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Artifact production in the assay of anhydroecgonine methyl ester in serum using gas chromatography–mass spectrometry

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Abstract

The detection of the pyrolysis product anhydroecgonine methyl ester (AEME, methylecgonidine) after cocaine smoking using gas chromatography–mass spectrometry is hampered by the artifactual production of AEME. The amount of AEME increases with the amount of cocaine used producing false positive values in authentic samples. A method for the correction of quantitative values was established using calibration of pyrolysis and estimation of the artifactual AEME. Authentic AEME in serum was differentiated from the artifact above 3.5 μg/l, 99% prediction limits of the quantitation were ±3.1 μg/l. In 16 serum samples and five postmortem blood samples, cocaine and AEME were detected, but after application of the correction method only ten were truly positive for AEME.

Keywords: Artifact production; Forensic toxicology; Anhydroecgonine methyl ester; Cocaine

1. Introduction

Anhydroecgonine methyl ester (AEME, methylecgonidine) is the principal thermal breakdown product of cocaine, it is not formed metabolically [1,2] and therefore recognized as a specific marker for smoked cocaine [1,3–8]. The distinction between the inhalation of cocaine and nasal insufflation or injection has forensic and medical implications, e.g. the high potential for addiction [9] and the development of various cardiovascular and pulmonary diseases [10,11]. The analysis of biological samples (blood, urine, hair, saliva, sweat, meconium and postmortem material) [3,5,6,12–15] for AEME is usually included into standard screening procedures for cocaine and its metabolites using gas chromatography–mass spectrometry (GC–MS). However, as a major drawback of this method, AEME is also formed by pyrolysis of cocaine in the injection port of the GC and may also be detected in cases where cocaine was not smoked.

Gonzalez et al. [16] investigated the influence of the injection technique on the degradation of cocaine. They found 2.6% AEME artifact with a common open liner and less than 0.1% using cool

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on-column injection. However, the average GC–MS instrument is equipped with a split/splitless injection device. Using the splitless injection, Cone et al. [5] and Kintz et al. [15] assessed the pyrolysis of the internal standard cocaine-d_3 as marker and considered a 1% degradation rate as not relevant. However, in both studies AEME was not detected in authentic samples. We had found that AEME concentration in serum can be very low [4] and a pyrolysis rate of 2.6% or less would produce false positive results. A procedure for the analysis of AEME and its metabolite anhydroecgonine (ecgonidine) in urine or post-mortem fluids was developed by Paul et al. [8] and Shimomura et al. [3], which consisted of two consecutive solid-phase extractions and three separate GC–MS analyses. To avoid AEME as an artifact the injection of the sample was performed at 140 °C and no pyrolysis of COC was observed under these conditions.

In a previously published procedure for the assay of AEME in serum [4], artifact production was not important because serum samples were collected in devices without stabilizing agents leading to a complete degradation of cocaine prior to analysis. The aim of the present work was to modify that procedure to detect and determine AEME in the presence of cocaine. This procedure may be applied to the analysis of authentic stabilized serum samples and should provide information on the concentration of AEME in serum from crack users.

2. Experimental

2.1. Chemicals and reference standards

Solutions of the reference standards (1 mg/ml) cocaine (COC), ecgonine methyl ester (EME), anhydroecgonine methyl ester (AEME) and of the corresponding deuterated internal standards (0.1 mg/ml) cocaine-d_3 (COC-d_3), benzoylecgonine-d_3 (BZE-d_3) and ecgonine methyl ester-d_3 (EME-d_3) were from Cerilliant (Promochem, Wesel, Germany), the derivatization reagent *N*-methyl-*N*-(tert.-butyldimethylsilyl)trifluoroacetamide (MBDSTFA) from Macherey and Nagel (Düren, Germany). All other reagents and organic solvents were of analytical grade and from Merck (Darmstadt, Germany).

