Studies on Hydrolytic and Oxidative Metabolic Pathways of Anhydroecgonine Methyl Ester (Methylecgonidine) Using Microsomal Preparations from Rat Organs

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During smoking of cocaine-base (crack), anhydroecgonine methyl ester (AEME, methylecgonidine) is formed in large amounts as a pyrolysis product of cocaine and is absorbed in the lungs. The metabolism of AEME was studied in the present investigation using microsome preparations from rat liver, lung, kidney, and brain. Potential metabolites of AEME were synthesized and used as substrate to complement the experiments. Analysis of the incubation mixtures was performed using gas chromatography−mass spectrometry and nanoelectrospray multiple-stage mass spectrometry. Screening for metabolites was focused on postulated oxidative pathways, chemical and enzymatic hydrolysis, and ethanol dependent transesterification as known from cocaine metabolism. Enzymatic hydrolysis of AEME to anhydroecgonine (AE), which was inhibited by sodium fluoride, was found in all microsomal preparations. Liver microsomes exhibited the highest activity, brain microsomes the lowest. Anhydronorecgonine methyl ester (ANEME) and anhydroecgonine methyl ester *N*-oxide were identified as AEME metabolites of liver and lung microsomes only. In the presence of ethanol AEME was metabolized to anhydroecgonine ethyl ester and anhydronorecgonine ethyl ester. Further metabolism of AE or ANEME was not observed. No *N*-hydroxy-anhydronorecgonine derivatives were found which could represent precursors of cytotoxic metabolites as known to be formed from cocaine.

Introduction

During crack smoking, cocaine (COC)[1] and its main thermal breakdown product anhydroecgonine methyl ester (AEME, methylecgonidine) are absorbed in the lungs (*1*). Though there are some reports on potential metabolic products of AEME, the metabolism of AEME has not yet been studied. It is known that AEME can be hydrolyzed enzymatically to anhydroecgonine (AE, ecgonidine) in human liver homogenate (*2*). In vitro degradation of AEME to AE has been observed in sheep plasma (*3*) and in human plasma due to butyryl cholinesterase and nonenzymatic processes (*4*). Metabolites of oxidative pathways have also been identified: anhydroecgonine methyl ester *N*-oxide (AEMENO) has recently been detected in incubations with rat liver microsomes and in blood and urine samples from crack users (*5*). Anhydronorecgonine methyl ester (ANEME) was found in a urine sample from a cocaine user (*6*), however, it cannot be excluded that this metabolite was an artifact produced from norcocaine during analysis by gas chromatography.

AEME may be metabolized by similar routes as COC. The main in vivo metabolism of COC is its hydrolysis to benzoylecgonine by liver carboxylesterase (in humans: hCE-1). In the presence of ethanol, this enzyme also catalyzes the ethyl transesterification to cocaethylene (*7*). Dean et al. (*8*) reported that the supernatants of rat liver, lung, kidney, and heart homogenates also exhibited cocaine methyl esterase and ethyl transferase activities. COC is also metabolized through oxidative pathways leading to hepatotoxic metabolites through a multistep cytochrome P450-dependent *N*-oxidative pathway [reviewed by Boelsterli and Göldlin (*9*)]. Through this pathway, COC is *N*-demethylated to norcocaine, which is further oxidized to *N*-hydroxynorcocaine and to norcocaine nitroxide. This pathway was observed following COC injection in mice (*10*−*12*), COC incubation with rat, hamster or mice liver microsomes (*12*−*14*), and rat brain microsomes (*15*). Pellinen et al. (*16*) demonstrated that the first step of cocaine bioactivation is catalyzed by the P450 3A enzymes in both murine and human liver microsomes and that cocaine-induced liver injury in mice may be prevented by the administration of inhibitors of these enzyme isoforms.

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[1] Abbreviations: AEME, anhydroecgonine methyl ester; AEME-d_3, anhydroecgonine methyl ester-d_3; AE-d_3, anhydroecgonine-d_3; AE, anhydroecgonine; ANEME, anhydronorecgonine methyl ester; ANE, anhydronorecgonine; AEMENO, anhydroecgonine methyl ester *N*-oxide; AENO, anhydroecgonine *N*-oxide; AEEE, anhydroecgonine ethyl ester; ANEEE, anhydronorecgonine ethyl ester; COC, cocaine; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; NaF, sodium fluoride; nanoESI-MS^n, nanoelectrospray multiple-stage mass spectrometry.

