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Separation of ortho, meta and para isomers of methylmethcathinone (MMC) and methylethcathinone (MEC) using LC-ESI-MS/MS: Application to forensic serum samples

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Aim: Separation and identification of positional isomers is an important issue in forensic toxicology. This study presents a sensitve and selective LC-MS/MS method to separate the ortho, meta and para isomers of methylmethcathinone (MMC) and methylethcathinone (MEC). Validation of the method was carried out according to international guidelines recommended for analysis of rare analytes. Retrospective measurements were performed on samples with suspicion of a recent MMC or MEC consumption collected in the period from June 2014 to August 2016. **Methods:** For sample preparation, 200 µl of the serum sample was fortified with 10 µl butylone-d_3 [1 µg/ml] and a subsequent protein precipitation was done using 200 µl methanol. After vortexing and centrifugation, 50 µl of the supernatant was diluted with 150 µl water. Chromatographic separation of the isomers was achieved using a Restek Raptor^{TM} Biphenyl column (100 mm x 2.1 mm, 2.7 µm particle size). The mobile phase consisted of (A) 0.1% formic acid in water/methanol (95:5, v/v) and (B) 0.1% formic acid in methanol. **Results:** By using this method, complete separation of the ortho, meta and para isomers of MMC and MEC could be achieved. Investigation of recommended validation parameters proved selectivity, linearity, accuracy, precision, processed sample stability and appropriate quantification and detection limits as well as appropriate matrix effects and recovery. Application of the method to real serum samples revealed the proof of a recent MMC or MEC consumption, respectively, in eight cases. Isomers of MMC could be detected in three of these eight cases, of which two were positive for 3-MMC and one was positive for 2-MMC. The other samples were tested positive for 3-MEC. In none of the samples 4-MMC, 2-MEC or 4-MEC could be detected. **Discussion:** Reliability of the method was confirmed under consideration of the validation parameters selectivity, linearity, accuracy, precision, analytical limits, matrix effects, recovery and processed sample stability. Only substances that were not governmentally controlled at that point of time could be detected, reflecting the rapid response of the recreational drug marked to newly enacting drug laws. **Conclusion:** A reliable and selective LC-MS/MS method for the separation and clear identification of the ortho, meta, and para isomers of MMC and MEC was developed and validated.

1. Introduction

Separation and identification of positional isomers is an important issue in forensic toxicology for various reasons. Despite their structural similarity, positional isomers often show different pharmacological properties [1-3]. Morevoer, compounds with similar structure can show dramatic differences with respect to their toxicity [4]. Apart from these pharmacological and toxicological effects, the legal status is also of great importance [5].

This study aims to present a new LC-MS/MS method for separation and identification of the ortho, meta and para isomers of methylmethcathinone (MMC) and methylethcathinone (MEC). Additionally, the applicability of the method is illustrated with reference to real cases examined at the Institute of Forensic Medicine of Bonn.

2. Material and Methods

2.1. LC-ESI-MS/MS analysis

Analyses were carried out by LC-electrospray ionization (ESI)-MS/MS in the multiple reaction monitoring (MRM) mode using the following specific ion transitions: *m/z* 178.1 → 145.1 and 178.1 → 160.0 for MMC isomers, *m/z* 192.1 → 174.0 and 192.1 → 144.0 for MEC isomers and *m/z* 225.1 → 176.9 for butylone-d_3. The LC system was equipped with a Restek Raptor™ Biphenyl analytical column (100 mm x 2.1 mm, 2.7 µm particle size). The mobile phase consisted of (A) 0.1% formic acid in water/methanol (95:5, v/v) and (B) 0.1% formic acid in methanol. A gradient program starting at a composition of 5% B and ramped to 17% B from 1 to 12 min with a flow rate of 0.5 ml/min was applied. Column washing at 98% B was maintained for 8 min and than ramped down to starting conditions with a subsequent re-equilibration step (20-23 min). Additionally, a five minutes equilibration step prior to sample injection was implemented to ensure constant retention times of the analytes. The injection volume was 10 µl and the temperature-controlled column oven was kept at 50°C.

2.2. Sample collection

The sample collective consisted of real serum samples that were previously analyzed at the Institute of Forensic Medicine of Bonn within the frame of routine traffic control. Retrospective measurements were performed on samples obtained in the period from June 2014 to August 2016. Selection of samples was based on GC-MS findings indicating the presence of MMC or MEC isomers, respectively, and/or on informations from the police reports.

2.2. Sample preparation

Prior to LC-ESI-MS/MS analysis, whole blood was centrifuged at 1248 *g* for 10 min and serum was separated from the red blood cells immediately. 200 µl of each serum sample were fortified with 10 µl butylone-d_3 [1 µg/ml] and a subsequent protein precipitation was done using 200 µl methanol. After vortexing and centrifugation (8 min, 1625 *g*), 50 µl of the supernatant were diluted with 150 µl water. Samples were stored at -20°C until analysis.

3. Results and Discussion

3.1. Separation of MMC and MEC isomers

Mass spectrometry revealed that no specific fragments for the individual isomers could be identified but both the MMC and the MEC isomers could be chromatographically separated and identified using the developed LC-MS/MS method (see figure 1). Calculation of the chromatographic resolution (R) revealed that by using this gradient program R was >1.5 for all closely eluting isomers, confirming baseline, or complete, separation between all analytes.

