Sofie R. Vande Casteele[1]
Marie-Paule L. Bouche[2]
Jan F. Van Bocxlaer[1]

[1]Laboratory of Medical Biochemistry and Clinical Analysis, Vakgroep Bio-analysis, Ghent University, Ghent, Belgium
[2]Formerly Laboratory of Medical Biochemistry and Clinical Analysis, Vakgroep Bio-analysis, Ghent University, Ghent, Belgium

LC-MS/MS in the elucidation of an isomer of the recreational drug methylenedioxy ethylamphetamine: Methylenedioxy dimethylamphetamine

This paper describes the surplus value of a quadrupole-orthogonal acceleration TOF mass spectrometer, coupled to a liquid chromatographic separation system, for the unequivocal identification and structural elucidation of an unknown compound in the field of designer drugs. In a patient sample set (blood, tissues, vitreous humor, *etc.*), analyzed with a dedicated liquid chromatographic–fluorescence detection method for the determination of methylenedioxy amphetamine, methylenedioxy methamphetamine, and methylenedioxy ethylamphetamine (MDEA), a "strange" inexplicable peak appeared at a retention time not corresponding to any of our reference materials. Based on the identical excitation and emission wavelengths in detection, and a retention behavior comparable to MDEA, it was assumed that this unknown compound was an isomer of the recreational drug MDEA. With a simple and straightforward methodological crossover between LC fluorescence detection and LC-MS/MS, additional information for structural elucidation was easily obtained. Chromatographic separation was achieved on a Hypersil BDS C18 column (fluorescence detection part) and on a Hypersil BDS phenyl column (mass spectrometric detection part). MS showed that the unknown compound's molecular mass was identical to that of MDEA, and, in addition, its fragmentation pattern too proved quite similar to that of MDEA. A thorough literature overview and study of the fragmentation pattern by means of the MS/MS spectrum led to an evidence-based hypothesis of 3,4-methylenedioxy *N*,*N*-dimethylamphetamine (MDDM) being the unknown compound. To confirm this hypothesis, MDDM was synthesized and its presence in our biological sample was finally demonstrated by co-injection with alternatively synthesized MDDM and MDEA. This application shows the synergism between LC and MS in the elucidation of unknown compounds, nevertheless emphasizing the essence of chromatographic separation when dealing with isomers.

Key Words: Identification; Methylenedioxy dimethylamphetamine; Quadrupole time-of-flight mass spectrometry

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1 Introduction

The designer drugs 3,4-methylenedioxy methamphetamine (MDMA; ecstasy), 3,4-methylenedioxy ethylamphetamine (MDEA; eve), and 3,4-methylenedioxy amphetamine (MDA) are all phenylethylamine derivatives of the methylenedioxy type. In the last decades, these drugs have gained tremendous popularity as recreational drugs and are chiefly used for their agreeable stimulating effects [1]. Until recently, the determination of MDMA, MDEA, and MDA in biological samples has mainly been based on GC-MS, which provides excellent sensitivity and selectivity. However, MDMA and its analogs require derivatization before performing gas chromatographic determination to improve their chromatographic properties [2, 3]. The use of LC has grown over the years because derivatization is virtually never necessary. In the field of methylenedioxy amphetamines, LC combined with fluorescence detection (LC-FI) proved very suitable as a simple and sensitive

Correspondence: Sofie R. Vande Casteele, Laboratory of Medical Biochemistry & Clinical Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium. E-mail: sofie.vandecasteele@UGent.be.
Fax: +32-9-264-8197.