2.2. Sample preparation

One ml of serum was mixed with 4 ml of 0.1 *M* phosphate buffer pH 6.0 and 100 μl of internal standard solution (1 ng/μl EME-d_3, COC-d_3 and BZE-d_3 in acetonitrile) and the mixture was vortexed. The diluted samples were analyzed according to the published procedure [4] using 3 ml Bond Elut Certify HF 300 mg solid-phase extraction cartridges from Varian (Darmstadt, Germany) and the extraction robot RapidTrace from Zymark (Idstein, Germany). The extracts were evaporated to dryness using the Zymark TurboVap LV with 25 °C bath temperature, the residues were transferred with 2× 100 μl methanol into autosampler vials and mixed with 50 μl of 0.1 *M* hydrochloric acid in 2-propanol and evaporated to dryness at 60 °C under a nitrogen stream. The dried extracts were derivatized with 40 μl MBDSTFA for 30 min at 60 °C.

2.3. GC–MS analysis

Gas chromatographic-mass spectrometric (GC–MS) analysis was performed on a Hewlett-Packard (Waldbronn, Germany) HP6890 GC equipped with an autosampler HP6890 ALS and interfaced to a HP5973 MSD mass spectrometer. The analytical column was an OPTIMA-1-MS capillary column (30 m×250 μm I.D., 0.25 μm film thickness) from Macherey and Nagel (Düren, Germany), the carrier gas was helium with a flow-rate of 0.7 ml/min. The GC conditions were as follows: splitless injection mode, 280 °C injection port temperature, temperature program: 55 °C for 2 min, increasing with 20 °C/min to 170 °C, increasing with 12 °C/min to 310 °C and held for 5 min. The MS conditions were as follows: 280 °C transferline temperature, 70 eV ionization energy and 250 °C ion source temperature. Data analysis was performed using HP ChemStation software (Rev. B.01.00).

For analysis of samples, 1 μl was injected and the following analytes were measured in SIM mode (internal standards first, quantifiers underlined): EME-d_3 tBDMS *m/z* <u>85</u>, 185, 316, EME tBDMS *m/z* <u>82</u>, 182, 313, AEME *m/z* <u>152</u>, 181, 122, AEME-d_3 *m/z* <u>155</u>, 184 (artifact from COC-d_3), COC-d_3 *m/z* <u>85</u>, 185, 306, COC *m/z* <u>82</u>, 182, 303, BZE-d_3 tBDMS *m/z* <u>85</u>, 285, 406, BZE tBDMS *m/z* <u>82</u>, 282, 403.

Pooled drug free serum was used for calibration. AEME (2, 5, 10, 20 and 50 μg/l) was calibrated separately from COC, EME and BZE. One ml of the calibrators and of blank serum were analyzed as described above and a linear regression analysis (area ratios of analyte/internal standard) was performed. In the analyses of the COC containing calibrators AEME was produced as artifact. A linear regression was calculated from the area ratios of *m/z* 152 (AEME) and 82 (COC) to establish a calibration curve of COC pyrolysis within a series of analyses (external calibration of pyrolysis).

2.4. Influence of matrix and insert liner quality on the artifact production

To extracts of blank serum (1 ml) COC (50, 100, 250, 500 and 1000 ng) and 100 μl internal standard solution were added. After evaporation the residue was derivatized with 40 μl MBDSTFA and analyzed by GC–MS as described above. In another experiment the same amounts of COC and internal standard solution were mixed, derivatized and analyzed in the same way.

For determination of the intra-assay precision of the artifact production, a solution containing 1000 ng COC and 100 ng of the internal standards in 40 μl MBDSTFA was analyzed five times; the inter-assay precision was tested by analysis of the same solution every 2 days during 2 weeks. A clean liner was inserted before the first injection and was maintained in the gas chromatograph until the end of the experiment. In all experiments the area ratios of *m/z* 152 (AEME) and 82 (COC) and the area ratios of *m/z* 155 (AEME-d_3, derived from COC-d_3) and 85 (COC-d_3) were determined and compared to the COC amounts. A quantitation of the AEME amounts was performed against a calibration of AEME solutions (2, 5, 10, 20 and 50 ng) in 40 μl of MBDSTFA containing 100 ng of the internal standards. The pyrolysis rate was calculated as percentage of the molar ratios of AEME vs. COC.

