Determination of the enantiomeric composition of amphetamine standards

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Aim: From a forensic point of view, it is important to be able to discriminate between the intake of illicit (racemic mixture) and legally prescribed amphetamine (e.g. lisdexamphetamine). For the quantification of these substances, internal reference standards of d,l-amphetamine are used and the question arose whether these were in fact 1:1 racemic mixtures. **Methods:** Two racemic reference standards and two "enantiopure" reference standards were analyzed with a GC-MS method in full scan mode after derivatization with (R)-(-)-α-Methoxy-α-(trifluoromethyl) phenylacetyl chloride (R-MTPCl) to obtain diastereomers. **Results and discussion:** The analyses of the two racemic reference standards showed that they actually present an enantiomeric ratio of 1:1. However, the two "enantiopure" reference standards showed traces or even higher concentrations of the other enantiomer. These traces, respectively impurities (up to 4%) could potentially cause false results when using these reference standard for calibration. Consequently, this could also affect the interpretation of the results. **Conclusion:** Due to the traces and impurities of the supposedly "enantiopure" reference standards available, quantification of d- and l-amphetamine after chiral separation should be performed with a calibration using the racemic reference standards with an enantiomeric ratio of 1:1.

1. Introduction

According to the United Nations Office on Drugs and Crime's World Drug Report 2019, amphetamines and prescription stimulants have been consumed by 29 million people in the past years, being the third largest group of users, directly after cannabis and opioids. It furthermore showed that the form of amphetamines used differs remarkably from region to region. Whereas the non-medical use of prescription stimulants and methamphetamine predominate in North America, amphetamines prevail in Western and Central Europe [1].

Amphetamines comprise a class of synthetic drugs which are structurally and functionally close to endogenous amines. The leading compound of this drugs is amphetamine (α-methylphenethylamine), which occurs in two stereoisomeric forms: the more potent d-amphetamine and l-amphetamine (see figure 1) [2]. Illicit amphetamine is most commonly synthesized by the Leuckart method, using benzyl methyl ketone (BMK, P2P, phenylacetone) as a precursor and reagents such as formic acid, ammonium formate or formamide, yielding a racemic mixture of the d- and l-enantiomers. An "enantiopure" synthesis by reduction of the appropriate diastereoisomers of norephedrine or pseudoephedrine is much less common [3]. However, amphetamine has also been used as a legal drug for many decades. It has made its first appearance 1937 in the Bradley report, using a racemic amphetamine sulfate for the treatment of children and adolescents with attention-deficit/hyperactivity disorder (ADHD).

Nowadays, amphetamines are increasingly prescribed as maintenance therapy for ADHD and narcolepsy in adults [4,5]. Furthermore, the use of prescription stimulants has also made its entrance into schools and higher education, especially, but not exclusively, among medical students all over the United States and Europe. It is used as a performance/cognitive enhancer (increase of concentration) and reduces the need for sleep (less fatigue) and nervousness [6-10].

Fig. 1. Chemical structure of d-amphetamine (left) and l-amphetamine (right).

Whereas illicit amphetamine is usually in the form of its racemic mixture, the licit drug is prescribed as "enantiopure" d-amphetamine (e.g. dexamphetamine hemisulfate known as Attentin® in Germany) or as the prodrug lisdexamphetamine (e.g. Elvanse® in Germany and Switzerland), which is completely metabolized to d-amphetamine after intake. From a forensic point of view, it is important to be able to discriminate between the intake of illegal amphetamine (racemic d/l-mixture) or legally prescribed lisdexamphetamine for the treatment of ADHD or narcolepsy. Therefore, our Institute has developed a method for the quantitative determination of d- and l-amphetamine in blood, serum or urine.

During the assessment of the Institute for the accreditation of the SN EN ISO/IEC 17025: 2018, the question arose whether the internal standards for quantification were actually racemic mixtures, respectively "enantiopure". The corresponding data sheets provided by the suppliers contain no precise information about this matter. On request from one of the manufacturers, it was stated that the synthesis pathway leads to a 1:1 racemic mixture (by Knoevenagel condensation of benzaldehyde with nitroethane yielding 1-phenyl-2-nitropropene followed by reduction with $LiAlH_4$). However, its enantiomeric ratio has not been confirmed by analytical methods, and no data were available on the certificates of the reference material. That is why we carried out an examination of the reference standards to evaluate their racemic purities.

2. Material and Methods

2.1. Chemicals and reagents

The following reference standards (racemic mixtures and "enantiopure" substances) were tested.

