The 2C-series – A new Class of Designer Drugs

Studies on the Identification of Metabolites, and Toxicological Analysis as well as on Cytochrome P450 and MAO Isoforms Involved in Major Metabolic Steps

Kumulative Dissertation
zur Erlangung des Grades
des Doktors der Naturwissenschaften
der Naturwissenschaftlich-Technischen Fakultät III -
Chemie, Pharmazie und Werkstoffwissenschaften
der Universität des Saarlandes

von
Denis Stephan Theobald
Saarbrücken
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	Univ.-Prof. Dr. R.W. Hartmann

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und meinen Eltern

TABLE OF CONTENTS

1 GENERAL PART

1.1 INTRODUCTION

1.1.1 The 2C-series-Phenethylamine Derived Designer Drugs

Consumption of drugs of abuse is a widespread problem in societies all over the world. Especially, so-called designer drugs are becoming more and more popular among young people. The most frequently abused drugs are amphetamine, methamphetamine and their derivatives, such as 3,4-methylenedioxyamphetamine (MDA), 3,4-methylene-dioxymethamphetamine (MDMA, "Ecstasy"), para-methoxyamphetamine (PMA), and para-methoxymethamphetamine (PMMA). However, during the 1990s, the illicit drug market for recreational drugs changed considerably with several new types of drugs appearing on the drug scene. Information about these new drugs is readily available and they can even easily be purchased via the internet.[1]

One of these new classes of drugs of abuse are the so-called 2Cs. Typical 2Cs are 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B), 4-iodo-2,5-dimethoxy-β-phenethylamine (2C-I), 2,5-dimethoxy-4-methyl-β-phenethylamine (2C-D), 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E), 4-ethylthio-2,5-dimethoxy-β-phenethylamine (2C-T-2), and 2,5-dimethoxy-4-propylthio-β-phenethylamine (2C-T-7). Their chemical structures are shown in Fig. 1.

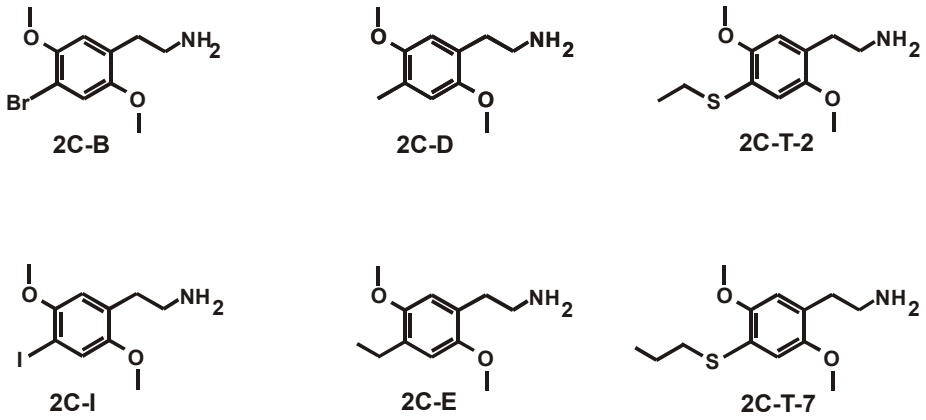

Fig. 1: Chemical structures of phenethylamine-derived designer drugs.

They all have in common a phenethylamine backbone with two methoxy groups in positions 2 and 5 of the aromatic ring and further contain different lipophilic 4-substituents.

Introducing new substituents allows the drug abusers to create “legal” products which are not scheduled as controlled substances. Most of the 2Cs were synthesized by Alexander Shulgin and described in his compilation “PIHKAL”.[2] This work contains the structures of the 2Cs, their hallucinogenic potency, effects, dosage and their synthesis. Because of this it is relatively easy for the illicit drug producers to manufacture a new 2C entity when another one is scheduled. Although many 2Cs were first synthesized during the 1970s and 1980s and appeared on the illicit drug market, they gained increasing popularity in the 1990s after the publication of “PIHKAL”. 2C-B was one of the first compounds of the 2C type entering the illicit drug market in the mid 1980s,[3] followed by the S-alkyl compounds 2C-T-2 and 2C-T-7 and the iodo analogue 2C-I in the 1990s.[4] They were sold in so-called “smart shops” alone or in mixture with other designer drugs in form of tablets, powder or liquid preparations. This trend was accompanied by seizures by the police of tablets containing 2Cs or combinations of them with other drugs.[5-10] During 2000 and 2001 fatalities after the consumption of 2C-T-7 were reported. Because of these increasing problems with the 2Cs, many of them were scheduled in most countries.[4,11] At present, new members of the 2C series, such as 2C-D and 2C-E, which are not scheduled are entering the illicit drug market, as indicated by seizures and experience reports on so-called drug information web sites (http//:www.erowid.org, http//:www.lycaeum.org; October 2006).

Only little information is available on pharmacological and toxicological properties of the members of the 2C-series, but it is known, that they show affinity to 5-HT_2 receptors, and act as agonists or antagonists at different receptor subtypes.[12-18] For 2C-B, partial agonism at α_1-adrenergic receptors was described.[19,20] Because of these properties, radioactive 2C-I was synthesized as a label for the 5-HT_2 receptor and as a potential brain scanning agent for nuclear medicine.[12,21] The chemical structure responsible for hallucinogen-like activity comprises a primary amine functionality separated from the phenyl ring by two carbon atoms ("2C"), the presence of methoxy groups in position 2 and 5 of the aromatic ring, and a hydrophobic 4-substituent (alkyl, halogen, alkylthio, etc.).[15] Furthermore, several quantitative structure-activity relationships (QSAR) studies were published about hallucinogenic β-phenethylamines.[22-29] Using the results of these

analyses, predictions of the hallucinogenic potency of new β-phenethylamines should be possible.

