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Short communication

Gas chromatographic–mass spectrometric detection of anhydroecgonine methyl ester (methylecgonidine) in human serum as evidence of recent smoking of crack

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Abstract

The discrimination between smoking of crack and other routes of cocaine application has forensic implications. The pyrolysis product anhydroecgonine methyl ester (AEME, methylecgonidine) has been found to be a marker for smoked cocaine. An improved method for the determination of AEME in serum was developed, consisting of mixed phase solid-phase extraction and GC–MS. Special care was taken for the volatility of AEME and *tert*.-butyldimethylsilylation was used for derivatization. Thus AEME could be determined for the first time in 13 serum samples from living subjects. The concentrations found were in a range of 3 to 34 ng/ml, a correlation with the storage time of the samples or with benzoylecgonine concentrations could not be found.

Keywords: Anhydroecgonine methyl ester; Methylecgonidine; Cocaine

1. Introduction

The widespread abuse of cocaine has forensic implications, e.g., in crimes and in cases of driving under the influence of drugs. Cocaine is usually administered by nasal insufflation (snorting), injection or inhalation (smoking). The inhalation of the cocaine base (''crack'') exhibits basic differences when compared with the other routes of administration, e.g., under forensic aspects: (1) inhalation and snorting of cocaine leave no marks on the body like injections, (2) the occurrence of psychic effects after nasal application is substantially delayed (0.5 h) compared to injection or inhalation (few minutes), and (3) inhalation of cocaine [1] is supposed to have a higher potential for addiction [2], moreover, a higher rating for ''high'' and craving is reported [2].

Anhydroecgonine methyl ester (AEME, methylecgonidine) has been proposed as an analytical marker for heated cocaine [3–6]. It is produced only in the process of smoking cocaine and is not formed metabolically. AEME has been detected in urine [3–5,7,8], saliva [5,9], sweat [5] and hair [5,7] from crack smokers but up to now it has only been detected in two blood samples from corpses [4]. The amount of AEME formed in the process of crack smoking is dependent on the temperature applied, 2% to 89% of a cocaine dose may reach the alveoli

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in the lungs as AEME [10]. It is excreted into urine, saliva and sweat indicating that significant amounts are absorbed into the blood circulation. This paper describes a sensitive procedure for the assay of AEME in blood/serum samples with gas chromatography–mass spectrometry (GC–MS); for the first time AEME concentrations in serum samples of living subjects are reported.

2. Experimental

2.1. Chemicals, reference standards and apparatus

Solutions of the reference standards (1 mg/ml) cocaine (COC), benzoylecgonine (BZE), ecgoninemethylester (EME), AEME, anhydroecgonine (AE), ecgonine (ECG) and of the corresponding deuterated internal standards (0.1 mg/ml) cocaine-d_3 (COC-d_3), benzoylecgonine-d_3 (BZE-d_3) and ecgoninemethylester-d_3 (EME-d_3) were from Radian (Promochem, Wesel, Germany), the derivatization reagents *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MBDSTFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*-methyl-bis-(trifluoroacetamide) (MBTFA) and pentafluoropropionic acid anhydride (PFPA) from Macherey and Nagel (Dueren, Germany). All other reagents and organic solvents were of analytical grade and from Merck (Darmstadt, Germany).

GC–MS analyses were performed on a Hewlett-Packard (Waldbronn, Germany) HP6890 GC system equipped with an autosampler HP6890 ALS and interfaced to a HP5973 mass-selective detector. The GC conditions were as follows: HP-5 MS capillary column (30 m×250 μm I.D., 0.25 μm film thickness), helium as carrier gas with a flow-rate of 0.7 ml/min, splitless injection mode, 250°C injection port temperature, temperature program: 55°C for 2 min, with 20°C/min to 170°C, with 12°C/min to 310°C and hold for 5 min. The MS conditions were as follows: 280°C transferline temperature, 70 eV ionization energy, 250°C ion source temperature. Data analysis was performed on a Windows NT 4.0 computer with HP ChemStation software (Rev. B.01.00).