Tab. 1. Amphetamine standards.

Standard	Supplier	Art. no.	Concentration	Solvent
(±)-amphetamine	Cerilliant	A-007	1000 µg/mL	Methanol
d,l-amphetamine	Lipomed	AMP-95-HC-1LM	1000 µg/mL	Methanol
R-(-)-amphetamine	Cerilliant	A-049	1000 µg/mL	Methanol
S-(+)-amphetamine	Cerilliant	A-008	1000 µg/mL	Methanol

For sample preparation and analysis, the following chemicals and reagents were used: the derivatisation agent (R)-(-)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (R-MTPCl) was obtained from Sigma-Aldrich (Buchs, Switzerland), acetonitrile (HPLC gradient grade, 99.9%) was purchased from Acros Organics (Geel, Belgium), ethyl acetate (LiChrosolv®) from Merck (Darmstadt, Germany) and methanol (absolute, HPLC grade) from Biosolve (Dieuze, France).The reference standards (see table 1) were acquired from Cerilliant (Round Rock, TX, USA) and Lipomed (Arlesheim, Switzerland).

2.2. Sample preparation

The sample preparation is based on an already published method of Rasmussen et al. [11]: The reference standards are diluted from 1000 µg/mL to 10 µg/mL by adding 10 µL of each standard to 990 µL of acetonitrile in separate glass vials. Thereupon, 10 µL of the derivatisation

agent R-MTPCl are diluted in 200 µL of acetonitrile and 25 µL of this solution are added to each sample. The glass vials are sealed with a crimp cap and heated for 2 h at 80 °C in a block thermostat. As soon as the samples have reached room temperature, 100 µL of methanol are added. The samples are resealed and heated for another 15 min at 70 °C. Once the samples have reached room temperature again, they are evaporated to total dryness under a gentle nitrogen stream. The sample is redissolved in 500 µL of ethyl acetate and analyzed directly by GC-MS.

2.3. Chemical reaction

The separation of the two enantiomers is based on an acylation of the amphetamine with a chiral reagent (acid chloride). The following reaction takes place (Fig. 2).

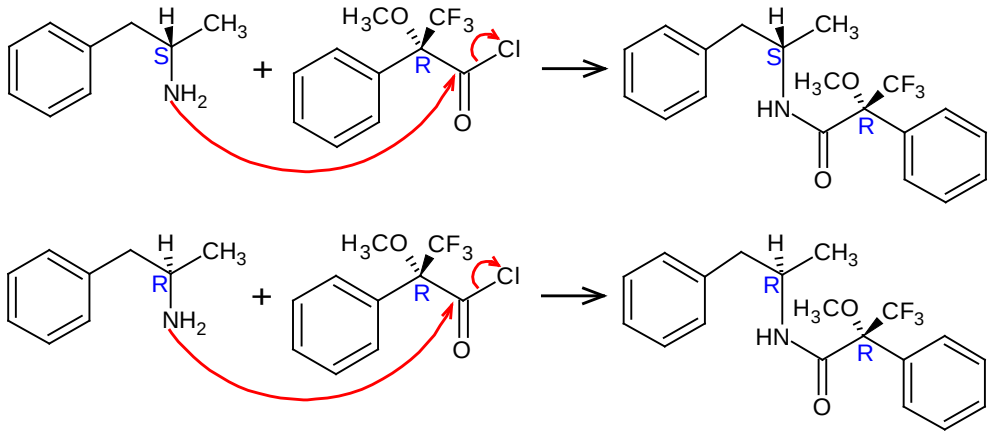

Fig. 2. Acylation reaction: conversion of S-(+)-/R-(-)-amphetamine (racemic mixture) with R-MTPCl.

Through nucleophilic substitutions (SN1 reaction) two molecular compounds are formed which differ in their spatial arrangement (S/R & R/R) (diastereomers) and can thus be separated by means of GC-MS.

2.4. GC-MS instrumentation

The GC-MS consisted of a 6890N GC system coupled to a 5973 *inert* mass selective detector. Samples were injected automatically by means of a 7683 auto sampler and a 7683B injector. MSD ChemStation Software version E.02.02.1431 was used for data acquisition and analysis (all Agilent Technologies, Santa Clara, CA, USA).