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The aim of the present paper was a systematic study of the metabolism of AEME, AE, and ANEME using microsome preparations from liver, lung, kidney, and brain of rats.

Experimental Procedures

Chemicals, Reference Standards. The solutions of the reference standards (1 mg/mL acetonitrile) AEME, AE, and norcocaine were from Cerilliant (Promochem, Wesel, Germany), AEME-d_3 and AE-d_3 were from Lipomed (Arlesheim, Switzerland), and the derivatization reagent MSTFA was from Macherey & Nagel (Dueren, Germany). All other reagents and organic solvents were from Merck (Darmstadt, Germany), and biochemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) in the highest purity available.

Reference substances for the postulated metabolites ANEME, ANE, AEMENO, AENO, AEEE, and ANEEE were synthesized as described below.

Apparatus. 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. The GC conditions were as follows: Macherey & Nagel OPTIMA-1-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 at 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, and 250 °C ion source temperature. Data analysis was performed on a Windows computer using HP ChemStation software (Rev. B.01.00).

Nanoelectrospray multiple-stage mass spectrometric (nanoESI-MS^n) experiments were performed with a LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark). Analysis was performed in the positive ion ESI mode, using self-made and gold-coated glass capillaries (opening 1−5 μm), in which the liquid samples (4 μL) were introduced. The capillaries were made with a Micropipet Puller Model P-97 (Sutter Instruments Co., Novato, CA), and gold covering was performed with an EMITECH K550.

Incubations with Rat Tissues Microsomes. Three male Sprague−Dawley rats (3 months old) were anesthetized with enflurane, and liver, kidneys, lungs, and brain were excised and immediately frozen in liquid nitrogen. After homogenization of the pooled organs in 3 vol of 0.1 M phosphate buffer (pH 7.4), microsomes were isolated by centrifugation at 10000*g* (30 min) and at 100000*g* (70 min), resuspended in 0.1 M phosphate buffer (pH 7.4), and again centrifuged at 100000*g* (70 min). The pellets formed were stored at −80 °C until use. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany) and applying bovine serum albumin as standard. The microsome preparation from liver contained 7.9 mg of protein/mL, from kidneys 4.7 mg/mL, from lungs 2.8 mg/mL, and from brain 2.1 mg/mL, respectively. Incubation of AEME or its metabolites with microsomes was essentially performed as described by Kraemer et al. (*17*). The incubation mixture consisted of an amount of microsome preparation equivalent to 0.75 mg of protein, 150 μM substrate (AEME, AE, ANEME, or ANE as 1 mg/mL solution in acetonitrile), 1.2 mM NADP, 2 units of isocitrate dehydrogenase, 5 mM isocitrate, and 5 mM magnesium chloride in 0.1 M phosphate buffer (pH 7.4) up to a total volume of 500 μL. Incubations of AEME with liver microsome preparation were carried out for 15, 30, and 60 min at 37 °C to find out if product formation was linear. Incubations of AEME were carried out for 60 min at 37 °C with microsome preparations from each tissue with or without 200 mM sodium fluoride (n = 5 each) and incubations without substrate or without microsomes were used as control. Incubations of AE, ANEME, or ANE with liver microsome preparation were carried out for 60 min at 37 °C. The reaction was stopped by adding 500 μL of acetonitrile followed by centrifugation for 10 min at 14000*g*. The supernatant was used for GC/MS and nanoESI-MS^n analysis.

The amounts of AE formed in the presence of sodium fluoride (NaF) were used as control for the nonenzymatic hydrolysis as 200 mM NaF completely inhibit the esterases according to Dean et al. (*8*). Enzymatic hydrolysis was calculated by subtraction of the nonenzymatic component of the reaction.

Tests for ethyl transesterification of AEME in rat liver microsomes were also performed in the absence and presence of 200 mM NaF with 5 mM AEME and 250 mM ethanol, according to the optimal incubation conditions for COC as determined by Boyer and Petersen (*18*).

