ON THE METABOLISM OF THE AMPHETAMINE-DERIVED ANTISPASMODIC DRUG MEBEVERINE: GAS CHROMATOGRAPHY-MASS SPECTROMETRY STUDIES ON RAT LIVER MICROSONES AND ON HUMAN URINE

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ABSTRACT:

We describe gas chromatography-mass spectrometry studies of the metabolism of the antispasmodic drug mebeverine [Duspatal, (MB)]. MB is the veratric acid (VA) ester of 4-(ethyl-[2-(4-methoxyphenyl)-1-methylethyl]amino)butan-1-ol (MB-OH), which is an N-substituted ethylamphetamine derivative. The metabolites were first identified in rat liver microsome incubates and then detected in urine samples of volunteers through the use of electron impact and positive chemical ionization gas chromatography-mass spectrometry. Urinary conjugates were enzymatically cleaved before analysis. The following phase I metabolites of MB could be identified: VA, O-demethyl VA (vanillic and/or isovanillic acid), O-bis-demethyl VA (protocatechuic acid), MB-OH, hydroxy MB-OH, O-demethyl MB-OH, O-demethyl-hydroxy MB-OH, N-desethyl MB-OH, N-desethyl-O-demethyl MB-OH, N-de(hydroxybutyl) MB-OH (methoxy-ethylamphetamine), N-de(hydroxybutyl)-O-demethyl MB-OH (hydroxy-ethylamphetamine), and N-bisdealkyl MB-OH (p-methoxy-ampetamine, known as the designer drug PMA). The following, partly overlapping metabolic pathways of MB could be postulated: ester hydrolysis, O-demethylation, ring hydroxylation, N-deethylation, and N-de(hydroxybutylolation). The latter pathway led to ethylamphetamine derivatives and bisdealkylation led to PMA, which are substances of forensic interest. The metabolites containing alcoholic or phenolic hydroxy groups were partly excreted into urine as conjugates.

Mebeverine [Duspatal (MB)] is a musculotropic antispasmodic drug that is widely used in the treatment of irritable bowel syndrome (Baume, 1972; Ritchie and Truelove, 1980; Chapman et al., 1990; Evans et al., 1996). It is the veratric acid (VA) ester of 4-(ethyl-[2-(4-methoxyphenyl)-1-methylethyl]amino)butan-1-ol (MB-alcohol, MB-OH; Fig. 1). Kristinsson et al. (1994) described gas chromatography-mass spectrometry (GC-MS) studies on the metabolism of MB. They only found the hydrolysis products MB-OH and VA and the 1- and/or 2-fold O-demethylated compounds. However, because we have found p-methoxy-ampetamine (which is known as the designer drug PMA) and the ethylamphetamine (EA) derivatives methoxy-EA (MO-EA) and hydroxy-EA (HO-EA) several times in the urine of patients receiving MB therapy, we reinvestigated the metabolism of MB in rat liver microsome incubates and in humans with the use of GC-MS in the electron impact (EI) and chemical ionization (CI) modes. The aim of our study was to clarify whether MO-EA, HO-EA, and PMA are indeed metabolically formed from MB and whether there are additional metabolites that have been overlooked in previous studies.

Experimental Procedures

Materials. Isocitrate dehydrogenase, isocitrate, and S-adenosyl-methionine were obtained from Sigma-Aldrich (Deisenhofen, Germany). NADP was obtained from BIOMOL (Hamburg, Germany). Ethyl acetate, methanol, magnesium chloride, and all other chemicals were obtained from Merck (Darmstadt, Germany). All chemicals used were of analytical or biochemical grade. Duspatal tablets (each containing 135 mg of MB) were obtained from a local pharmacy.