GC was performed with a (5%-phenyl)-methylpolysiloxane (HP-5MS) column (30 m x 0.25 mm x 0.25 µm) from Agilent Technologies (Santa Clara, CA, USA). A 1 µL aliquot of the prepared sample was injected in splitless mode. The temperature program of the oven was as follows: holding 50 °C for 3 min, then increase from 50 °C to 150 °C at a rate of 5 °C/min, followed by an increase from 150 °C to 275 °C at a rate of 25 °C/min and isotherm at 275 °C for 4 min (total analysis time: 32 min).

Mass spectrometric data were acquired in full scan mode with a mass range of 50 – 500 amu and EI-ionisation at 70 eV.

The resulting diastereomers were identified by the extracted m/z-values 91, 189, 260 (Fig. 3) and by by comparison with the mass spectra from literature [11]. The enantiomeric ratios were calculated according to their corresponding peak areas.

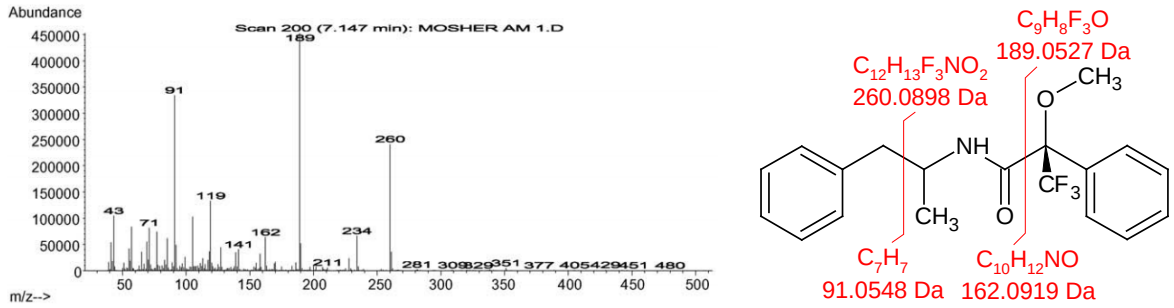

Fig. 3. Structure and mass spectrum of amphetamine derivatized with R-MTPCl (according to Rasmussen et al. [11]).The reaction results in a R-MTP derivative of amphetamine.

3. Results and Discussion

The analyses of the racemic reference standards (±)-amphetamine from Cerilliant and d,l-amphetamine from Lipomed resulted in two racemic mixtures, containing each 50% of (+)/d- and (-)/l-amphetamine (Fig. 4).