2.2. Solid-phase extraction procedure for basic analytes in blood/serum

Blood samples were centrifuged for 10 min at 1900 *g* for serum separation. One ml of serum was diluted with 4 ml of 0.1 *M* phosphate buffer, pH 6.0, 100 μl of internal standard solution (1 ng/μl Coc-d_3, BZE-d_3, EME-d_3 in acetonitrile) was added and finally vortexed. The diluted samples were extracted 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 extraction protocol was as follows: conditioning with 2 ml methanol and 3 ml phosphate buffer, 5 ml of sample was loaded onto the column at 1 ml/min, the column was rinsed with 2 ml 0.1 *M* acetic acid and 3 ml methanol at 1.5 ml/min and the analytes were eluted with 3 ml of daily prepared methylene chloride–2-propanol–ammonium hydroxide solution (80:20:2, v/v/v) at 1 ml/min. The extracts were evaporated to dryness using the Zymark TurboVap LV with 25°C bath temperature and the residues were transferred with 2×100 μl methanol into autosampler vials, 50 μl of 0.1 *M* hydrochloric acid in 2-propanol was added and evaporated at 60°C under a nitrogen stream.

2.3. Optimization of the derivatization

Drug-free serum was spiked with AEME to 5 ng/ml and two aliquots were extracted as described above. One extract was derivatized for 30 min at 60°C with 40 μl MSTFA and another extract with 40 μl MBDSTFA. One μl of both extracts was analyzed by GC–MS in the selected ion monitoring (SIM) mode for AEME (*m/z* 152, 181, 122, 138, 166).

2.4. Method for the quantitative assay of AEME with GC–MS

The dried extracts were derivatized with 40 μl MBDSTFA for 30 min and 1 μl of the solutions was analyzed by GC–MS in SIM mode for the following analytes (internal standards first, quantifiers underlined): COC-d_3 *m/z* 85, 185, 306, COC *m/z* 82, 182, 303, BZE-d_3 *m/z* 85, 285, 406, BZE *m/z* 82, 282, 403, AE *m/z* 224, 252, 281, ECG *m/z* 356, 398, 413,

EME-d_3 *m/z* 85, 99, 316, EME *m/z* 82, 96, 313, AEME *m/z* 152, 181, 122. Fragments of injection port artifacts of COC-d_3 (AEME-d_3 *m/z* 155, 184) and of BZE-d_3 (AE-d_3 *m/z* 227) were also detected.

For calibration, a pooled drug-free serum was spiked with AEME to 2.5, 5, 10, 25, 50, 125 and 250 ng/ml. One ml of the calibrators and of blank serum was analyzed as described above and a linear regression analysis (analyte/internal standard area ratios) was performed. The analytical recovery was determined using five 1-ml portions of a blank serum spiked with AEME to 100 ng/ml. For determination of the accuracy and of the intra-day and inter-day precision of the method, five 1-ml portions each of a blank serum spiked with AEME to 20 ng/ml were used.

2.5. Relevance of AEME as artifact in the GC injection port

To study the dependence and the amount of AEME produced as artifact from cocaine in the GC injection port, solutions of cocaine were injected in varying amounts (10, 25, 50, 75, 100, 250, 500, 750 and 1000 ng cocaine in 40 μl acetonitrile with each solution containing 100 ng COC-d_3). To study whether AEME is produced as an artifact from derivatives of EME, 2 μg of EME were derivatized with 40 μl of MSTFA, MBDSTFA, MBTFA and PFPA for 30 min at 60°C. One μl of the solutions was analyzed by GC–MS for the following analytes in SIM mode (quantifiers underlined): COC-d_3 *m/z* 85, 185, 306, COC *m/z* 82, 182, 303 and AEME *m/z* 152, 181, 122. In addition, *m/z* 155 was recorded which derives from the COC-d_3 artifact AEME-d_3. The amounts of the artifact AEME were quantitated after area correction with the internal standard COC-d_3 using a calibration with solutions of 5, 10 and 25 ng AEME and 100 ng COC-d_3 in 40 μl acetonitrile.