Synthesis of AEMENO and AENO. AEMENO and AENO were synthesized according to the method of Cymerman and Purushothaman (*19*) for synthesis of *N*-oxides from tertiary amines. Due to the thermolability of these compounds, analysis was performed by nanoESI-MS^n, and the identity of the synthesis products was confirmed by interpretation of the product ion spectra as described by Fandiño et al. (*5*).

Synthesis of AEEE. AEEE was synthesized according to the method of Kozikowski et al. (*20*). To 3 g of COC was added 36 mL of 1 N HCl, and the solution was heated at 100 °C for 21 h. The resulting solution was cooled to room temperature and extracted three times with 9 mL of dichloromethane. The organic phase was discarded and the aqueous phase was lyophilized. $POCl_3$ (12 mL) was added to the dry residue, and the mixture was heated at 100 °C for 20 h. The excess $POCl_3$ was removed under reduced pressure, and the receptacle with the resulting dark oil was cooled using a mixture of liquid nitrogen and ethanol (−40 °C). The dark oil was then carefully treated with 7.5 mL of anhydrous ethanol and was stirred for 67 h. After the oil was warmed to room temperature, it was stirred for another 20 min and concentrated under reduced pressure. The residue was dissolved in 6 mL of water (alkalinized with ammonia, pH 10) and extracted with dichloromethane. The aqueous phase was discarded, and the dried organic phase (sodium sulfate) was concentrated and distilled under reduced pressure. The synthesized AEEE was purified and isolated by high performance liquid chromatography [Bruker LC21 pump (Bremen, Germany) with Merck-Hitachi UVD detector L-4000 (Darmstadt, Germany)] using a 16 × 250 mm Eurospher RP 18 column (100 Å, 7 μm particle size, Macherey & Nagel, Dueren, Germany). Elution was performed with a gradient of 0.1% trifluoroacetic acid in water (A) and methanol (B) from 0 to 35% within 35 min and to 100% for another 10 min. Fractions were collected during 7 min intervals. AEEE (absorption maximum at 220 nm) was eluted between 28 and 35 min and extracted with ether at pH 10. The identity of the substance was confirmed by GC/MS analysis (Figure 1a).

Synthesis of ANE, ANEME, and ANEEE. To 1 mg of norcocaine in a 20 mL vial was added 2 mL of concentrated HCl, and the sealed vial was heated at 110 °C for 24 h. After the solution cooled to room temperature, it was extracted twice with 1 mL of dichloromethane to remove benzoic acid. The organic phase was discarded, and the aqueous phase evaporated to dryness under a stream of air. The identity of the product ANE was confirmed after trimethylsilylation using MSTFA by GC/MS analysis (Figure 1b).

For the preparation of ANEME or ANEEE, ANE was dissolved in methanol (for the synthesis of the methyl ester) or in ethanol (for the synthesis of the ethyl ester), and 0.25 mL of boron trifluoride ethyl etherate (BF3·Et2O) was added. The sealed vials were incubated at 50 °C for 4 h and after cooling to room temperature were evaporated to dryness. The identity of both substances was confirmed after trimethylsilylation using MSTFA by GC/MS analysis (Figure 1, panels c and d).

Extraction Procedure for the Analysis of AEME and Its Metabolites in Microsome Incubation Mixtures. To the microsome incubation mixture were added 4 mL of 0.1 M HCl and 100 ng of internal standards (AE-d_3, AEME-d_3), and they