Abbreviations: LC-FI, liquid chromatography fluorescence detection; **MDA**, 3,4-methylenedioxy amphetamine; **MDDM**, 3,4-methylenedioxy *N,N*-dimethylamphetamine; **MDEA**, 3,4-methylenedioxy ethylamphetamine; **MDMA**, 3,4-methylenedioxy methamphetamine; **MDMPA**, methylenedioxy methylpropylamphetamine; **Q-TOF**, quadrupole time-of-flight mass spectrometer; **TMS**, tetramethylsilane

technique. Also, suitable selectivity is obtained when the native fluorescence of methylenedioxy amphetamine analogs is used [1]. In such an approach, however, compound identification is highly dependent on the comparison of the retention characteristics of an unknown compound with those of reference materials determined under identical experimental conditions. Taking into consideration the relatively limited separation power of standard LC, the time dimension of chromatography only often does not provide the desired absolute certainty regarding the identity of a targeted compound, despite some added selectivity from, *e.g.*, fluorescence detection. Furthermore, in the case of an unknown compound eluting at a retention time not corresponding to any of the reference material, identification becomes, at best, an educated guess [4].

Clearly, for unequivocal identification of unknown compounds, mass spectrometric detection has become an almost indispensable tool. The synergistic combination of the separation capability of chromatography and the identification power embedded in a mass spectrum is consequently advantageous in many analytical applications [4].

LC-MS and LC-MS/MS using atmospheric pressure ionization techniques have evolved from a research subject into a robust and routinely applicable analytical tool [5, 6]. These ionization approaches are most often coupled to triple quadrupole or IT mass analyzers. Popular as they are for their ease-of-use and relatively low cost, they still do have their limitations. This mainly pertains to the elucidation of the structure of unknown compounds on the basis of low resolution MS [7–10]. Alternatively, a quadrupole-orthogonal acceleration TOF mass spectrometer (Q-TOF) can be used for the identification of unknown compounds. The Q-TOF consists of a first mass analyzer and collision region adapted from a triple quadrupole instrument, and a reflectron-type orthogonal acceleration TOF analyzer as the second mass analyzer [11, 12]. The enhanced "full scan" sensitivity of the TOF analyzer allows the acquisition of product-ion spectra from trace components at levels that would be impossible using a conventional triple quadrupole analyzer. For MS/MS acquisitions, a precursor ion is selected in the first quadrupole and the mass analysis of the fragment ions occurs in the TOF analyzer. All TOF-based mass analyses are at high resolution and provide accurate mass measurements. On the basis of these accurate masses of precursor (single MS) and/or fragment ions (MS/MS), structural elucidation can be performed [11].

In this paper, we illustrate the manifest added value of LC combined with mass spectrometric detection, in our case a quadrupole-orthogonal acceleration TOF mass spectrometer (LC-QTOF), for the structural elucidation and identification of an unknown compound, using a case from the field of forensic toxicology. In our laboratory, we were involved in a collaborate study on MDMA, MDEA, or MDA abuse and toxicokinetics. For this purpose, the numerous biological samples (blood, tissues, vitreous humor, *etc.*) are routinely analyzed using a validated method based on HPLC with fluorescence detection [1]. In a particular forensic case, a user deceased from an MDMA overdose (as was confirmed later on), a peak was observed in the chromatogram at a retention time close to that of an MDEA standard. Having only a 1-D (fluorescence) trace, it was at first almost mistakenly taken as MDEA. The high reproducibility, however, of the methylenedioxy amphetamines within the LC-FI system and indeed the unchanged retention time of MDMA and MDA, present in the sample, and the internal standard alarmed us to a potential case of mistaken identity. Because the exitation and emission wavelengths of the methylenedioxy amphetamines are rather specific, we soon realized this might be a new designer drug of the methylenedioxy amphetamine family and further investigation was clearly mandatory. To investigate the identity of our unknown compound, an LC-QTOF approach was used. To that end, a simple and straightforward methodological crossover between LC-FI and LC-MS/MS was applied [1].