For some 2Cs, analytical data are available.[3,30-37] Screening for and validated quantification of several 2Cs in human blood plasma has been published using gas chromatography-mass spectrometry (GC-MS).[38] Furthermore, a GC-MS procedure was presented for detection of 2C parent compounds in urine.[39] However, for developing toxicological screening procedures, especially in urine, it is a prerequisite to know the metabolism of the compounds in question, especially if they are excreted in urine primarily or even exclusively in form of metabolites. Furthermore, data on the metabolism are needed for toxicological risk assessment, because the metabolites may play a major role in the toxicity of a drug. Some studies have been published about the metabolism of psychoactive phenethylamines.[40-47] For 2C-B, Kanamori et al. investigated qualitative and quantitative metabolism in rat urine and qualitative metabolism in rat hepatocytes.[42,44,47] De Boer et al. reported the only available human data of metabolites of 2C-B in human urine, but only in form of preliminary data.[41] Carmo et al. studied 2C-B metabolism in hepatocytes of six species including humans as well as in mice.[45,48] For 2C-T-2, Lin et al. described qualitative metabolism in rats.[43]

1.1.2 The Cytochrome P450 System

Most drugs are metabolized by a variety of enzymes, and these metabolic processes can generally produce metabolites that are usually less toxic than the parent compound. The metabolites may also be more reactive, producing toxic effects. The metabolic profiling of drugs is, therefore, necessary to assess their effects and toxicity.[49] Cytochrome P450 (CYP) enzymes are responsible for oxidative and, to a minor extent, reductive metabolic transformations of drugs, environmental chemicals and natural compounds. Over its long history of more than 3.5 billion years, the CYP superfamily of enzymes has developed remarkable versatility. The primary catalytic function of CYPs was identified as transfer of one oxygen atom from molecular oxygen into various substrates (Fig. 2). A coenzyme, cytochrome P450 oxidoreductase (OR), is essential for CYP catalytic function, and cytochrome b_5 can stimulate catalytic activities of some enzymes.[50]

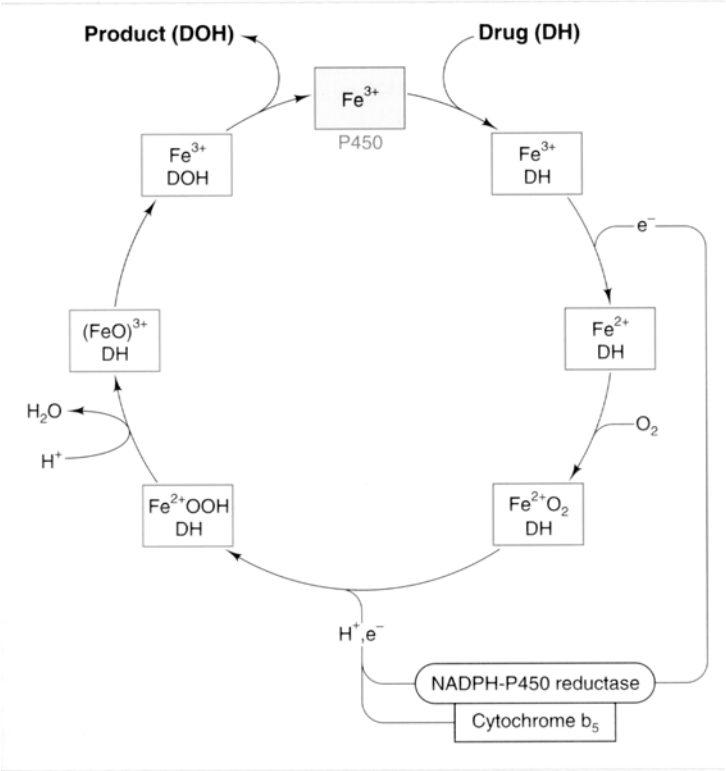

Fig. 2: The cytochrome P450 redox cycle.

Single electron shifts are frequently responsible for the formation of reactive intermediates or allow the leakage of free radicals capable of causing toxicity. When a CYP enzyme activity is modified by induction or inhibition, the biological activity of the xenobiotic substrate can be altered considerably. Such effects are called drug-drug, drug-chemical or chemical-chemical-interactions. Such interactions can modify the disposition of xenobiotics.[51-53] CYPs are heme-containing, membrane-bound enzymes ("heme-thiolate proteins") detected in both prokaryotes and eukaryotes. The enzymes were given their names because their complexes with carbon monoxide under reductive conditions show an absorbance maximum at about 450 nm. In mammals the enzymes can be identified in nearly every tissue, being most abundantly present in the liver. The CYP superfamily has been classified in different families in accordance to the degree of homology of amino acid sequence in their protein structures. CYP enzymes having $\leq$ 40% homology in their amino acid sequence are classified in different families which are designated by Arabic numbers, for example, CYP1. Each family is further divided into subfamilies of enzymes. The enzymes within a mammalian subfamily have $\geq$ 55% sequence homology and are designated by capital letters, for example, CYP1A. An

Arabic number is used for designating individual enzymes within a subfamily, for example, CYP1A2.[51] In humans, 18 CYP families with 43 subfamilies and 57 CYP isoenzymes are known so far, of which only 3 families with 7 subfamilies and 12 CYP isoenzymes are relevant for drug metabolism (Fig. 3),[54] namely CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5.[55]