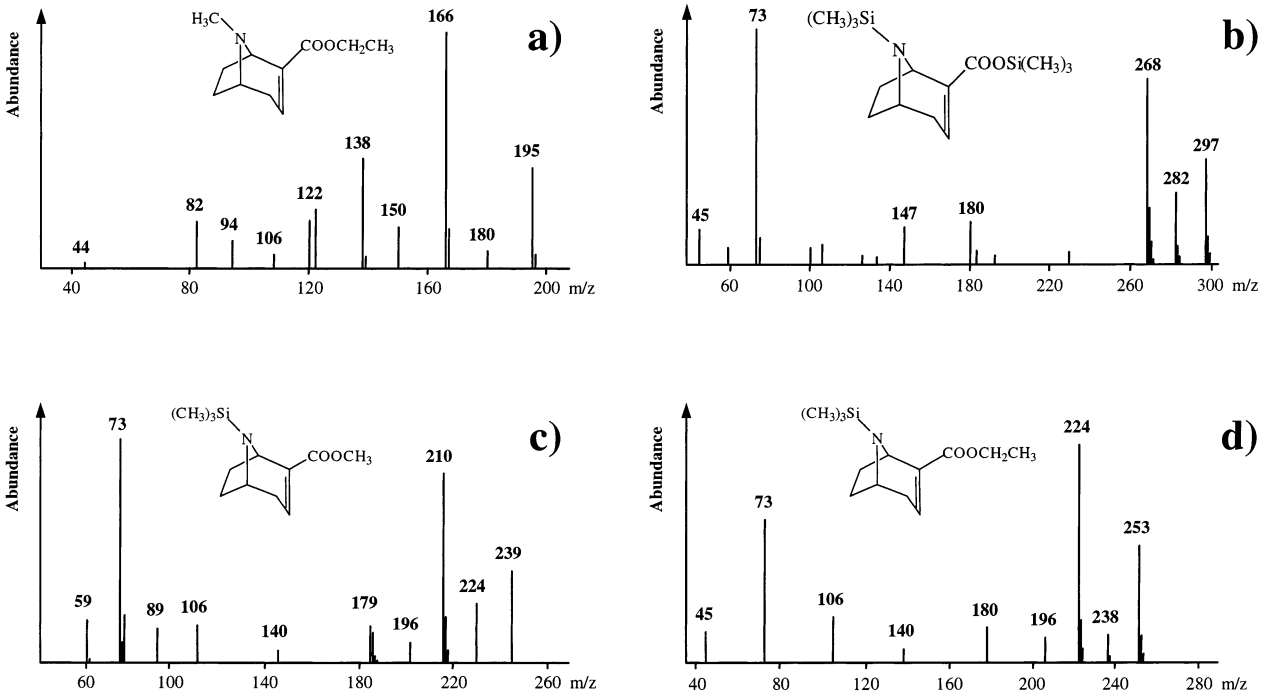

Figure 1. Electron impact mass spectra of trimethylsilylated (a) anhydronorecgonine methyl ester, (b) anhydroecgonine ethyl ester, (c) anhydronorecgonine ethyl ester, (d) anhydronorecgonine.

were vortex-mixed. Extraction was performed using 3 mL of Bond Elut Certify HF 300 mg of 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 of methanol and 3 mL of 0.1 M HCl, loading of the sample onto the column at 1 mL/min, rinsing with 2 mL of 0.25 M acetic acid and 3 mL of methanol at 1.5 mL/min, and elution of the analytes with 3 mL of freshly prepared methanol-ammonium hydroxide solution (98:2, 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 acetone into 1.5 mL reaction tubes. The solutions were centrifuged for 20 min at 14000*g*, and the supernatant was transferred into autosampler vials, and 50 μL of 0.1 M hydrochloric acid in 2-propanol was added and evaporated at 25 °C under a nitrogen stream.

Qualitative and Quantitative Assay of AEME and Its Metabolites with GC/MS. The dry extracts of the microsome incubation mixtures were derivatized with 40 μL of MSTFA for 30 min at 90 °C and 1 μL was injected. GC/MS analysis was first performed in SCAN mode for screening and in selected ion monitoring (SIM) mode for quantifying of the following analytes (internal standard first, quantifiers underlined): AEME-d_3 *m/z* 155, 184, 125, AEME *m/z* 152, 181, 122, ANEME *m/z* 210, 224, 239, AEEE *m/z* 166, 195, 122, ANEEE *m/z* 224, 238, 253, AE-d_3 *m/z* 213, 227, 242, AE *m/z* 210, 224, 239, ANE *m/z* 268, 282, 297. For calibration, phosphate buffer (pH 7.4) was spiked with AEME and AE to 0, 1, 2, 5, 10, 20, 30, 50, 100, 200, 500, and 1000 ng/mL. One milliliter was analyzed as described above, and a linear regression analysis was performed (analyte area/internal standard area). The responses were linear over the range tested with regression coefficients being greater than 0.994. The limit of detection for AEME was 1 ng/mL and for AE 5 ng/mL. The extraction yield was 81±3% for AEME (100 ng/mL, $n = 5$) and 47 ± 7% for AE (100 ng/mL, $n = 5$). The intra-day precision for AEME analysis was 3% and for AE 5% (250 ng/mL serum, $n = 5$), and the corresponding accuracies were 3% for AEME and 8% for AE. The inter-day precision for AEME was 5% and for AE 9% (250 ng/mL serum, $n = 5$ days).

