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Stereoselective confirmation of amines by chiral derivatization and UPLC-MS/MS

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Abstract

In doping control, a urine sample with adverse analytical finding from a screening method has to be confirmed by separate analysis. The laboratories should be capable of separating L and D-enantiomers of amphetamine and methamphetamine, e.g. in order to distinguish between prescribed use of selegelin and illegal use of methamphetamine.

A new method has been developed to separate the enantiomeric forms (D and L) of amphetamine and methylamphetamine.

The method utilizes the chiral reagent, (S-) *N*-(trifluoracetyl)-prolyl-chloride, to convert the amines to diastereomers, and the stable derivatives can be separated on traditional reversed phase columns. Extracted and derivatized samples are injected to a UPLC-MS/MS system and the sensitivity of the method is below the ppb level (< 1 ng/ml).

Introduction

According to the WADA prohibited list [1] the laboratories should be capable of separating L- and D-enantiomers of amphetamine and methylamphetamine, e.g. in order to distinguish between prescribed use of selegeline and illegal use of methamphetamine. Enantiomers are mirror image compounds that contain one chiral carbon with the same physical/chemical properties, but different biological effects [2]. By converting the enantiomers, with a chiral derivatization agent, to diasteriomers, (Fig. 1) a chromatographic separation can be performed without the need of a chiral column. The method was originally developed for GC-MS analysis and further transferred to UPLC-MS/MS.

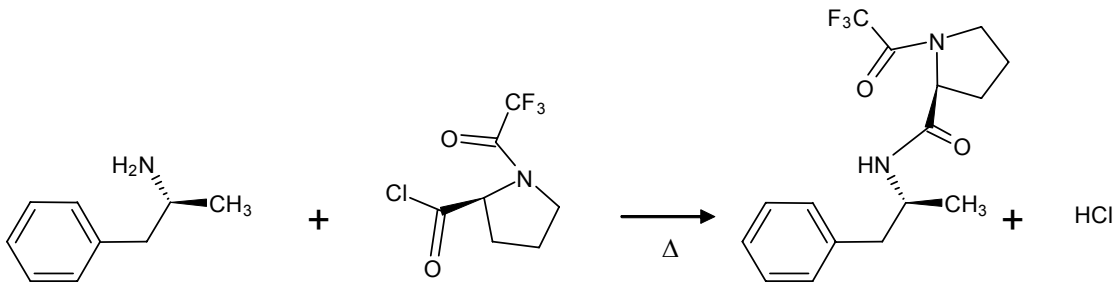

Figure 1. Schematic of derivatization mechanism

Materials and Methods

Chemicals

Amphetamine-D8, amphetamine (D and L) and methylamphetamine (D and racemic) were purchased from Cerilliant (Round Rock, Texas USA). Butylacetate (BuOAc), methanol (MeOH) and methyl *tert.*-butyl methyl ether (TBME) were obtained from Fisher Scientific (Leicestershire, UK). Ammonium acetate (NH_4Ac), formic acid (HCOOH) and sodium hydroxide were purchased from Merck KGaA (Darmstadt,Germany).
(S-) *N*-(trifluoracetyl)-prolyl-chloride (TFAP-Cl) was obtained from Sigma Aldrich (ST Louis, Missouri USA).

Preparation of standard stock solutions

Three stock solutions were prepared in methanol containing; 1) internal standard (IS) 100 ng/ml amphetamine-D8, 2) racemate reference (DL-ref; D- and L-forms of amphetamine and methamphetamine, à 1000 ng/ml) and 3) L-form reference (L-ref; L-forms of amphetamine and methamphetamine, à 1000 ng/ml).

Preparation of calibrators

Two calibrator solutions (50 ng/ml) were prepared by diluting 50 µl DL-ref and 50µl L-ref stock solutions, respectively, to 1 ml with blank urine.

Sample preparation

An aliquote of 100 µl IS was added to 1 ml urine sample. Prior to the extraction 0.1 ml sodium hydroxide (0.2 M) was added to the sample and 2 ml TBME was used to extract the analytes. 20µl HCOOH was added to the organic phase and the TBME was evaporated to dryness by a stream of nitrogen at 60 °C .The residue was derivatized with 100µl of 2% TFAP-Cl dissolved in BuOAc at 60 °C for 15 min [3]. After evaporation (N_2, 60 °C) of the derivatized samples the residue was reconstituted by 5% MeOH in 10 mM NH_4Ac. An injection volume of 2 µl was used.