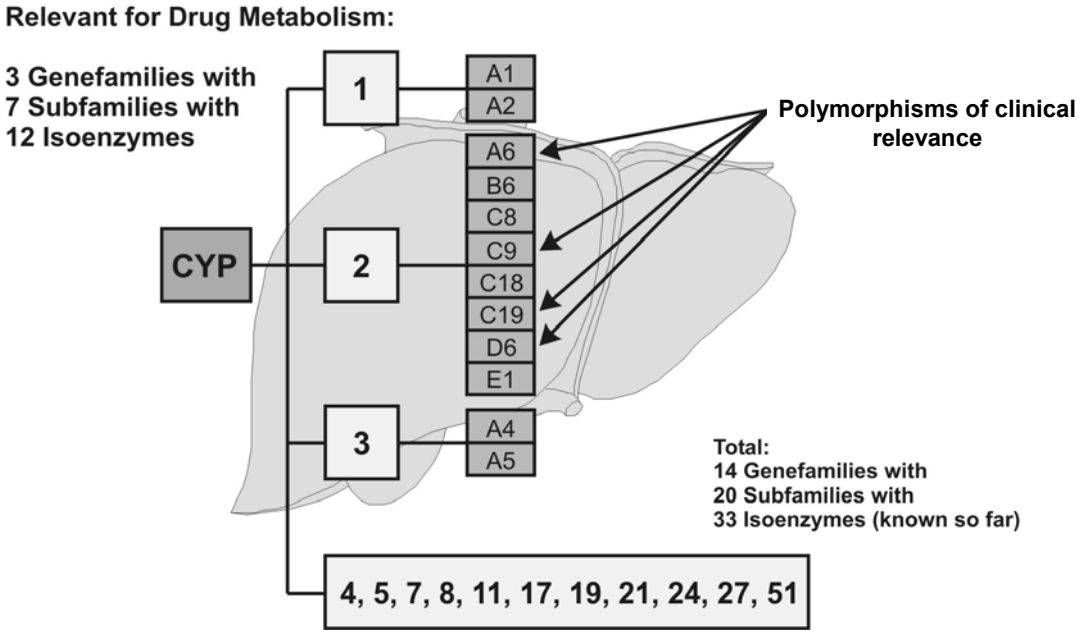

Fig. 3: Cytochrome P450s found in humans and their relevance in xenobiotic metabolism. Modified according to ref[55].

The remainder is responsible for the transformation of endogenous biomolecules, for which reason they are called “housekeeping enzymes”. Fig. 4 illustrates the abundances of CYPs in human liver and their importance in xenobiotic metabolism. Some CYP genes are polymorphically expressed, leading to variabilities in patterns of drug metabolism

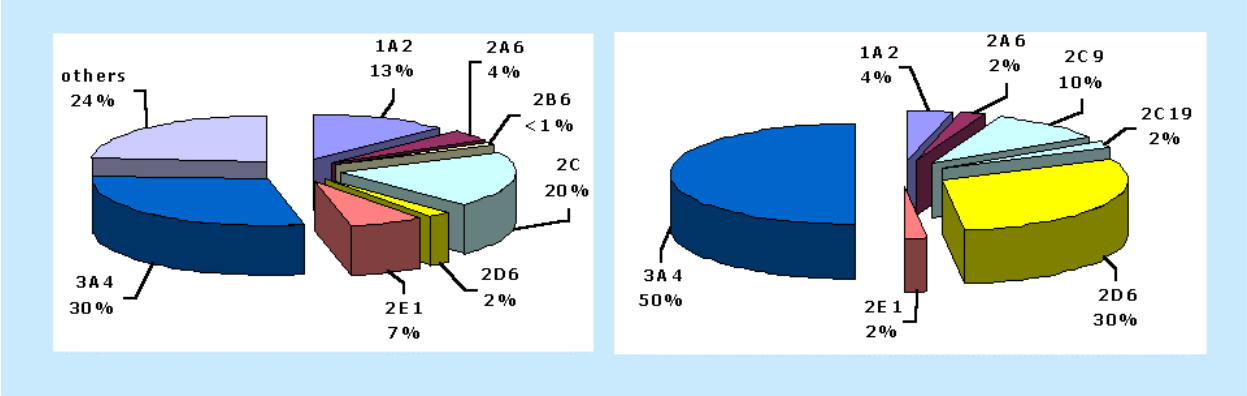

Fig. 4: Relative quantities of CYPs in human liver and their relevance in drug metabolism. Left side: human CYP-expression in the liver. Right side: involvement in xenobiotics metabolism.

1.1.3 Monoamine oxidases

Although the substrates of the monoamine oxidase (MAO) are primarily endogenous compounds, MAO can also contribute to the metabolism of xenobiotics, especially if these xenobiotics contain a primary amine function as in the case of the 2Cs.

The human monoamine oxidase catalyzes the oxidation of primary, secondary, and some tertiary amines to their corresponding protonated imines with concomitant reduction of O_2 to hydrogen peroxide.[56] The dissociated imine product is then nonenzymatically hydrolyzed to the corresponding aldehyde (Fig. 5). Monoamine oxidases are flavoproteins localized to the outer mitochondrial membranes of mammals, birds, fish, and a variety of lower animals and some fungi.[56] In humans and other mammals two isoforms, MAO-A and MAO-B, are expressed. The amino acid sequences of the two human isoforms are 71% identical, and each contains a flavin adenine

dinucleotide (FAD) cofactor covalently attached to a conserved cysteinyl residue via an 8-α-*S*-thioether linkage.[56] In humans, most tissues express both isoenzymes. The highest MAO levels are present in the liver and the placenta and the lowest in spleen. MAO isozymes are also present in most areas of the human brain. MAO-B appears to be predominantly localized in serotonergic neurons whereas dopaminergic neurons contain MAO-A.[57] MAO-A and MAO-B have received extensive attention as targets of antidepressants. MAO-B inhibitors are currently used synergistically with L-DOPA therapy in the treatment of Parkinson's disease.[56]