Due to the lack of pure reference standards, a calibration with ANEME, AEEE, ANEEE, and ANE was not possible. For comparison of the incubation mixtures with and without NaF, the relative responses were used (analyte area/AEME-d_3 area).

NanoESI-MS^n Analysis. For nanoESI-MS^n analysis, the dry extracts were dissolved in 100 μL of methanol/water (1:1, v/v) containing 0.1% formic acid. The capillary and tube lens voltages were optimized for analysis of AEMENO and were set to 13.54 and 5 V, respectively. The heated metal-transfer capillary was held at 150°C, and the ionspray voltage at 1 kV. Positive-ion-spectra were averaged over 20 scans, each scan consisting of three microscans. For consecutive MS^n experiments, the relative collision energy in the ion-trap was varied between 20 and 25% corresponding to the LCQ software settings defining the amplitude of the resonance excitation AC voltage, depending on the chemical nature of the precursor ion. Analysis was performed between *m/z* 50 and 400 in full scan mode.

Results

Microsomal Hydrolytic Metabolism. Nonenzymatic hydrolysis of AEME to AE as exhibited in NaF-treated controls was only 1.1 ± 0.1% (0.83 ± 0.09 nmol/incubation) in all tissue microsomes ($n = 20$, 4 tissues with $n = 5$ each) after 60 min of incubation. The formation of AE over 60 min of incubation with liver microsome preparation was linear (regression coefficient 0.993). The amount of AE produced in any incubation sample was reproducible with variation coefficients between 4 and 13% for AEME and between 4 and 11% for AE ($n = 5$ each). The amounts were significantly higher without NaF ($p < 0.01$) than in NaF containing incubation mixtures which indicated that all organs contained esterase activity. However, marked differences in these enzyme activities of the microsome preparations from the four organs were noted (Table 1). The enzymatic activities per milligram of microsomal protein decreased in the order liver > lung > kidney > brain, where liver exhibited 83-fold more activity than brain microsomes, lung 15-fold, and kidney 3-fold. If the protein yields of the microsome preparations (liver 1.4−3.3-fold higher than the others) and the organ weights (liver 7.2−8.7-fold higher than the others) are

Table 1. Enzymatically Produced Anhydroecgonine (AE) during Incubation of Anhydroecgonine Methyl Ester with Rat Tissue Microsomes (n = 5 each, AE concentration corrected for the nonenzymatically produced AE of the control incubations with 200 mM NaF)[a]

microsome preparation	organ weight (g)	protein yield (mg/g)	enzymatic AE (pmol/min/mg)
liver	50.00	20.5	748 ± 29
lung	5.85	8.5	139 ± 6
kidney	6.94	14.3	30 ± 4
brain	5.75	6.5	9 ± 3

[a] The weights of the pooled organs from three rats and the protein yields of the microsomal preparations are given for estimation of relative hydrolyzing capacity of the four organs.

multiplied the much higher esterase content of liver is evident (liver 2279-fold compared to brain, lung 21-fold and kidney 9-fold).

Transesterification with ethanol is another aspect of the hydrolytic metabolism and was studied with microsomes from liver only. In the incubation mixtures with AEME and ethanol, the metabolites AE, ANEME, AEEE, and ANEEE were identified using GC/MS, whereas in the presence of the esterase inhibitor NaF, AEEE and AE were hardly detectable. A quantification of the identified metabolites was not possible due to the lack of pure reference standards.