2.6. Authentic samples

Blood samples from a pool of forensic samples stored at 4°C without further stabilization were analyzed for AEME using the method as described above. Samples were selected from cases in which crack abuse was claimed (nine cases) and samples in which relatively high benzoylecgonine had been determined previously by routine screening procedures (20 cases).

3. Results and discussion

3.1. Influence of the derivatization

In order to detect AEME (mass spectrum shown in Fig. 1) together with cocaine and its metabolites in serum, a derivatization step is necessary. Trimethylsilylation with BSTFA+1% trimethylchlorosilane (TMCS) is mainly used (e.g., Refs. [3–5]), since hydroxyl- and carboxylic groups of all cocaine metabolites are readily derivatized. AEME is not derivatized under these conditions, however, we found that with trimethylsilylation matrix compounds were eluted in the same time range as AEME, which interfered considerably with sensitivity. Derivatization with *tert*.-butyldimethylsilyl groups by MBDSTFA is an alternative to trimethylsilylation because this procedure leads to derivatives with high molecular masses which exhibit appropriate fragmentation [11]. Through the higher masses of the derivatized matrix compounds, a better selectivity for AEME could be achieved (Fig. 2).

3.2. Extraction and quantitative determination of AEME in blood/serum

Solid-phase extraction with mixed phase columns like the Bond Elut Certify HF from Varian has been found to be useful for sensitive determination of basic analytes like cocaine, its metabolites or other

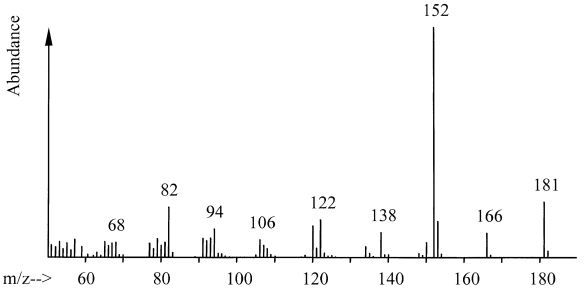

Fig. 1. Mass spectrum of anhydroecgonine methyl ester.