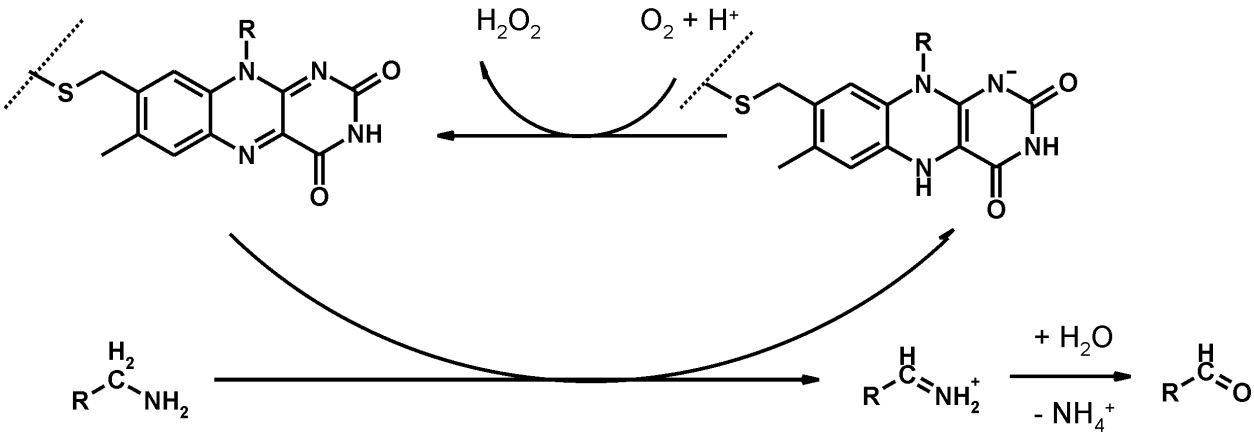

Fig. 5: Oxidation of amines by MAO-bound FAD. Modified according to ref[57].

Human liver derived enzyme preparations, e.g. human liver microsomes or human liver mitochondria, contain a natural mixture of CYPs or MAOs. Chemical inhibitors, immunochemical inhibitors, and/or correlation analyses with marker activities must be used to obtain information on which enzymes are performing specific biotransformations. In contrast, only a single active CYP or MAO is present in preparations of cDNA-expressed enzymes. Inhibitors and correlation analyses are not needed, because the mentioned assignments can be performed by direct incubation of the drug with a panel of individual CYPs or MAOs. However, the balance of enzymes, present in vivo, is lost.[50] Bacteria, yeast, baculovirus and several mammalian cells have been used to produce a wide range of catalytically active CYPs and MAOs. The baculovirus system offers high-level expression of both the CYP and OR or the MAO, and are therefore advantageous for metabolism studies of all kinds, especially for low turnover substrates. The development of the cDNA-bearing virus is relatively time-

consuming and labor-intensive, but baculovirus infected insect cell microsomes are commercially available. However, because the enzymes are produced transiently in the insect host cells, exact harvest time can have a pronounced effect on the activity of the final preparation.[58]

Identification of the human enzymes involved in the metabolism of specific drugs is becoming an increasingly important aspect of drug development. Such identifications should consider two processes involving the new drug: metabolism and inhibition. The identification of enzymes involved in metabolism of the new drug allows prediction, based on knowledge of the ability of co-administered drugs to inhibit the same enzymes, of which co-administered drugs may inhibit the metabolism of the new drug. This information can also be used to predict individual variability based on known metabolic polymorphisms.[50]

1.2 AIMS AND SCOPES

In clinical cases where an unknown substance was ingested (e.g. poisonings), the identity of this substance has to be clarified to be able to start suitable medical treatment and to make statements on the clinical outcome. Also in forensic cases, intake of an illegal drug has to be proven. Usually, a general unknown screening is performed in urine, where the concentrations of the parent compound/and or its metabolites are higher than in blood or plasma and the taken drugs or toxicants can be detected for several hours or even days after ingestion, in contrast to blood analysis which covers only a few hours.[59,60] Knowledge about metabolic steps is a prerequisite for developing toxicological screening procedures, especially, if the compounds are excreted in urine only in form of their metabolites.

The knowledge of the involvement of particular isoenzymes such as CYP or MAO in the biotransformation of a new drug is a prerequisite to predict possible drug-drug-interactions, inter-individual variations in pharmacokinetic profiles and increased appearance of side effects and serious poisonings.[61] However, such risk assessment is

typically performed for substances intended for therapeutic use, but not for drugs of the illicit market. In addition, there is good evidence that genetic variations in drug metabolism have important behavioral consequences that can alter the risk of drug abuse and dependence.[62] The 2Cs were not yet investigated in any of these respects, so that the aims of the presented studies were:

- Investigation of the metabolism of the 2Cs
- Development of toxicological analysis procedures
- Identification of MAO and CYP isoenzymes involved in one of the major metabolic steps (deamination).

2 PUBLICATIONS TO THE RESULTS

The results of the studies were published in the following papers:

2.1 NEW DESIGNER DRUG 2,5-DIMETHOXY-4-PROPYLTHIO-β-PHENETHYLAMINE (2C-T-7): STUDIES ON ITS METABOLISM AND TOXICOLOGICAL DETECTION IN RAT URINE USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY[63]

JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2005; 40: 105–116
Published online inWiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/jms.784

2.2 NEW DESIGNER DRUG 2,5-DIMETHOXY-4-ETHYLTHIO-β-PHENETHYLAMINE (2C-T-2): STUDIES ON ITS METABOLISM AND TOXICOLOGICAL DETECTION IN RAT URINE USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY[64]

JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2005; 40: 1157–1172
Published online 22 July 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/jms.890

2.3 NEW DESIGNER DRUG 4-IODO-2,5-DIMETHOXY-β-PHENETHYLAMINE (2C-I): STUDIES ON ITS METABOLISM AND TOXICOLOGICAL DETECTION IN RAT URINE USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY[65]

JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2006; 41: 872–886
Published online inWiley InterScience
(www.interscience.wiley.com)
DOI: 10.1002/jms.1045

2.4 STUDIES ON THE METABOLISM AND TOXICOLOGICAL DETECTION OF THE DESIGNER DRUG 4-ETHYL-2,5-DIMETHOXY-β-PHENETHYLAMINE (2C-E) IN RAT URINE USING GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC TECHNIQUES[66]

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Studies on the metabolism and toxicological detection of the designer drug 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E) in rat urine using gas chromatographic–mass spectrometric techniques☆

Denis S. Theobald, Hans H. Maurer*

Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Saarland, D-66421 Homburg (Saar), Germany

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Abstract

The phenethylamine-derived designer drug 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E) was found to be mainly metabolized in rats by *O*-demethylation, *N*-acetylation, hydroxylation of the ethyl side chain at C2′ or at C1′ followed by oxidation at C1′ to the corresponding ketone, by deamination followed by reduction to the corresponding alcohols or by oxidation to the corresponding acids, and finally combinations of these steps. Most of the metabolites were excreted in conjugated form. The authors' systematic toxicological analysis (STA) procedure using full-scan GC–MS allowed the detection of an intake of a dose of 2C-E in rat urine that corresponds to a common drug users' dose. Assuming similar metabolism, the described STA procedure should be suitable for proof of an intake of 2C-E in human urine.