Microsomal Oxidative Metabolism. In the incubation mixtures of kidney and brain microsomes, no metabolites of oxidative pathways were detected. *N*-Demethylation was observed with liver microsomes and could be increased by inhibition of the hydrolytic metabolism with NaF. In lung microsomes, *N*-demethylation of AEME was observed in the presence of NaF only. Analysis of thermolabile metabolites (e.g., *N*-oxides) using nanoESI-MS^n in the full scan mode revealed an ion with *m*/*z* 198 in addition to *m*/*z* 182 (precursor AEME) and *m*/*z* 168 (subsequent fragmentation gave a mixed spectrum containing the product ions of AE and ANEME). The ion with *m*/*z* 198 was not found in the controls or kidney and brain microsome incubations but only with liver and lung microsomes and was identified to be AEMENO by its product ion spectrum. In the analysis of liver microsome incubation mixtures with AE as substrate using GC/MS and nanoESI-MS^n, no ANE or AENO were detectable.

As it is known for norcocaine, the metabolism to *N*-hydroxynorcocaine and further oxidations are responsible for the cocaine-induced hepatotoxicity (*21*). The extracts of the incubation mixtures were screened for further metabolites of AEME, however, neither in the incubation mixtures with AEME nor in those with the hypothetical precursors ANEME or ANE was further metabolism to *N*-hydroxylated derivatives observed.

Discussion

During the process of crack smoking, the cocaine pyrolysis product AEME is absorbed in considerable amounts together with cocaine. The present paper describes the results of metabolic studies using rat liver, lung, kidney, and brain microsome preparations. As AEME contains some of the structural features similar to cocaine, which are the target of metabolizing enzymes, similar metabolic reactions could be expected. It is known that COC undergoes hydrolysis and transesterification (*7*, *8*), aryl oxidation (*22*), *N*-demethylation, and further *N*-oxidation (*9*).

In the incubation mixtures of rat liver and lung microsomes, the AEME metabolites AE, ANEME, and AEMENO were identified. In the incubation mixtures of rat kidney and brain microsomes, only AE was detected, indicating that these organs contained negligible microsomal oxidizing capacity for AEME. However, it

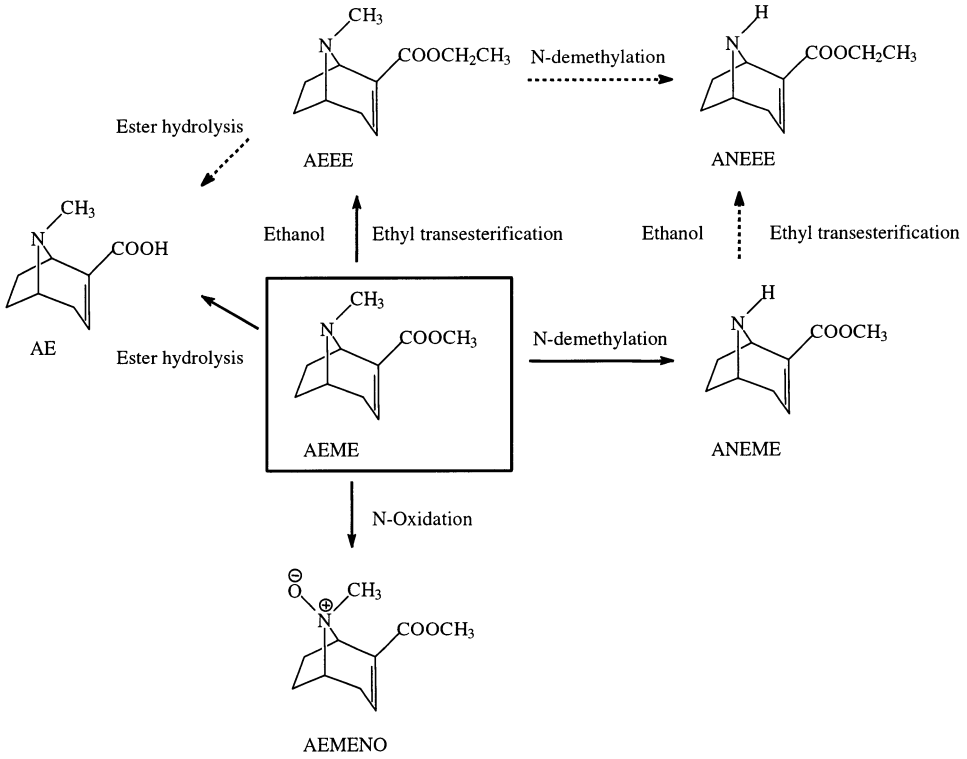

Figure 2. Metabolic pathways of anhydroecgonine methyl ester. Solid arrows show proven pathways, dashed arrows indicate postulated pathways.