2 Materials and methods

2.1 Materials

All reagents and chemicals were of analytical grade and were obtained from Aldrich (St. Louis, MO, USA). All solvents were of HPLC grade (Biosolve, Valkenswaard, The Netherlands). MDA, MDMA, and MDEA pure standards were obtained from Sigma (St. Louis, MO, USA). Methylenedioxy methylpropylamphetamine (MDMPA), synthesized in-house [1], was used as internal standard. A 3,4-methylenedioxy *N*,*N*-dimethylamphetamine (MDDM) standard was also synthesized and characterized in-house. Stock solutions (1.0 mg/mL) of these active substances were prepared in methanol. The stock solutions were stored in the dark at -20°C and were stable for at least 1 year. Suitable working dilutions were prepared in methanol and stored under the same conditions but discarded after 6 months.

2.2 MDDM synthesis

A mixture of dimethylamine hydrochloride, 3,4-methylenedioxy phenylacetone, and sodium cyanoborohydride was prepared. The mixture was stirred and acidified with a 1 : 1 mixture of concentrated HCl and MeOH for 2 days to allow the reduction to take place. In a next step, most of the MeOH solvent was evaporated, and the reaction mixture was diluted with H_2O until 250 mL. Next, the mixture was made strongly acidic with an excess of HCl. After washing with 2×100 mL of CH_2Cl_2, the aqueous phase was made basic using 25% NaOH, and extracted with

3×100 mL of CH_2Cl_2. Subsequent evaporation of the organic phase on a rotavapor yielded a nonviscous solution instead of the nearly colorless oil, which was expected. As a consequence, analytical TLC was performed with Alugram Sil G/UV plates (Macherey-Nagel, Germany). Next, purification was performed on preparative column chromatography with ICN Silica (63–200 mesh), type 60 A, and 70/29/1 CH_2Cl_2/MeOH/ammonia in MeOH 7 N as mobile phase. All fractions were tested on TLC; only fractions containing our product were collected. Evaporation of the organic eluent resulted in a yellow oil, which was resolubilized by addition of 5 mL of CH_2Cl_2. Final addition of 4 mL of 1 M HCl in diethyl ether yielded crystallization. The obtained solid product was collected by filtration. After air drying, 450 mg MDDM was obtained. The identity and purity of the synthesized product, MDDM, were obtained on the basis of ^{1}H NMR spectroscopy and MS (LC-MS/MS). The ^{1}H NMR spectrum (600 mHz) of the hydrochloride salt (in D_2O and with external tetramethylsilane (TMS)) was completely compatible with the expected structure. The signals were (*d*): 1.25, 1.37 (d) CCH_3, 3H; $ArCH_2$ under the $N(CH_3)_2$, 2.8, 8H; CH (m) 3.65; CH_2O_2 (s) 5.9 2H; ArH 6.93 (3H).

2.3 Samples and extraction procedure

The particular biological sample set (blood, tissues, vitreous humor, *etc.*) was obtained within the framework of a forensic toxicological postmortem examination. Samples were extracted using an extraction method described in an earlier report [1]. In short, after addition of the MDMPA internal standard to a 250-μL aliquot of the sample (liquid matrix, tissue homogenate, or calibrator), it was diluted with 1 mL of distilled water, and the pH was adjusted to 9.5 with 1 M aqueous K_2CO_3. After liquid–liquid extraction (LLE) (10 min), the organic phase was acidified with 50 μL of methanolic hydrochloric acid and evaporated to dryness (N_2). Finally, the dry residue was redissolved in 100 μL of HPLC eluent. For LC-FI analysis, 25-μL aliquots were injected whereas for the Q-TOF system 30 μL were used.

2.4 Instrumentation

For LC-FI analysis, the HPLC unit was composed of a ternary low-pressure gradient pump, an autosampler with a 25-μL loop (both from Kontron Instruments, Milano, Italy), and equipped with a solvent degassing module (Shodex, Tokyo, Japan). We used a spectrofluorometric detector (RF-10Axl; Shimadzu, Kyoto, Japan) linked to a Kromasystem 2000 data system (Kontron Instruments) for data acquisition. The LC-MS analyses were carried out on a Q-TOF hybrid mass spectrometer (Waters, Manchester, UK) equipped with an orthogonal electrospray source (Z-spray®) operated in the electrospray positive ion mode (ESI+). The chromatographic system consisted of an Alliance 2790 separation module (Waters) controlled by Masslynx software (Waters).