Chromatographic Separation

Waters Acquity UPLC system (Waters Assoc, Milford, MA) was used to perform the separation on Waters Acquity UPLC BEH Shield RP18 column 50 mm x 2.1 mm with 1.7-µm particles. Column temperature and the flow rate were 60 °C and 0.5 ml/min, respectively. The mobile phases were aqueous solution of 10 mM NH_4Ac (A) and MeOH (B). Separation was performed by a gradient according to table 1.

Mass spectrometric method

The amine-derivatives were detected by positive ion electrospray multiple reaction monitoring (MRM) by a Waters Quattro Premier triple quadrupole instrument (Waters Assoc., Manchester, UK). Three diagnostic ions for each substance were used and the selected precursor/product ion transitions are listed in Table 2.

Table 1. Gradient table

Time	**% mobile phase B**
0.0	5
7.5	50
8.0	95
8.5	95
8.6	5

Table 2. MRM ion transitions

Substance	**Precursor ion (m/z)**	**Product ions (m/z)**	**Collision offset (eV)**
Amphetamine	329	119; 91; 166	14; 26; 16
Amphetamine-D8	337	97	34
Methylamphetamine	343	91; 166; 225	32; 30; 12

Results and Discussion

Baseline separation of the derivatives was obtained. Chromatograms of a calibrator, amphetamine positive sample containing both enantiomers and a selegeline positive sample containing the L-forms of amphetamine and methamphetamine are shown in Fig. 2a, 2b and 2c, respectively. Calibration curves of the diastereomeric derivates were constructed by spiked urine samples at concentration range of 5-1000 ng/ml. The correlation was found to be better than 0.99. Limit of detection was determined to 0.25 ng/ml (s/n > 3) corresponding to 5 pg of the injected derivative. The recovery was found to be 60% (by comparing the area of the internal standard spiked in water with the area of the internal standard spiked in urine). The precision was determined by ten consecutive preparations of control samples containing 100 ng/ml of amphetamine and methylamphetamine (50 ng/ml of each enantiomeric form) and found to be from 2.1% (L-amphetamine) to 17% (L-methylamphetamine) .

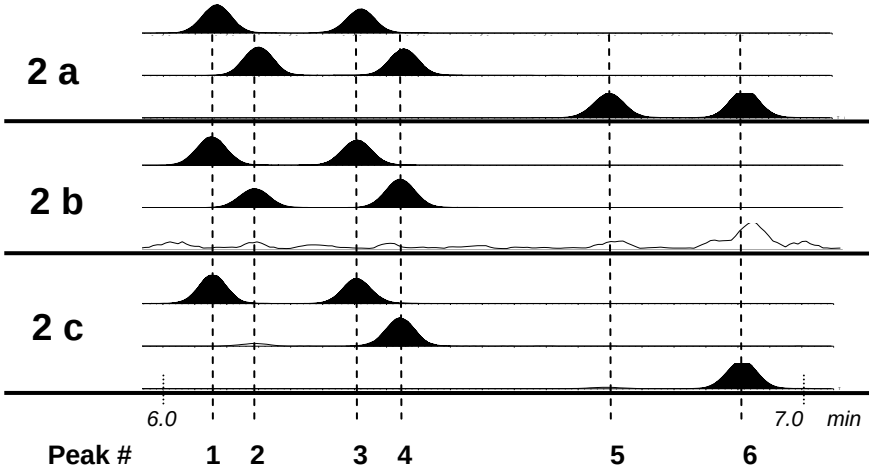

Figure 2. Ion chromatograms of a calibrator (2 a), an amphetamine positive sample (2 b) and a selegilin positive sample (2 c). Peak 1-6 corresponds to (D-) amphetamine-D8 TFAP, (D-) amphetamine TFAP, (L-) amphetamine-D8 TFAP, (L-) amphetamine TFAP, (D-) methylamphetamine TFAP and (L-) methylamphetamine TFAP, respectively.

Conclusions

This rapid method can be used to distinguish between the enantiomeric L-forms and the racemates for amphetamine and methylamphetamine with the same chromatographic system as the screening method used at Doping Control Laboratory Karolinska University Hospital, allowing immediate confirmation. The sensitivity of the method is far below the minimum required performance limit (MRPL) according to WADA technical document TD2004MRPL. The drawback of the method is the racemization of approximately 10% during the derivatization step [4], however the method is sufficient for distinguishing between the isomeric and the racematic form. Stability test of samples stored at ambient temperature for three weeks showed no extended racemerization. By using isotope labelled methylamphetamine the method precision for these enantiomers can be improved.

References

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