cannot be excluded that in specific cells (e.g., proximal or distal tubular cells in the kidney) AEME may be metabolized by P450 enzymes, which was not detected under the conditions used (microsomes were prepared from the whole organs). In the incubation mixtures of liver microsomes with AE, ANEME, or ANE, only the parent compound was detected, and no other metabolites were observed. Figure 2 shows the proposed metabolic pathways for AEME according to our study results.

The highest yield of the hydrolysis product AE was found in liver microsome incubates. If microsomal protein yield, organ weight, and esterase activity of the four organs are considered, it appears that liver has more than 100-fold more hydrolyzing capacity than the lung, kidney, or brain (cf. Table 1). The nonenzymatic hydrolysis at physiological pH was negligible in comparison to the enzymatic route. These findings are in agreement with data from investigations of mammalian carboxylesterases by Satoh and Hosokawa (*23*) who found that the highest hydrolase activity of all tissues studied occurred in the liver. In a study on the stability of AEME in human serum (*4*), slow enzymatic and chemical hydrolysis of AEME was found where 50% of the initial AEME was hydrolyzed to AE within 5 days. It can be concluded that from the four studied tissues, the liver plays the most important role in the hydrolytic metabolism of AEME in vivo.

Like cocaine is metabolized to cocaethylene in the presence of ethanol AEME also undergoes ethyl transesterification to form AEEE. The addition of the esterase inhibitor NaF decreased the formation of AEEE, indicating that a microsomal esterase is involved. Whether the new oxidative metabolite ANEEE is formed from ANEME by ethyl transesterification or from AEEE by *N*-demethylation has still to be investigated.

The enzyme responsible for methyl ester cleavage of cocaine in humans has been shown to be the hepatic microsomal carboxylesterase isoform 1 (*7*). It was also found that the removal of the *N*-methyl group decreases the affinity of COC, benzoylecgonine, or cocaethylene to the enzyme, since the kinetic inhibition constants (K_i) were substantially higher for the *N*-demethylated derivatives norcocaine, benzoylnorecgonine, and norcocaethylene. This can explain our findings that ANE, the hypothetical hydrolysis product of ANEME and ANEEE, was detected neither in incubates of AEME with rat tissue microsomes with and without the addition of ethanol nor in the incubate of ANEME with rat liver microsomes. These results indicate that ANEME has a very low affinity to microsomal esterases.

From the oxidative metabolic pathways assumed for cocaine, only *N*-demethylation to ANEME and *N*-oxidation to AEMENO was observed. The amounts of ANEME produced with liver microsomes were markedly larger than with lung microsomes; in brain and kidney microsomal incubation mixtures no ANEME was detected, probably because the levels of the P450 in brain and of the P450 3A isoform in kidney are only 10% of those in the liver (*15*, *24*). A quantitative determination of AEMENO using nanoESI-MS in addition to the qualitative detection was not possible due to the large variation of quantitative results (poor precision) therefore the amounts of AEMENO produced in the two tissues could not be compared. GC/MS results are also not reliable as the thermal labile metabolite AEMENO is degraded to AEME and carbomethoxycycloheptatriene isomers during analysis (*5*). Therefore, it was not possible to perform a material balance and assess the relative importance of oxidation vs hydrolysis.

In the liver microsomal incubation mixtures with AE as substrate, no oxidative metabolites (ANE, AENO, or others) were detected, which confirms findings of Thompson et al. (*10*) who reported that the polar metabolites benzoylecgonine and ecgonine are *N*-demethylated by liver microsomes much more slowly than cocaine.

In the present study, further oxidative metabolism of ANEME was not observed in any of the incubation mixtures; therefore, a metabolic pathway of AEME leading to hepatotoxic metabolites, like that of cocaine, could not be established. However, up to now nothing is known on the toxicological properties of AEMENO.

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