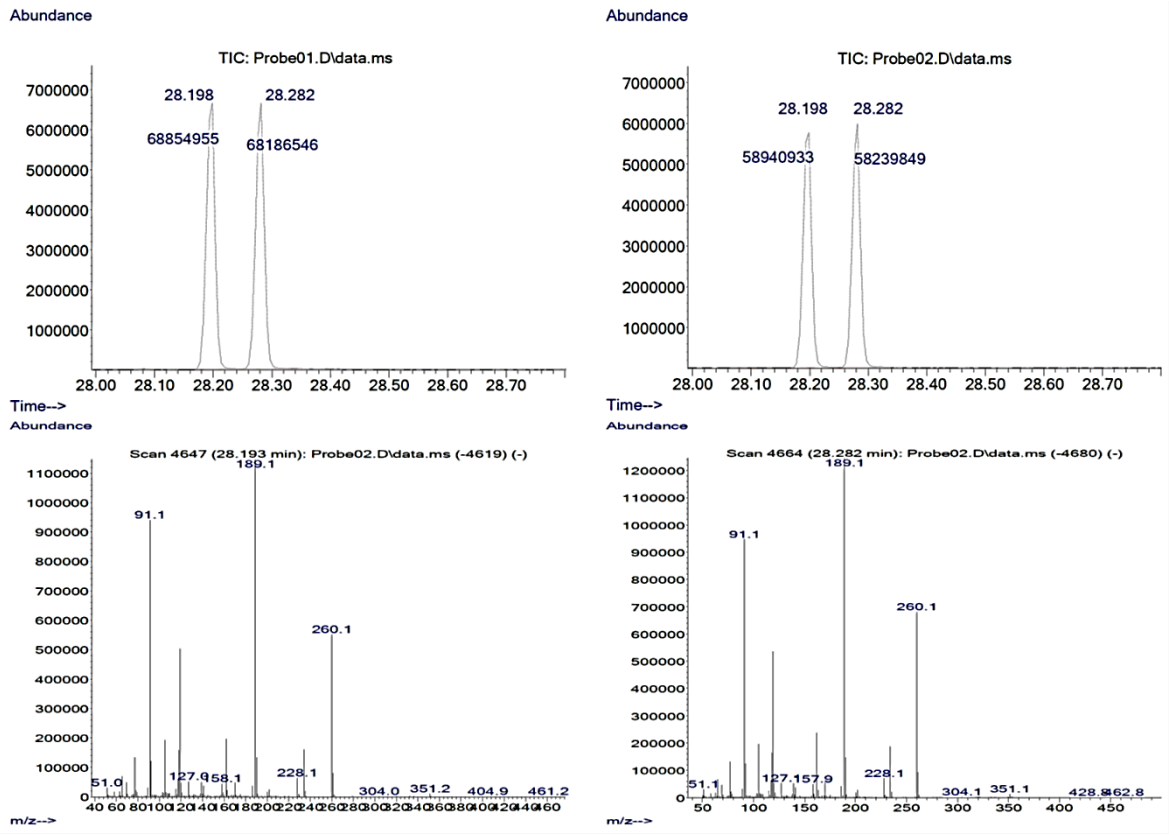

Fig. 4. Chromatograms and corresponding MS spectra of R-MTP-derivatives of (±)-amphetamine (top left) and d,l-amphetamine (top right). 28.198 min: R-(-)/l-amphetamine (R/R configuration after derivatization); 28.282 min: S-(+)/d-amphetamine (S/R configuration after derivatization). Enantiomeric ratio: 50.2% : 49.8%.

Considering the analyses of the "enantiopure" reference stan¬dards R-(-)- and S-(+)-amphetamine from Cerilliant, traces of the S-(+)-enantiomer were found in the "enantiopure" R-(-)-amphetamine. The supposedly "enantiopure" S-(+)-amphetamine even showed a concentration of 4% of the R-(-)-enantiomer (Figs. 5 and 6).

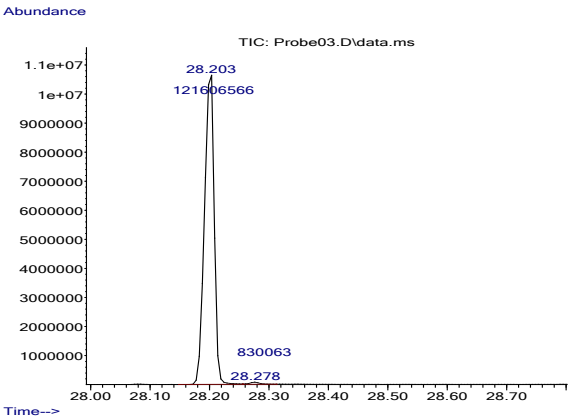

Fig. 5. Chromatogram of R-MTP-derivative of R-(-)-amphetamine. 28.203 min: R-(-)-amphetamine (R/R configuration); 28.278 min: S-(+)-amphetamine (S/R configuration). Enantiomeric ratio: 99.3% : 0.7%.

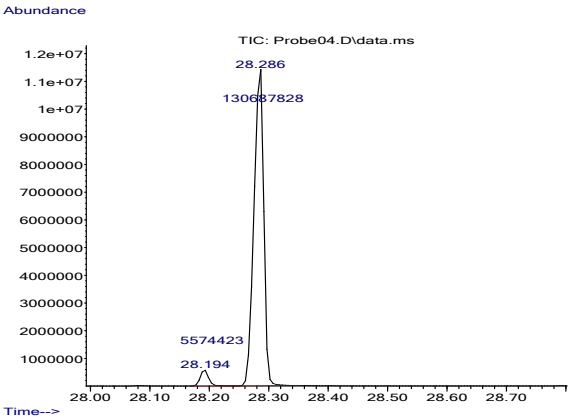

Fig. 6. Chromatogram of R-MTP-derivative of S-(+)-amphetamine. 28.194 min: R-(-)-amphetamine (R/R configuration); 28.286 min: S-(+)-amphetamine (S/R configuration). Enantiomeric ratio: 4.1% : 95.9%.

This impurity could potentially cause false results when using this reference standard for calibration. Consequently, this could also affect the interpretation of the results. It can be excluded that these are contaminations from the previous analyses, since a methanol blank was run between two analyses and these did not show any traces of the compounds.

4. Conclusions

The two racemic reference standards (±)-amphetamine and d,l-amphetamine from Cerilliant and Lipomed respectively are racemic mixtures with a enantiomeric ratio of 1:1.

Due to the impurities of the supposedly "enantiopure" reference standards available, it makes sense to perform the quantification after chiral separation with a calibration using the racemic reference standards (ratio 1:1) for the calibration.

5. References

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