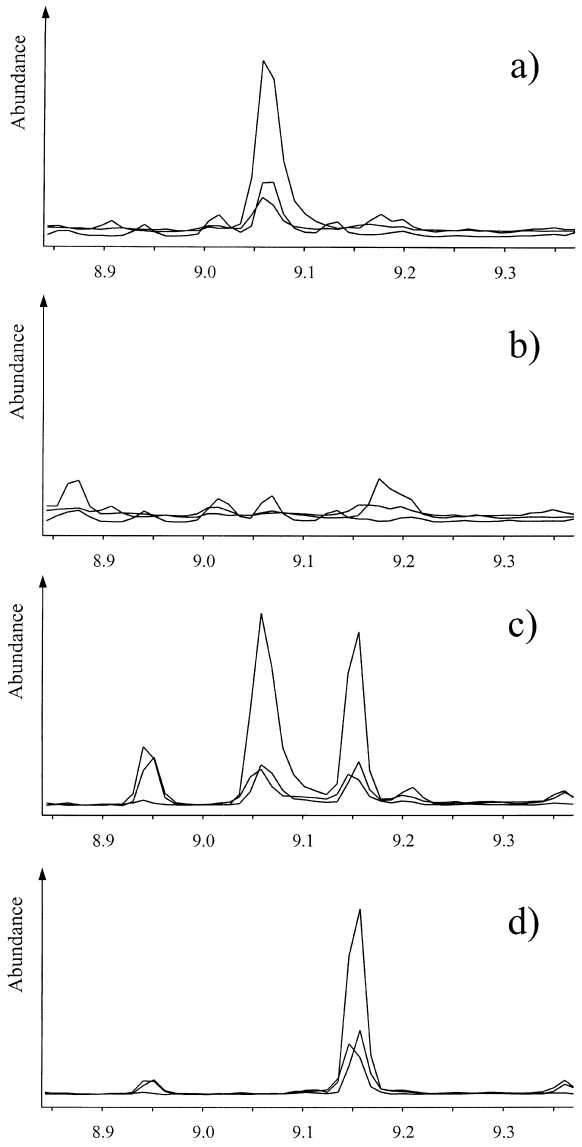

Fig. 2. Mass fragmentograms (m/z 152, 181, 122) of AEME in extracts of (a) 5 ng/ml AEME in blank serum, derivatized with MBDSTFA, (b) blank serum, derivatized with MBDSTFA, (c) 5 ng/ml AEME in blank serum, derivatized with MSTFA, (d) blank serum, derivatized with MSTFA; abundances are in equal scale in all graphs.

drugs of abuse (reviews: [12,13]). For the quantitation of AEME, EME-d_3 was selected as internal standard, since both compounds were found to be volatile. In the absence of hydrochloric acid more than 98% of 2 μg AEME (in 200 μl methanol) was lost during evaporation at 60°C, the same was observed for EME. Therefore special care was taken for their volatility during the evaporation steps (25°C, 0.1 *M* hydrochloric acid in 2-propanol). A relevant hydrolysis of AEME, COC, BZE or EME in the presence of hydrochloric acid at 60°C was excluded.

In the present procedure, the analytical recovery of AEME was 86±2% ($n=5$). The calibration was limited to 250 ng/ml as higher values were not to be expected. The calibration was linear in a range of 2.5 to 250 ng/ml ($y=0.181\cdot x$, correlation coefficient 0.998). The limit of detection was found to be 1 ng/ml ($S/N>3$). For the determination of accuracy and precision, a concentration of 20 ng/ml AEME was selected. The accuracy was assayed to be 6.5%, the intra-day precision 5.6% and inter-day 13.3% ($n=5$ each).

3.3. Relevance of AEME as a GC injection port artifact

AEME is produced during thermal decomposition of cocaine by elimination of benzoic acid when heated to over 170°C [14]. This occurs if crack is smoked, but also in the hot GC injection port (250°C). Cone et al. [3] suggested to measure the artifact production by recording AEME-d_3, which is an injection port artifact from the internal standard COC-d_3. They calculated the area ratio of m/z 155 (AEME-d_3) and 185 (COC-d_3) which they found to be consistently less than 1% and therefore considered to be not relevant. Since he observed this phenomenon only in extracts, which contained 100 ng COC-d_3 in 40 μl, we analyzed cocaine solutions of increasing concentrations to estimate to what extent artifact production during GC analysis might contribute to the amount of AEME deriving from pyrolysis during smoking. Cocaine was dissolved in 40 μl acetonitrile which was equal to the final volume of extracted serum samples. The artifact AEME was assayed and the ratios of the areas of m/z 152 and 182 were calculated according to Cone et al. [3]. In a regular serum analysis, the artifact AEME would be determined using a calibration curve from serum extracts and would be mistaken as original analyte. Therefore, the AEME produced in the present study can be interpreted as the original serum concen-

trations which are thus given in the following as ng/ml.

Our results differed slightly from those of Cone et al. [3] in the respect that the area ratio *m/z* 152/182 was found to be 1.5±0.7% ($n=9$). We also observed, that the AEME production in the injection port showed a linear correlation with cocaine concentrations up to 1000 ng/ml ($y=0.0061 \cdot x$, correlation coefficient 0.997). This was confirmed by the findings that all ratios of *m/z* 152/182 (AEME from different COC concentrations) were almost identical to the corresponding ratios of *m/z* 155/185 (AEME-d_3 from consistently 100 ng COC-d_3 internal standard). Thus from 100 ng/ml cocaine an amount of artificial AEME equivalent to 0.6 ng/ml can be expected. It appears that the extent of artifact production depends on the injection port type, so other analysts might experience a lower or higher degree.