Keywords: 4-Ethyl-2,5-dimethoxy-β-phenethylamine; 2C-E; Designer drug; Metabolism; GC–MS

1. Introduction

The members of the so-called 2C-series [1] belong to a class of substances abused as designer drugs that are all phenethylamine derivatives. β-Phenethylamine itself is not a common drug of abuse, because it is rapidly metabolized [2], but other compounds of this type like 3,4,5-trimethoxy-β-phenethylamine (mescaline), 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B), 4-iodo-2,5-dimethoxy-β-phenethylamine (2C-I), 4-ethylthio-2,5-dimethoxy-β-phenethylamine (2C-T-2), or 2,5-dimethoxy-4-propylthio-β-phenethylamine (2C-T-7) have obviously psychoactive properties and are often abused [1]. 2C-E was described in Alexander Shulgin's compilation "PIHKAL" as a hallucinogenic substance [2]. Further data are very limited, but descriptions and experience reports on internet web sites (http://www.erowid.org, http://www.lycaeum.org; February 2006) indicate that 2C-E plays a role among drug abusers. Furthermore, 2C-E was identified in several countries on the illicit drug market [3,4]. Lood and Eklund reported that they could identify 2C-E (parent drug) in urine samples of three males [5]. In most countries with exception of Sweden, 2C-E is not a controlled substance. This fact may enhance the spreading among drug abusers, because the more popular members of the 2C-series like 2C-B or 2C-T-7 are all scheduled now in many countries.

Only little information is available on pharmacological and toxicological properties of the members of the 2C-series, but it is known, that they show affinity to 5-HT_2 receptors, acting as agonists or antagonists at different receptor subtypes [6–12]. For 2C-B, partial agonism at α_1-adrenergic receptors was described [13,14]. The chemical structure responsible for hallucinogen-like activity comprises a primary amine functionality separated from the phenyl ring by two carbon atoms ("2C"), the presence of methoxy groups in positions 2 and 5 of the aromatic ring, and a hydrophobic 4-substituent (alkyl, halogen, alkylthio, etc.) [9]. For some of the substances that belong to the 2C-series, analytical data are available [15–23]. Screening for and

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* Corresponding author. Tel.: +49 6841 16 26050; fax: +49 6841 16 26051.
E-mail address: hans.maurer@uniklinikum-saarland.de (H.H. Maurer).

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validated quantification of 2C-E itself in human blood plasma has been published using gas chromatography–mass spectrometry (GC–MS) [24]. Furthermore, a GC–MS procedure was presented for detection of 2C-E parent compound in urine [5]. However, for developing toxicological screening procedures, especially in urine, it is a prerequisite to know the metabolism of the compounds in question, especially if they are excreted in urine primarily or even exclusively in form of metabolites. Furthermore, data on the metabolism are needed for toxicological risk assessment, because the metabolites may play a major role in the toxicity of a drug. Some studies have been published about the metabolism of psychoactive phenethylamines [25–35]. The aim of the study presented here was to identify the 2C-E metabolites in rat urine using GC–MS in the electron ionization (EI) and positive-ion chemical ionization (PICI) modes. In addition, the detectability of 2C-E and its metabolites within the authors' systematic toxicological analysis (STA) procedure in urine by GC–MS was studied [25,26,36,37].