2.5 Chromatography

Chromatographic separation in LC-FI mode was achieved on a Hypersil BDS C18 column (100 mm × 2.1 mm ID, particle size 3 μm; Alltech, Lokeren, Belgium). The mobile phase consisted of water/methanol/ACN (80:10:10) containing 0.1 M ammonium acetate (solvent A) and water/methanol/ACN (5:45:45), again supplemented with 0.1 M ammonium acetate (solvent B). The chromatographic conditions were as follows: 100% A for 6 min, followed by a linear gradient from 0 to 70% B within 14 min at a flow rate of 0.2 mL/min. After completion of the chromatographic run, the pump was programmed to return to the initial conditions within 0.5 min, and equilibrated for 8 min yielding a total run-time of 28.5 min.

LC-MS was carried out using a Hypersil BDS phenyl column (100 mm × 2.1 mm ID, particle size 5 μm; Alltech). Gradient elution was performed, starting at 100% water/methanol/ACN (80:10:10) containing 0.1 M ammonium acetate (solvent A) for 10 min, followed by a linear gradient from 0 to 35% water/methanol/ACN (5:45:45) again supplemented with 0.1 M ammonium acetate (solvent B) within 13 min. The final eluent composition was maintained for 5 min. Subsequently, the system was programmed to regain its initial conditions over a 0.5-min time interval, followed by a 3.5 min equilibration time prior to the next injection. This resulted in an overall run-time of 32 min. The flow rate was 0.2 mL/min.

2.6 Fluorescence detection

The excitation and emission wavelengths of the fluorescence detector were 288 and 324 nm, respectively (bandwidth was 15 nm for both slits).

2.7 MS

Optimization of the MS conditions was performed using MDEA. The capillary voltage and cone voltage were optimized at 3000 and 23 V, respectively. The source and desolvation temperatures were kept constant at 145 and 395°C, respectively. The protonated molecules $[M + H]^+$ were selected in the first quadrupole and transported to the hexapole collision cell, which used argon as collision gas. The collision energy was optimized for each compound, in order to obtain an abundance of 10% for the protonated molecule compared to the base peak, and varied from 14 to 16 eV. MS as well as MS/MS data (see Table 1) were acquired over a mass range of 50–500 Da; the MCP detector setting was to 2800 V. TOF-MS resolution was approximately 5000 (FWHM). Nitrogen acted both as the nebulizing and desolvation gas.

Table 1. Overview of the compounds, their major fragment ions, and retention time (LC-MS system)

Compound	Ion transitions (*m/z*)	Retention time, min
MDA	180.10 → 105 + 133 + 135 + 163	8.5
MDMA	194.10 → 105 + 133 + 135 + 163	10.5
MDEA	208.10 → 105 + 133 + 135 + 163	15.5
? (MDDM)	208.10 → 105 + 133 + 135 + 163	17.6
MDMPA (IS)	236.10 → 105 + 133 + 135 + 163	22.1

3 Results and discussion

3.1 Fluorescence detection

In this analytical method, the retention times of MDA, MDMA, MDEA, and the internal standard MDMPA were 13.1, 14.2, 15.2, and 17.3 min, respectively. In the particular forensic sample set, an idiosyncratic peak was found at a retention time of 16.5 min. The retention time equivalence of the other methylenedioxy amphetamines in these runs ruled out the identification of this peak as MDEA. Fluorescence detection is, however, relatively selective. The ring-substituted amphetamines, dissolved in the chromatographic eluent mixture, exhibit a good native fluorescence with an excitation maximum at 288 nm. When the compounds were irradiated at 288 nm, an emission maximum at 324 nm is recorded. The observed fluorescence spectral behavior of the unknown compound explicitly pointed toward a methylenedioxy amphetamine structure. This is, however, as far as the examination and elucidation of such an unknown compound can be taken. A more informative detection technique is obviously required.