Our results indicate that artifact production in the GC injection port can influence the AEME assay. According to Cone et al. [3] the area ratio of *m/z* 155/182 (internal standard) should be used to estimate the actual amount of artifact produced. At high COC concentrations this poses a real problem. Therefore, exact AEME assay is possible in cases only, where no cocaine is present or its concentration is low.

Derivatives of EME could be considered as another potential source of AEME as an artifact during GC analysis. But after injection of even high concentrations of derivatized EME, no AEME was detected (2 μg EME trimethylsilylated, *tert*.-butyldimethylsilylated, trifluoroacetylated or pentafluoropropionylated).

3.4. Authentic serum samples

AEME had been found only twice in blood samples from corpses with concentrations of 44 and 63 ng/ml [4]. For a first screening approach, samples from forensic cases were selected and reanalyzed specifically for AEME. Cases were selected where the abuse of crack was claimed and cases where high benzoylecgonine concentrations had been detected. In a total of 29 samples, AEME has been detected in 13 (Table 1). The presence of AEME artifact could be excluded, as cocaine was present only in two samples (1 and 16 ng/ml, Table 1, Nos. 2 and 10), a consequence of the lack of a stabilization reagent (in Germany, forensic blood samples usually do not contain fluoride). The concentrations of AEME were in a range between 3 and 34 ng/ml with a median at 11 ng/ml. The highest serum concentrations were still lower than those reported in the two cases (post mortem blood). In eight of the nine cases where crack abuse was claimed, the presence of AEME could be confirmed. In the case not confirmed, an acute cocaine abuse was not proved.

Table 1
AEME, COC, BZE and EME concentrations in 13 positive cases out of 29 authentic samples which have been reanalyzed for AEME (sorted by AEME concentration in ascending order)[a]

No.	Claims on arrest	Storage time	AEME (ng/ml)	COC (ng/ml)	BZE (ng/ml)	EME (ng/ml)
1	3–4 stones	3	3	0	45 (440)	7
2	Crack	0	4	16	1692	225
3	Crack	16	5	0 (5)	2 (440)	1
4	No comment	0	7	0	595	145
5	Crack pipe found	0	7	0	328	52
6	Crack	3	8	0	19 (100)	10
7	No comment	1	11	0	1250 (1750)	143
8	Crack	22	13	0	2 (820)	3
9	One stone	19	13	0 (3)	2 (620)	3
10	No comment	1	13	1	1247 (1750)	143
11	No comment	0	23	0	232	165
12	Four stones	6	29	0 (2)	17 (1890)	7
13	No comment	0	34	0	740	140

[a] Results of the previous analyses are given in parentheses; the storage time before reanalysis of the samples is given in months.

In six samples which had been stored for more than three months before reanalysis, a considerable decrease in the concentrations of COC, BZE and EME (Table 1, Nos. 1, 3, 6–10, 12) was found, which is a common problem for these analytes [15–18]. It is surprising that the AEME concentrations are not correlating with the storage time of the serum samples or the BZE concentrations. Even at a low BZE concentration, a relatively high AEME concentration was found (Table 1, No. 11). In four samples stored for more than six months (Table 1, Nos. 3, 8, 9, 12), the AEME concentrations were even higher than the residual amounts of BZE or EME, suggesting that AEME is more stable than COC, BZE and EME. This can be explained by the fact that EME undergoes chemical hydrolysis in samples with pH values of 7.4 and above [15,19]. On the other hand the methylester group in AEME is resonance-stabilized, probably the reason for its higher hydrolytic stability. In an incubation study with 2 μg AEME in blank serum at ambient temperature over two weeks, we found a decrease of 19% per week, which is very slow in comparison to cocaine [15,16].

The assay proposed differs in two aspects from previously published methods [3–5]: care for the volatility of AEME and the derivatization with MBDSTFA. These improvements enabled the assay of AEME in serum samples of living subjects. In cases where crack abuse was claimed and recent cocaine abuse was proved, this could be confirmed by the detection of AEME. This compound seems to be stable towards hydrolysis, because it is possible to detect it even in old serum samples.

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