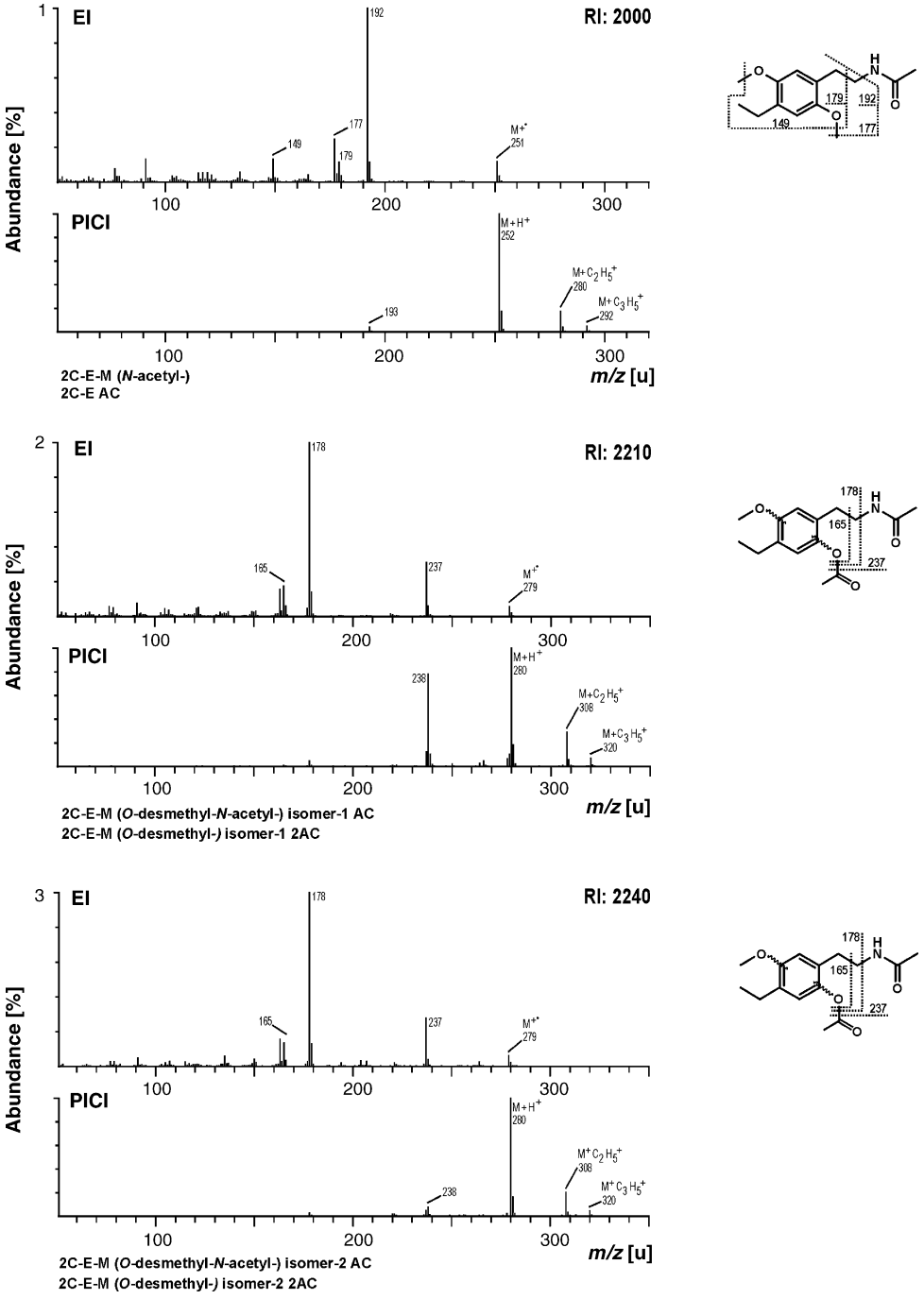

Fig. 1. EI and PICI mass spectra, RIs, structures and predominant fragmentation patterns of 2C-E and its metabolites after acetylation, methylation plus acetylation, trifluoroacetylation, or propionylation. The numbers of the spectra correspond to those of the structures and peaks shown in Figs. 2 and 3.

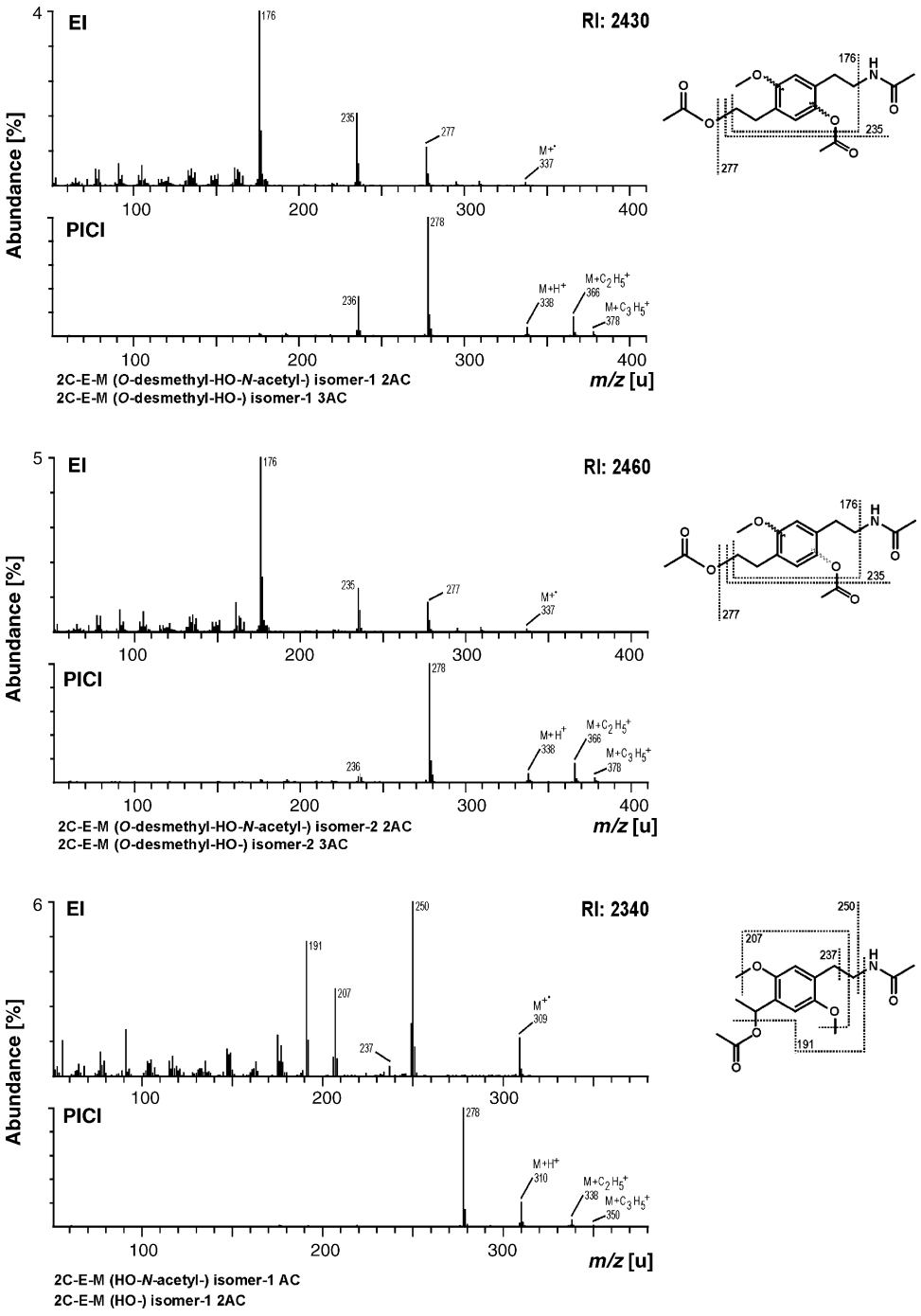

Fig. 1. (*Continued*)

2. Experimental

2.1. Chemicals and reagents

2C-E HCl was provided by Dejachem (Schwendi, Germany) for research purposes. *N*-Methyl-bis(trifluoroacetamide) was obtained from Fluka (Taufkirchen, Germany). All other chemicals and biochemicals were obtained from Merck (Darmstadt, Germany). All chemicals and biochemicals were of analytical grade.