2.5. Study on the accuracy of the AEME determination

To assess the accuracy of the AEME determination in the presence of COC, extracts of blank serum containing combinations of AEME (0, 2, 5, 10 and 20 μg/l) and COC (0, 50, 100, 500 and 1000 μg/l) were prepared and analyzed as described above on five (0 μg/l AEME), three (2, 5 and 10 μg/l AEME) or two (20 μg/l AEME) occasions. The AEME concentrations in these samples were estimated by two methods: internal standardization and external calibration of the pyrolysis rate. For internal standardization the area of *m/z* 152 of the AEME artifact in a specific sample was estimated by multiplication of the area ratio *m/z* 155/85 (AEME-d_3/COC-d_3, where AEME-d_3 is the pyrolysis product of COC-d_3) with the area of *m/z* 82 (COC). This value was subtracted from the measured area of *m/z* 152 resulting in the value of authentic AEME in the sample. Using this area (negative values were set to zero) the corrected AEME concentration was calculated from the AEME calibration curve (using EME-d_3 as internal standard). For the calculation of AEME in a sample using an external calibration of the pyrolysis rate a linear regression of the area ratios of *m/z* 152/82 in the COC containing calibrators was prepared as described above. The area of *m/z* 152 (AEME artifact) in a specific sample was estimated using the area of *m/z* 82 (COC) in the sample as parameter for the regression equation. By subtraction of the result from the measured area of *m/z* 152 the area of the authentic AEME was estimated and the corresponding concentration determined as described above. From the spiked AEME concentrations and the determined concentrations a linear trend and the 99% prediction interval were calculated using the program SigmaPlot V8.0 (SPSS Inc., Chicago, IL).

2.6. Biological samples

Blood samples from former opiate addicts (now on methadone) were collected at an institution for therapy of drug addicts (Frankfurt/Main, Germany) as part of a health-care program. In cases of supposed crack use an additional blood sample (5.5 ml) was collected in devices (Monovette®, Sarstedt AG and Co, Nümbrecht, Germany) containing potassium fluoride (1.0 mg/ml) and EDTA (1.2 mg/ml) to assess the extent of the cocaine use. The samples were immediately centrifuged and acidified using 30 μl of 2 *M* acetate buffer (pH 4.0) per ml of serum

and stored at −18 °C. In addition blood samples from five autopsy cases were obtained and stored at −18 °C until analysis. The causes of death could be attributed to heroin intoxication by toxicological analysis, all had also used cocaine. Analyses for COC, EME and BZE were performed, the apparent (uncorrected) AEME concentration was determined and a correction for AEME artifact by the internal standardization method was performed as described above.

3. Results and discussion

Since AEME is considered to be a marker of cocaine smoking, the analytical demonstration of its presence in biological samples should be beyond reasonable doubt. AEME is almost exclusively analyzed using GC–MS after solid-phase extraction and derivatization, but this methodology can produce AEME as artifact from COC. The determination of AEME using a calibration curve from extracted serum samples without correction for AEME artifact results in concentrations which are higher than the amounts of AEME originally present in the sample (Fig. 1). In previous reports [5,6,15] this problem of AEME artifact was not considered to be relevant, but it is known that AEME concentrations, particularly in blood or serum, may be lower than 10 μg/l [3,4]. It is evident that pyrolysis of COC during analysis has an adverse effect on the assay of AEME.