Preparation of Rat Liver Microsomes. Adult male Wistar rats were obtained from Charles River (Sulzfeld, Germany). Liver samples were homogenized in 2 volumes of 1.15% KCl solution. Microsomes and cytosol were isolated after centrifugation at 10,000 and 100,000 g. The microsomes were resuspended in 1.15% KCl solution for washing and recentrifuged at 100,000 g. The microsome pellets and the cytosol samples were stored at −80°C before incubation. Microsomal and cytosolic protein concentrations were determined by the Bio-Rad protein assay kit (Muenchen, Germany) using a BSA standard solution. Total cytochrome P-450 levels were determined according to Omura and Sato (1964).

Microsome Incubation. Microsomes (1.5 mg protein/ml) were incubated...
with substrate [3 μl of methanolic or aqueous stock solution (10 ng/ml), 1.2 mM NADP, 2 U isocitrate dehydrogenase, 5 mM isocitrate, and 5 mM MgCl2 in 0.1 M phosphate buffer (pH 7.4) for 90 min at 37°C. The whole sample (1 ml; final concentration, 30 μg/ml) was used for analysis. The reaction was stopped by the addition of the organic extraction mixture (see later). Incubation without the addition of NADP was performed to check whether incubation products were indeed enzymatically formed. Additional microsome samples were incubated after the addition of cytosolic preparation (1.5 mg protein/ml) and 0.58 mM -adenosyl-methionine. Microsome preparations without substrate were prepared to check for possible interferences.

**Drug Administration and Urine Sampling.** Urine samples. After four healthy volunteers were informed of the Declaration of Helsinki and provided written consent, they received a single oral dose of 405 mg of MB, which corresponds to a usual daily dose. Urine samples were collected every 4 h for 3 days. The samples were stored at −20°C before analysis. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

Sample preparation of urine. A 5-ml portion of urine was adjusted to pH 5.2 with acetic acid and incubated at 38°C for 12 h with 100 μl of a mixture of β-glucuronidase and arylsulfatase (Helix pomatia; 100,000 Fishman U/ml) and then extracted with 5 ml of a dichloromethane/isopropanol/ethyl acetate mixture (1:1:3, v/v/v). After phase separation through centrifugation, the organic layer was transferred and evaporated to dryness, and the residue was acetylated with 50 μl of an acetic anhydride/pyridine mixture (3:2, v/v) for 10 min under microwave irradiation (Kraemer et al., 1997b; Maurer, 2000). After evaporation of the acetylation mixture, the residue was methylated using ethereal diazomethane solution (Maurer, 2000). After evaporation, the residue was dissolved in 50 μl of methanol, and 2 μl of this solution was injected into the GC-MS apparatus.

Another urine sample was adjusted to pH 8 to 9 with 1 M sodium hydroxide solution after cleavage of conjugates. The sample was extracted with 5 ml of a dichloromethane/isopropanol/ethyl acetate mixture (1:1:3, v/v/v). After phase separation through centrifugation, the organic layer was transferred and evaporated to dryness, and the residue was acetylated with 50 μl of an acetic anhydride/pyridine mixture (3:2, v/v) for 10 min under microwave irradiation (Kraemer et al., 1997b; Maurer, 2000). After evaporation, the residue was dissolved in 50 μl of methanol, and 2 μl of this solution was injected into the GC-MS apparatus. The same procedures, except for enzymatic hydrolysis, were used to study whether metabolites of MB were excreted in an unconjugated form.

Sample preparation of microsomal incubation mixtures. The microsomal incubation mixtures with and without cytosol were extracted and derivatized in the same manner as the urine samples. Cleavage of the conjugates was omitted.

**Apparatus.** All extracts were analyzed using a Hewlett Packard (HP; Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5989B MS engine mass spectrometer and HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were as follows: splitless injection mode; cross-linked methylsilicone, 330-nm film thickness; injection port temperature, 280°C, and 0.2 mm i.d.), cross-linked methylsilicone, 330-mm film thickness; injection port temperature, 280°C, carrier gas, helium; flow rate 1 ml/min; column temperature, programmed from 100–310°C at 30°/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode; EI ionization mode: ionization energy, 70 eV; CI using methane, positive chemical ionization mode (PCI): ionization energy, 230 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C.