3.2 Mass spectrometric detection

In earlier work of our laboratory, a simple and straightforward methodological crossover between LC-FI detection and LC-MS/MS was already described [1]. The same column and chromatographic conditions were used on both systems, and the samples could be injected on both systems after a common extraction procedure. Because we have encountered this kind of analytical challenges before, it is customary to us to develop LC methods with volatile buffers, thus MS compatible eluents, no matter which detection technique the method is intended for. In this particular case, the methodological crossover was not entirely complete as we choose to use a phenyl column, and matching, slightly divergent chromatographic conditions to achieve a better separation between MDEA and the unknown compound.

A typical mass chromatogram, obtained in MS/MS conditions, of a 50 ng/mL MDA, MDMA, and MDEA calibrator supplemented with the internal standard MDMPA is shown in Fig. 1. It should be noticed that a Q-TOF system has such an excellent full scan sensitivity that full scan spectra are always acquired in the TOF section, either in single MS or in MS/MS. Mass chromatograms are then reconstructed either from one or more ions (single MS) or from one or more transitions (MS/MS). All peaks are well separated. The retention times of MDA, MDMA, MDEA, and the internal standard MDMPA were 8.7, 11.9, 15.9, and 22.2 min, respectively. The matching MS/MS reconstructed ion chromatogram of the forensic sample (left pleura) is shown in Fig. 2. It is clear from the tailing and shifted retention time that the peak for MDMA is heavily overloaded. This MS analysis was initially performed to exclude the presence of MDEA. Much to our surprise, the

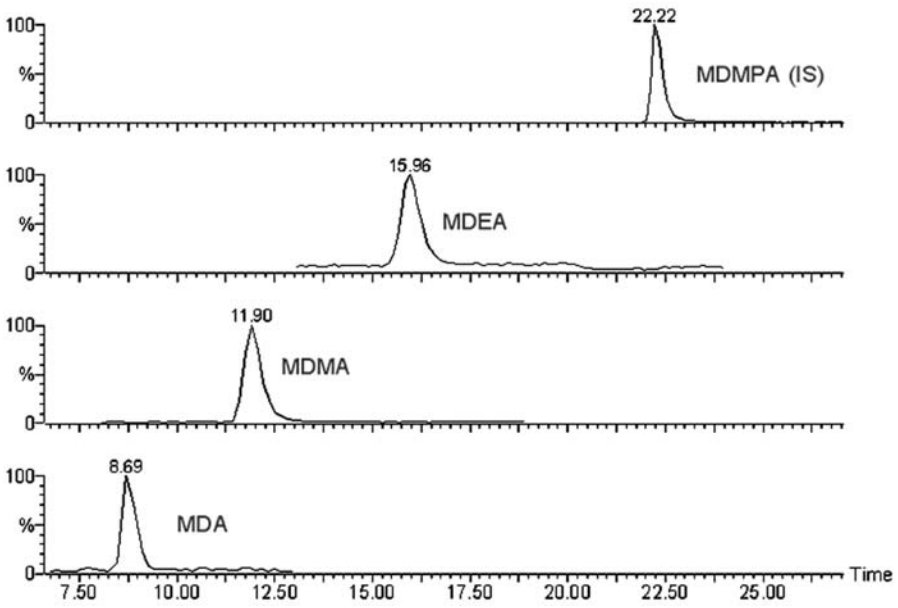

Figure 1. Reconstructed ion chromatogram of a calibrator containing 50 ng/mL MDA, MDMA, MDEA, and internal standard MDMPA. Table 1 tabulates the respective ion transitions illustrated.