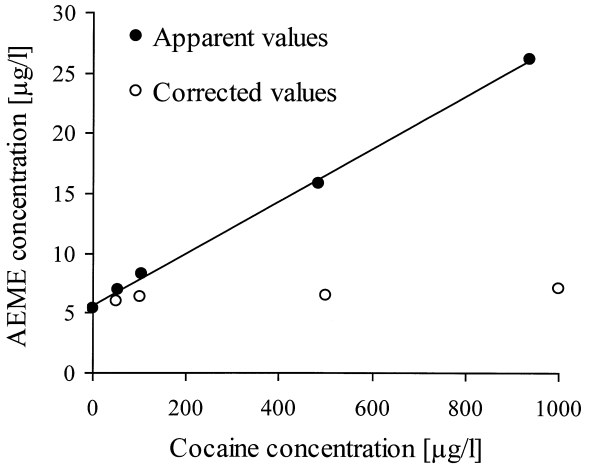

Fig. 1. Results of AEME determination in five serum samples each containing only 5 μg/l of AEME and increasing concentrations of COC. The closed circles represent the apparent concentrations as calculated from the calibration curve of extracted calibrators. The regression line indicates the linear increase with the COC concentration (regression coefficient 0.999). The open circles demonstrate the quantitative estimates obtained from artifact correction using internal standardization.

3.1. Factors influencing artifact production

The results of the experiments indicated a linear relationship of the amount of AEME artifact with the amount of COC injected (cf. Fig. 1). When the AEME artifact was quantitated molar ratios of the concentration of AEME artifact and COC were found to be in a range of 1.0–6.8% which corresponds to results published by Gonzalez et al. [16]. The pyrolysis rate in the presence of serum was not significantly different from that in the absence of serum (3.9±0.5% vs. 4.9±1.4%, each $n=5$ various COC concentrations). The pyrolysis rate of 1000 ng COC in 40 μl MBDSTFA varied by 1.7% in five consecutive analyses, but the rate increased remarkably in six injections every 2 days during 2 weeks of continuous usage yielding a variation coefficient of 40.7% (range of molar ratios: 1.2–4.5%).

From these results we conclude that the degree of pyrolysis depends on the state of the insert liner and that in analyses for AEME the use of a clean insert liner is recommended to minimize artifact production.

3.2. Correction of quantitative results for AEME artifact

Pyrolysis can be neglected in the determination of COC concentration, as pyrolysis proportionally affects the COC calibration and is balanced by the internal standard. However, to determine the authentic AEME concentration in a sample in the presence of COC requires the subtraction of the area of the AEME artifact from the total AEME area before using the calibration of AEME. There are two methods to estimate the area of AEME artifact: assessing the actual pyrolysis rate by using an internal standard or by using an external calibration. Gonzalez et al. [16] stated that the thermal degradation of the internal standard balances the degra-

dation processes, therefore internal standardization can be accomplished by measuring the pyrolysis rate of the internal standard COC-d_3 to its artifact AEME-d_3 as first described by Cone et al. [5]. However, with solutions of COC and COC-d_3 (100 or 500 ng/40 μl) it was verified, that the pyrolysis rates of COC and COC-d_3 were equal ($n=5$ each, Student's *t*-test). For an external calibration of the pyrolysis rate extracts with increasing COC concentrations should be analyzed and the area of AEME artifact measured. From both methods a pyrolysis rate can be determined, which can be used in combination with the COC area in a specific sample to estimate the area of AEME artifact in this sample. However, internal calibration would be most desirable, because it measures pyrolysis in an individual sample, although low amounts of AEME-d_3 artifact may be difficult to assay, for example in matrix-laden extracts from post-mortem material. In these cases external calibration of the pyrolysis rate may be an alternative. Whether the correction is sufficiently reliable to distinguish between AEME-negative and -positive samples, this problem has been approached by extraction of a series of serum samples containing no or low AEME amounts and no or increasing COC amounts. Results of the correction method using internal standardization or external calibration are demonstrated for one AEME concentration in Fig. 1 and are shown in Fig. 2 with 99% prediction intervals. As expected, internal calibration produced slightly more precise results than external calibration. At 0 μg/l AEME and increasing COC concentration ($n=5$ concentrations on 5 days) false positive results were obtained in 56% of the analyses with external calibration (values up to 1.1 μg/l) and in 28% of the analyses with internal standardization (values up to 2.0 μg/l). At a 2-μg/l AEME level 20% of the results were false negative ($n=5$ COC concentrations on 3 days), but AEME concentrations of 5 μg/l or more always produced positive results ($n=5$ COC concentrations on 3 days). However, from the upper 99% prediction levels it may be concluded that corrected AEME concentrations of more than 3.0 μg/l (internal calibration) or more than 4.6 μg/l (external calibration) are highly indicative for smoking of cocaine. From the lower 99% prediction limits it can be concluded that AEME concentrations above 3.5 μg/l (internal standardization) or 4.8 μg/l (external calibration) may produce positive results in 99% of the cases. The slopes of the two curves (spiked vs. determined concentration) were both about unity indicating that there is no systematic error, but only statistical uncertainty. In the case of internal calibration the calculated 99% prediction limits were about ±3.1 μg/l of the mean which is not acceptable