**Results**

The MB metabolites were identified first in microsome incubates and then in urine with and without cleavage of conjugates, extraction and acetylation, and/or methylation by EI and PCI GC-MS. For identification in microsome preparations, cleavage was omitted because no phase II cosubstrates were added. Therefore, the microsomes could form only phase I metabolites. For detection of the metabolites in human urine samples, cleavage of conjugates was essential because the hydroxylated and/or N-desalkylated metabolites are usually conjugated. Extraction at slightly acidic pH and at pH 8 to 9 allowed the isolation of acidic, basic, and amphoteric compounds. The extraction solvent that was used has proved to be very efficient in extracting compounds with very different chemical properties from biomatrices (Ensslin et al., 1996; Maurer, 1996; Kraemer et al., 1997a). It is also routinely used for comprehensive screening of drugs, poisons, and their metabolites with very different physicochemical properties, the so-called systematic toxicological analysis (Maurer, 1992; Maurer et al., 1997).

**Identification of Metabolites in Rat Liver Microsomes.** The extracts of microsome incubates were analyzed by full-scan GC-MS. The detected metabolites were first identified through interpretation of the EI mass spectra of the postulated metabolites in correlation to that of the known parent compound and its hydrolysis products according to the rules described by McLafferty and Turecek (1993). In addition, GC and mass spectral data of the metabolites XIV and XII were known. Metabolite XIV corresponds to PMA, whose GC and MS data are published in our handbook and library (Pfleger et al., 2000a,b). MO-EA was recently synthesized by Marson et al. (2000), showing the identical GC and MS data as the proposed MB metabolite XII. Formation of the main fragments in the EI mass spectra is explained in Fig. 4. The main fragment ions m/z 72, 86, 114, 186, and 200 should be produced by β-cleavage; the fragment ions m/z 72 and 158 correspond to the loss of the acetyl group. Benzyl cleavage leads to the formation of the main fragments in the EI mass spectra is explained in Fig. 4. The main fragment ions m/z 72, 86, 114, 186, and 200 should be produced by β-cleavage; the fragment ions m/z 72 and 158 correspond to the loss of the acetyl group. Benzyl cleavage leads to the production of the main fragments in the EI mass spectra is explained in Fig. 4. The main fragment ions m/z 72, 86, 114, 186, and 200 should be produced by β-cleavage; the fragment ions m/z 72 and 158 correspond to the loss of the acetyl group.

Fragment ions for mass chromatography were selected from the mass spectra of the metabolites, which had been identified in microsome incubates. Mass chromatography with the masses m/z 72, 86, 114, 148, 158, 186, and 200 allowed us to indicate (acetylated) MB metabolites. In Fig. 3, such reconstructed merged mass chromatograms are shown recorded from an extract of a microsomal incubation mixture (bottom) and from an acetylated extract of a urine sample taken 4 h after the ingestion of 405 mg of MB (top). The peak numbers correspond to those in Figs. 2 and 5. As can be seen, there was less matrix background with the use of the microsome incubation mixture. All of the metabolites, which had been found in the extracts of the microsome incubations, could also be found in the urine samples of the volunteers. In addition, O-demethyl-hydroxy MB-OH (IX) could be detected in some urine samples. The hydroxy metabolites (II, IV-VI, VII-XI, and XIII) were partly excreted as conjugates cleavable by glucuronidase or arylsulfatase.

PMA (XIV) was detectable only in the 4-h samples of the volunteers. The N-dealkylated metabolites (X, XI, XII, and XIII) were detectable for 12 h to a maximum of 20 h after ingestion. As can be seen in the mass chromatograms in Fig. 3, the signals for these metabolites as well as for the hydroxylated ones (VII and IX) were quite small. MB-OH (II) and O-demethyl MB-OH (VIII) were even detectable up to 44 h after ingestion.