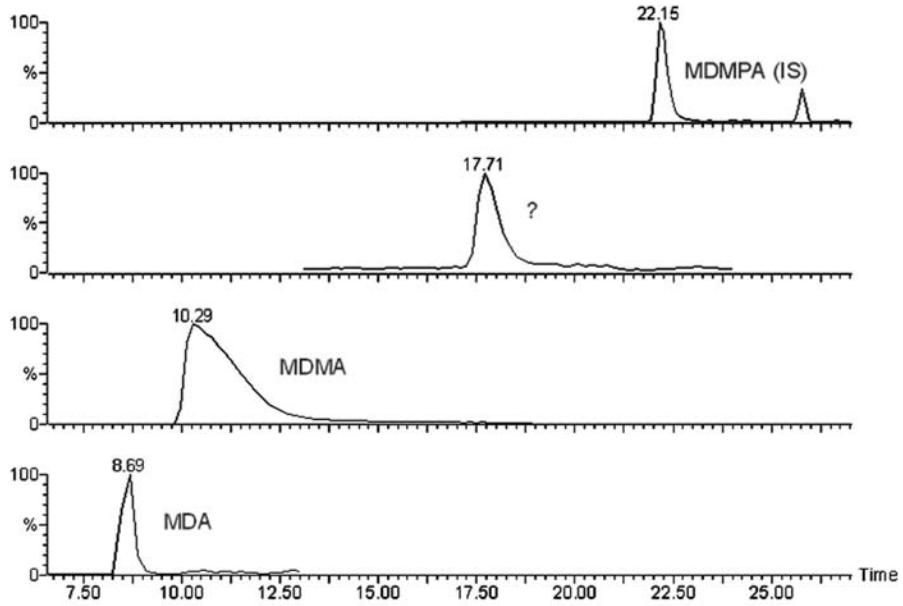

Figure 2. Reconstructed ion chromatogram of our sample containing MDA, MDMA, unknown compound (?), and internal standard MDMPA. See Table 1 for the respective ion transitions.

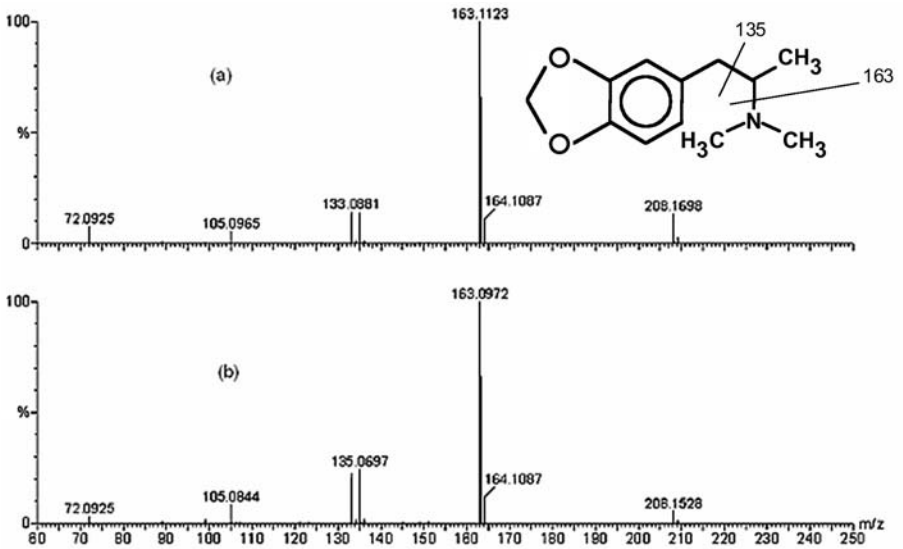

Figure 3. Product-ion spectra of the unknown compound (a) and MDEA (b). In the inset, the structure of the synthesized product, MDDM.

idiosyncratic peak we observed in the LC-FI runs again appears in the ion transition trace for MDEA and at a retention time of 17.7 min. As this is only possible on the condition that the precursor ion, *i.e.*, molecular mass (protonated), is that of MDEA (*m/z* 208.1), the peak either had to be of MDEA after all, or of an isomeric species.