2.2. Urine samples

The investigations were performed using urine of male Wistar rats (about one year old and 400 g body mass (BM), Ch. River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 20 mg/kg BM dose for metabolism studies or a 0.3 mg/kg BM dose for the STA study in aqueous suspension by gastric intubation ($n = 2$ for each dose). Urine was collected separately from the faeces over a 24 h period. All samples were

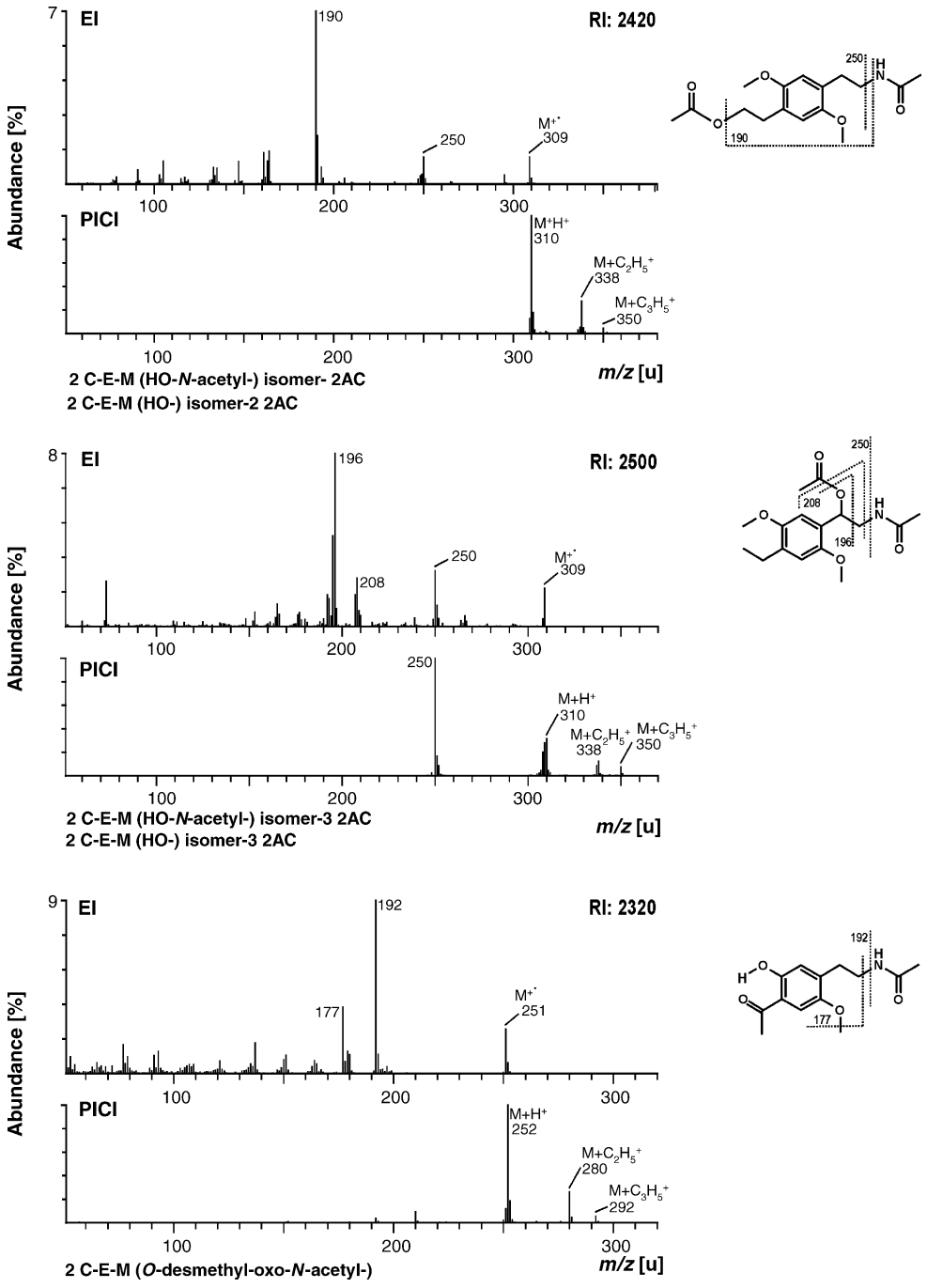

Fig. 1. (*Continued*)

directly analyzed. Blank rat urine samples were collected before drug administration to check whether they were free of interfering compounds.