3.2. Validation

Chromatographic selectivity of the method in human serum was demonstrated by the absence of endogenous interfering peaks at the retention times of the MMC and MEC isomers and butylone-d_3. By using a $1/x^2$ weighting, calibration curves for the MMC and MEC isomers were linear over the concentration range of 5-250 ng/ml in human serum. Accuracy (bias) was within ±15% of nominal value and precision was within 15% RSD. The limit of detection

(LOD) and lower limit of quantification (LLOQ) were <2 ng/ml and 5 ng/ml, respectively. Matrix effects were in the required range of 75-125% with acceptable %RSD (<25%). Recoveries were generally higher than 74% demonstrating a sufficient extraction technique for all analytes. Stability of the processed samples was verified for a time period up to 24 h.

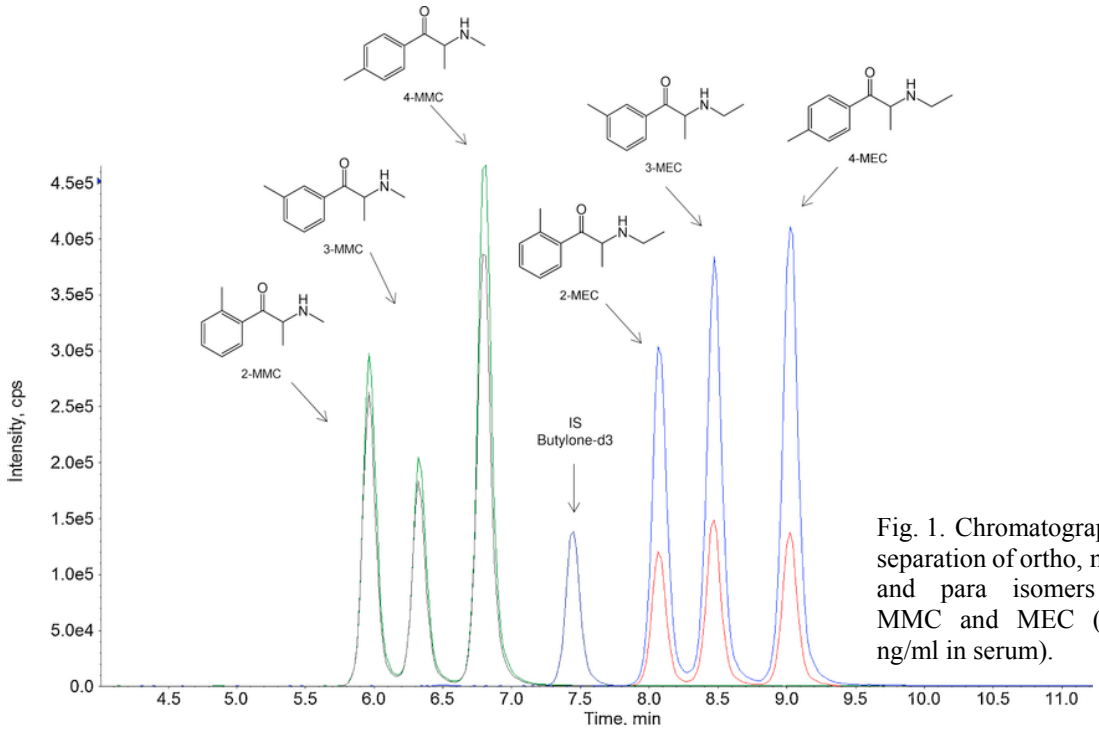

Fig. 1. Chromatographic separation of ortho, meta and para isomers of MMC and MEC (100 ng/ml in serum).

3.3. Retrospective measurements

Application of the method to real serum samples revealed the proof of a recent MMC or MEC consumption, respectively, in eight cases (see table 1). Isomers of MMC could be detected in three of these eight cases, of which two were positive for 3-MMC and one was positive for 2-MMC. The other samples were tested positive for 3-MEC.

Tab. 1. Results of the analyses of serum samples with suspicion of preceded MMC or MEC consumption. *Concentrations above the calibration range were determined by dilution of the sample with water (50:50, v/v).

Case	Date of blood sampling	Detected substance	Concentration [ng/ml]	Further substances detected
1	06/04/2014	3-MMC	13.7	methadone and metabolite, lorazepam
2	10/09/2014	3-MMC	39.9	-
3	04/05/2015	3-MEC	332*	dihydrocodeine, codeine and metabolite, methadone and metabolite, pregabalin
4	04/20/2015	2-MMC	12.6	morphine
5	05/03/2015	3-MEC	136	-
6	05/06/2015	3-MEC	268*	buprenorphine and metabolite, alcohol
7	11/06/2015	3-MEC	270*	methadone and metabolite, nordiazepam
8	08/19/2016	3-MEC	32.1	diazepam, nordiazepam

In none of the samples 4-MMC, 2-MEC or 4-MEC could be detected. Only substances that were not governmentally controlled at that point of time could be detected, reflecting the rapid response of the recreational drug marked to newly enacting drug laws.

4. Conclusion

A reliable and selective LC-MS/MS method for the separation and clear identification of the ortho, meta and para isomers of MMC and MEC was developed and validated. This method met regulatory requirements for selectivity, linearity, accuracy, precision, analytical limits and processed sample stability. Application of the method to real serum samples collected between June 2014 and August 2016 revealed the proof of a recent MMC or MEC consumption, respectively, in eight cases. Only substances that were not governmentally controlled at that point of time could be detected, reflecting the rapid response of the recreational drug market to newly enacting drug laws.

5. References

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