In a Q-TOF MS/MS analysis, additional information is available, namely, the fragmentation pattern of the unidentified substance. The product ions of the unknown compound proved quite similar to that of MDEA, as already noted from the reconstructed MS/MS chromatographic trace. The product-ion scan with *m/z* 208 as parent shows product ions at *m/z* 163, 135, 133, and *m/z* 105 (Fig. 3). Despite having the power of LC-MS/MS at our hand, the presence of MDEA could only be ruled out on the basis of its chromatographic behavior, stressing the importance of having LC in front of any MS system, no matter how powerful the MS system potentially is. The additional spectral information, however, allows structural elucidation. A thorough literature overview [13–16] and close inspection of the fragmentation pattern revealed small differences in ion intensities. The presence of the *m/z* 133 and 135 ions indicate an unmodified methylenedioxy phenylskeleton comparable to, *e.g.*, MDMA and MDEA. The latter is supported by the fluorescence spectral similarities. The presence of the *m/z* 163 ion, as in MDEA, indicates that the structural difference had to be situated at the substituted nitrogen atom. This difference, however, had to be conservative in terms of the molecular

mass. Based on all the data, the assumption of the MDEA isomer MDDM being the unidentified compound was made. The putative structure is shown in Fig. 3. There are unofficial reports that MDDM has been synthesized before, together with many other methylenedioxy amphetamine variations, but on testing, its psychedelic activity was considered minor.

To test our hypothesis with MDDM not being commercially available, we synthesized it ourselves. The identity and purity of the synthesized product were obtained on the basis of ^{1}H NMR spectroscopy and MS (LC-MS/MS). The purity was investigated with TLC and confirmed in the NMR spectrum. The MS/MS spectrum from the *m/z* 208 precursor not only affirmed that the synthesized product is the intended compound, MDDM, but also already provides a clear indication of the fact that our unknown compound is indeed MDDM. The MS/MS spectrum obtained for the unknown compound in our biological extracts (Fig. 3a) shows an exact similarity with that of the MDDM standard.

To finally confirm beyond any doubt the presence of MDDM in our biological samples, co-injection was performed. A mixture was made of our authentic sample extract, supplemented with an MDEA standard (10 μL MDEA 0.4 μg/mL + 25 μL sample (left pleura)). Two peaks appeared on the second trace of our reconstructed ion chromatogram sets, representing the transition for MDEA and MDDM, one peak at 15.4 min and a second at 17.7 min. A second mixture was prepared of our sample extract supplemented with the synthesized MDDM (10 μL MDDM 0.8 μg/mL + 25 μL sample (left pleura)). On the resulting chromatogram, only one peak appears at a retention time of 17.6 min. It is concluded that the synthesized MDDM and the unknown compound co-elute. Consequently, our unknown compound was identified as MDDM, an isomer of the recreational drug MDEA. The co-chromatography also ruled out the eventuality of a 2,3 regioisomer. It is known from literature [17, 18] that these regioisomers can hardly be differentiated mass spectrometrically but that they do behave differently in chromatographic separations.

MDDM has been hardly described until now, but was classified within the "new synthetic drugs in the European Union" [19]. Mass spectrometric detection paved the way for identification, but chromatographic separation was still considered a vital part of the identity confirmation process, as illustrated by the use of the old and trustworthy technique of co-chromatography.

4 Concluding remarks

From the obtained results, we conclude that the use of LC-MS(/MS), in particular Q-TOF-MS, is a useful tool for the identification of unknown analytes. Inevitably, LC-MS affords much more analytical information compared to, *e.g.*, LC-FI. LC-MS/MS offers the opportunity for structural elucidation of unknown compounds and positive, unequivocal identification and identity confirmation of suspect substances on the basis of their retention time and their unique MS/MS spectrum. In this particular case, our unknown compound is identified as MDDM. Being an isomer of the recreational drug MDEA, only the intelligent combination of chromatographic separation and mass spectrometric detection affords unequivocal identification.

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