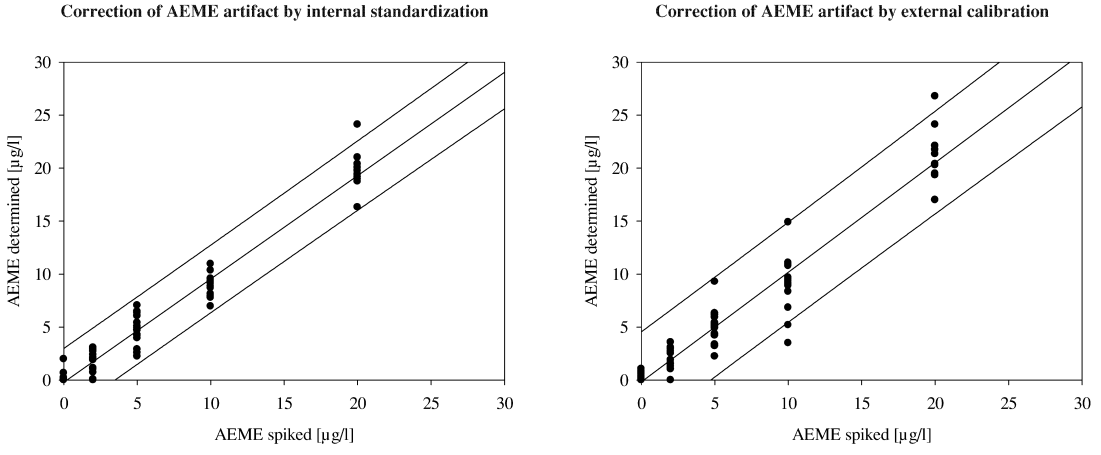

Fig. 2. Accuracy of the correction methods applying internal standardization or external calibration of the pyrolysis rate. Blank serum was spiked with different concentrations of AEME (0–20 μg/l) and each concentration was analyzed in the presence of increasing amounts of COC (0, 50, 100, 500 and 1000 μg/l). The AEME concentrations determined are plotted against the spiked concentration, the regression line with 99% prediction intervals (dashed lines) are shown.

at concentrations below 10 μg/l (30% accuracy), but acceptable at higher AEME concentrations. In the case of external calibration the 99% prediction limits were about ±4.5 μg/l of the mean which yields acceptable accuracies above 15 μg/l.

3.3. Analysis of authentic samples

German regulation requires that blood samples for the analysis of ethanol are collected in containers without any additives. As a consequence in cases of driving under the influence of drugs cocaine itself is rarely detected due to its in vitro degradation [17]. In the present study, COC and its metabolites were measured in 16 blood samples from cocaine/crack addicts who were treated daily with methadone for opiate dependence and in five postmortem blood samples of crack users. With respect to the detection of AEME, COC was regarded as the only source of AEME artifact in authentic serum samples as other aryl ester derivatives of COC like hydroxy-cocaine or cinnamoyl-cocaine isomers were not present in relevant concentrations (confirmed by MS screening in the full scan mode). However, in contrast to previous reports [4,16] we found that EME as tBDMS derivative may produce AEME artifact, but only at a very low rate (0.1%) which was neglected. Correction for AEME artifact was performed by internal standardization of pyrolysis, the quantitative results for COC, EME, BZE and AEME with and without correction, are given in Table 1.