**Discussion**

Sample Preparation. The MB metabolites were identified first in microsome incubates to exclude interferences and then in urine with and without cleavage of conjugates, extraction and acetylation, and/or methylation by EI and PCI GC-MS. For identification in microsome preparations, cleavage was omitted because no phase II cosubstrates were added. Therefore, the microsomes could form only phase I metabolites. For detection of the metabolites in human urine samples, cleavage of conjugates was essential because the hydroxylated and/or N-desalkylated metabolites are usually conjugated. Extraction at slightly acidic pH and at pH 8 to 9 allowed the isolation of acidic, basic, and amphoteric compounds. The extraction solvent that was used has proved to be very efficient in extracting compounds with very different chemical properties from biomatrices (Ensslin et al., 1996; Maurer, 1996; Kraemer et al., 1997a). It is also routinely used for comprehensive screening of drugs, poisons, and their metabolites with very different physicochemical properties, the so-called systematic toxicological analysis (Maurer, 1992; Maurer et al., 1997).

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Fig. 2. EI and PCI mass spectra and structures of the acetylated (Ac) or methylated metabolites of MB.

The numbers of the spectra correspond to those of the compounds used in the text. The mass fragments used for mass chromatography are underlined.
FIG. 2C.
FIG. 2D.

METHYLATED DERIVATIVES OF MEBEVERINE

CHART XI

CHART XII

CHART XIII

M + H + 264

M + C2H5+ + 264

M + C3H5+ + 276

M + H + 350

M + C2H5+ + 376

M + C3H5+ + 390

Fig. 2D.
Fig. 3. Merged mass chromatograms (ions given in the figure) indicating (acetylated) metabolites of MB in an extract of microsomal incubation (bottom) and in an extract of a urine sample of a volunteer (top).

The numbers correspond to those in Figs. 2 and 5.
fragments m/z 107, 121, and 149. The fragment ion m/z 148 can be explained as an α-cleavage product. Fortunately, the EI mass spectra were unequivocal in this case. Every metabolic alteration of the molecule corresponded to a change in the corresponding mass fragment of the known compounds (MB, MB-OH, and MO-EA). Thus, the mass spectra of the metabolites could unequivocally be interpreted, with the exception of those of the regioisomers. Unequivocal assignment of the hydroxy groups of the metabolites VII and IX to position 2 or 3 of the ring system was not possible, so we have renounced such assignment. Nevertheless, hydroxylation at position 3 seems to be more probable, taking into consideration standard knowledge of metabolic reactions.

The acidic metabolites gave strong molecular ions (methylated VA) or strong ions at M-42, indicating the loss of the acetyl group. Further signals indicated the loss of an OCH₃ fragment from the methyl ester group by α-cleavage. Unfortunately, not all the EI spectra gave distinct molecular peaks. Therefore, the PCI mass spectra were also used to ensure the identity of the metabolites, because they gave strong molecular peaks (MH +) with adduct ions typical for PCI using methane.

In accordance with the literature (Kristinsson et al., 1994), the parent compound (I) could not be found. It should be noted that cleavage of the ester bond can also occur under the conditions of sample preparation and analysis. Hydrolysis of MB also under physiological conditions has been discussed. Some authors (Dickinson et al., 1991; Sommers et al., 1997) stated that MB rapidly undergoes presystemic hydrolysis and does not reach the systemic circulation in measurable amounts. Sommers et al. (1997) concluded that the systemic effects of MB should be due to active metabolites.

