2.92
F.
164>133
2.96
Mephentermine
100
%
0
Time
2.00
3.00
4.00
5.00