Cocaine was present in all samples in a range of 5–1092 μg/l, but AEME was also detected in all samples. However, when these values were corrected for AEME artifact smoking of cocaine could be confirmed in ten of the 21 samples (12–21). In the 11 negative samples an increase of the apparent (uncorrected) AEME concentration with the COC concentration was noticed yielding values from 1 to 23 μg/l. The finding that concentrations in six of ten

Table 1
Concentrations of AEME with and without correction for artifact, COC, EME and BZE in serum samples (1–16) and postmortem blood samples (17–21). The apparent concentration of AEME was calculated using the uncorrected area of *m/z* 152 (AEME) with the AEME calibration curve. In cases, where the corrected AEME concentration was below 10 μg/l (30% accuracy) "+" indicates the presence of AEME (the calculated concentration is given in parentheses to illustrate the effect of the correction method)

Sample #	AEME (μg/l) (corrected)	AEME (μg/l) (apparent)	COC (μg/l)	EME (μg/l)	BZE (μg/l)
1	0	1	47	19	367
2	0	2	49	22	372
3	0	2	52	22	371
4	0	3	103	59	1033
5	0	5	227	200	2233
6	0	7	367	106	2066
7	0	7	389	58	1353
8	0	15	504	139	1825
9	0	10	505	172	2501
10	0	23	514	64	607
11	0	22	1092	214	3458
12	+(9)	13	43	41	1074
13	+(3)	5	46	43	596
14	+(7)	9	53	108	996
15	+(6)	11	72	102	548
16	+(8)	14	116	31	517
17	110	110	5	546	3475
18	14	14	7	72	685
19	26	26	12	113	1006
20	472*	475*	43	379	1752
21	80	94	389	1587	2885

*The value was above the linear range tested (250 μg/l, cf. [4]) and is given as an estimate.

AEME positive cases were below 22 μg/l illustrates again the necessity to consider AEME as artifact (cf. sample 11).

In the five samples from living subjects the quantitative estimates of AEME were below 10 μg/l (30% accuracy limit), but greater than 3 μg/l (upper 99% confidence interval) suggesting positive results. The quantitative estimates are within the range of concentrations previously reported [4,18]. The AEME concentrations in the five postmortem cases were all above 10 μg/l, three exceeded 63 μg/l [19] which is the highest value reported in blood up to date. As previous investigations suggested that AEME is rather stable in biological specimens [4,20,21] it may be assumed that no systematic difference exists between AEME concentrations in serum from living subjects or postmortem blood except for the plasma/whole blood ratio.

In the postmortem cases (17–21) the high concentrations of AEME indicate that large amounts of AEME can be absorbed and may still be present when COC is almost completely degraded. However, the lack of correlation in the concentrations of COC and AEME in the stabilized samples 12–14 and 20, where the COC concentrations are very similar, or in the samples 12, 15, 16 and 18, where the estimated AEME concentrations are very similar, indicates a marked interindividual variation in the processes of AEME absorption or elimination. In two samples with very high BZE concentrations (17 and 21) the AEME concentrations were also very high. This may be explained by the assumption of a repeated crack use in short intervals where BZE and possibly also AEME accumulate in the body.

In half of the authentic samples positive for AEME the AEME concentrations after correction for the artifact were below the 30% accuracy level. However, the correction of COC pyrolysis to AEME using the internal standardization method was found to be efficient in discriminating between smokers and non-smokers of cocaine and can be used for the quantitation of higher AEME concentrations.

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