Screening for and Detection of MB Metabolites in Urine Samples of Volunteers. For screening for possible metabolites, mass chromatography was used. With this technique, metabolites can be detected even in chromatograms with high background signals from the matrix. Fragment ions for mass chromatography were selected from the mass spectra of the metabolites that had been identified in microsome incubates. Mass chromatography with the masses m/z 72, 86, 114, 148, 158, 186, and 200 allowed us to indicate (acetylated) MB metabolites. The fragment masses m/z 72, 86, 114, 158, 200, and 186 were selected for screening, because they correspond to the (acetylated) product of the β-cleavage (cf. Fig. 4). The fragment ion m/z 148 was chosen to indicate the presence of PMA and MO-EA. The fragment masses corresponding to the products of the benzyl cleavage (m/z 121 for unchanged methoxy function and m/z 107 for the demethyl metabolites) were not used for screening, because they were not very specific. All the metabolites that had been found in the extracts of the microsome incubations could also be found in urine samples of volunteers. In addition, O-demethyl-hydroxy MB-OH (IX) could be detected in some urine samples. This is due to the fact that metabolites in the urine samples showed much higher concentrations than those in the incubates. To check whether this catechol (IX) was missed in microsomal incubates due to its instability, we incubated MB with both microsomes and cytosol plus S-adenosyl-methionine (Bickeboeller-Friedrich and Maurer, 1999). Thus, the catechol could

Fig. 4. Proposed mass spectral fragmentation pathways of MB metabolites.

Fig. 5. Proposed scheme of the metabolic pathways of MB in rats and humans. The numbers correspond to those in Figs. 2 and 3.
be stabilized in statu nascendi by catechol-O-methyl-transferase methylation. With the use of this technique, the peak for metabolite VII (hydroxy-methoxy compound) markedly increased, indicating that the catechol (IX) was altered if not derivatized during incubation.

The hydroxy metabolites (II, IV-VI, VII-XI, and XIII) were partly excreted as conjugates cleavable by glucuronidase or arylsulfatase. This is in accordance with the literature. Kristinsson et al. (1994) reported conjugation rates of more than 90% for MB-OH and the acidic metabolites.

It should be noted that the acidic metabolites may not be very specific because they may also appear in blank urine samples after the ingestion of certain food. However, because they were also found in the microosomal incubation mixtures after the addition of only MB, they also should be metabolites of MB.

The metabolites VII and IX-XIV were not identified by Kristinsson et al. (1994), even though the authors used similar extraction and derivatization procedures and measuring equipment. There could be several reasons for this: 1) lower dose (270 instead of 405 mg) and 2) sampling pattern of the urine samples. They collected urine for 24 h, whereas we collected separate urine samples every 4 h. PMA (XIV) was detectable only in the 4-h samples of the volunteers. The N-dealkylated metabolites (X, XI, XII, and XIII) were detectable for 12 to a maximum of 20 h after ingestion. As mentioned, these metabolites, as well as the hydroxylated ones (VII and IX), were present in the samples in only minor amounts. In diluted urine samples (collect ed over 24 h), detection may be impossible. In addition, Kristinsson et al. (1994) denounced the use of mass chromatography. However, in the total ion chromatograms of extracted urine samples, no obvious peaks for the MB metabolites can be seen, with the exception of the main metabolites. The same authors stated that only 5.5% of the alcohol moiety of MB could be recovered in urine. It can be assumed that the metabolites VII and IX-XIV should be part of the missing 94.5%.

Proposed Metabolic Pathways of MB. As shown in Fig. 5, five partially overlapping metabolic pathways in rats and humans could be postulated on the basis of the identified metabolites: ester hydrolysis, alteration of the phenyl ring by O-demethylation to the corresponding phenols and/or aromatic hydroxylation, and side chain degradation by N-deethylation or by N-dehydroxybutylation. The latter pathway led to EA derivatives (XII and XIII), whereas N-bisdealkylation led to PMA (XIV), which is known as a designer drug. The forensic implications of the detection of amphetamine derivatives in urine samples are discussed elsewhere (Kraemer and Maurer, 1998; Kraemer et al., 2000).

In conclusion, our reinvestigation on the metabolism of MB showed that MB is metabolized via five partially overlapping pathways. N-Deethylation, N-dehydroxybutylation, and ring hydroxylation could be postulated as new metabolic pathways of MB. The occurrence of amphetamine derivatives in urine should be considered when patients who are receiving MB therapy are tested for amphetamines, such as in workplace drug testing.

References
Mill Valley, CA.