2.3. Sample preparation for metabolism studies

A 5 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 mol/l) and incubated at 50 °C for 1.5 h with 100 μl of a mixture (100,000 Fishman units/ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1) from *Helix Pomatia*, L. Then pH was adjusted to 8–9 with 1 ml of 37% hydrochloric acid, 2 ml of 2.3 mol/l aqueous ammonium sulfate and 1.5 ml of 10 mol/l aqueous sodium hydroxide and the sample was extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was carefully evaporated to dryness at 56 °C under a stream of nitrogen. The residue was derivatized by one of the following three procedures. Acetylation was conducted with 100 μl of an acetic anhydride–pyridine mixture (3:2; v/v), propionylation with 100 μl of a propionic anhydride–pyridine mixture (3:2; v/v), or trifluoroacetylation with 50 μl of *N*-methyl-bis(trifluoroacetamide) for 5 min under

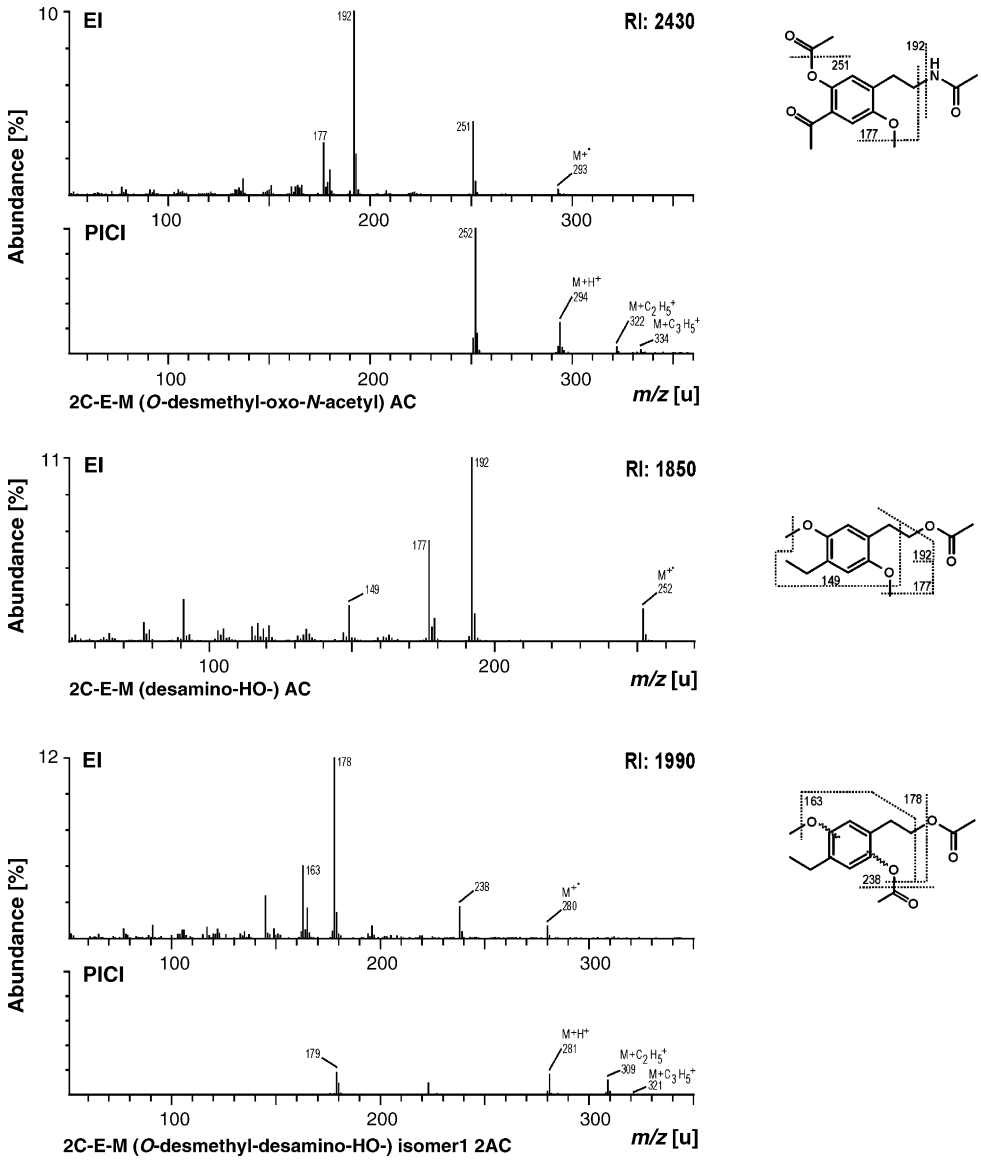

Fig. 1. (*Continued*)

microwave irradiation at approximately 440 W. After careful evaporation, the corresponding residue was dissolved in 100 μl of methanol (acetylation and propionylation) or 50 μl of ethyl acetate (trifluoroacetylation). Aliquots (2 μl) of the derivatized extracts each were injected into the GC–MS.

Another urine sample was worked up as described in the following. A 1 ml portion of urine was adjusted to pH 5.2 with acetic acid (1 mol/l) and incubated at 50 °C for 1.5 h with 100 μl of a mixture (100,000 Fishman units/ml) of glucuronidase (EC no. 3.2.1.31) and arylsulfatase (EC no. 3.1.6.1). The sample was then diluted with 2 ml of water and loaded on a solid-phase extraction (SPE) cartridge (Isolute Confirm HCX, 130 mg, 3 ml), previously conditioned with 1 ml of methanol and 1 ml of water. After passage of the sample, the cartridge was washed with 1 ml of water and 1 ml of 0.01 mol/l hydrochloric acid. The retained non-basic compounds were eluted into a 1.5 ml reaction vial with 1 ml of methanol and gently evaporated under a stream of nitrogen at 56 °C. After evaporation, the residue was dissolved in 50 μl of methanol and derivatized with 100 μl of a solution of diazomethane in diethyl ether, synthesized according to the procedure of McKay et al. [38]. The reaction vial was sealed and left at room temperature for 30 min. Thereafter, the mixture was gently evaporated to dryness under a stream of nitrogen at 56 °C. After evaporation to dryness, the sample was acetylated as described above. The final residue was dissolved in 50 μl of methanol and 2 μl were injected into the GC–MS. All work-up procedures were additionally performed without enzymatic hydrolysis to study which metabolites of 2C-E were excreted as glucuronides/sulfates.

2.4. Sample preparation for toxicological analysis

The urine samples (5 ml) were divided into two aliquots. One aliquot was refluxed with 1 ml of 37% hydrochloric acid for 15 min. Following hydrolysis, the sample was mixed with 2 ml of 2.3 mol/l aqueous ammonium sulfate and

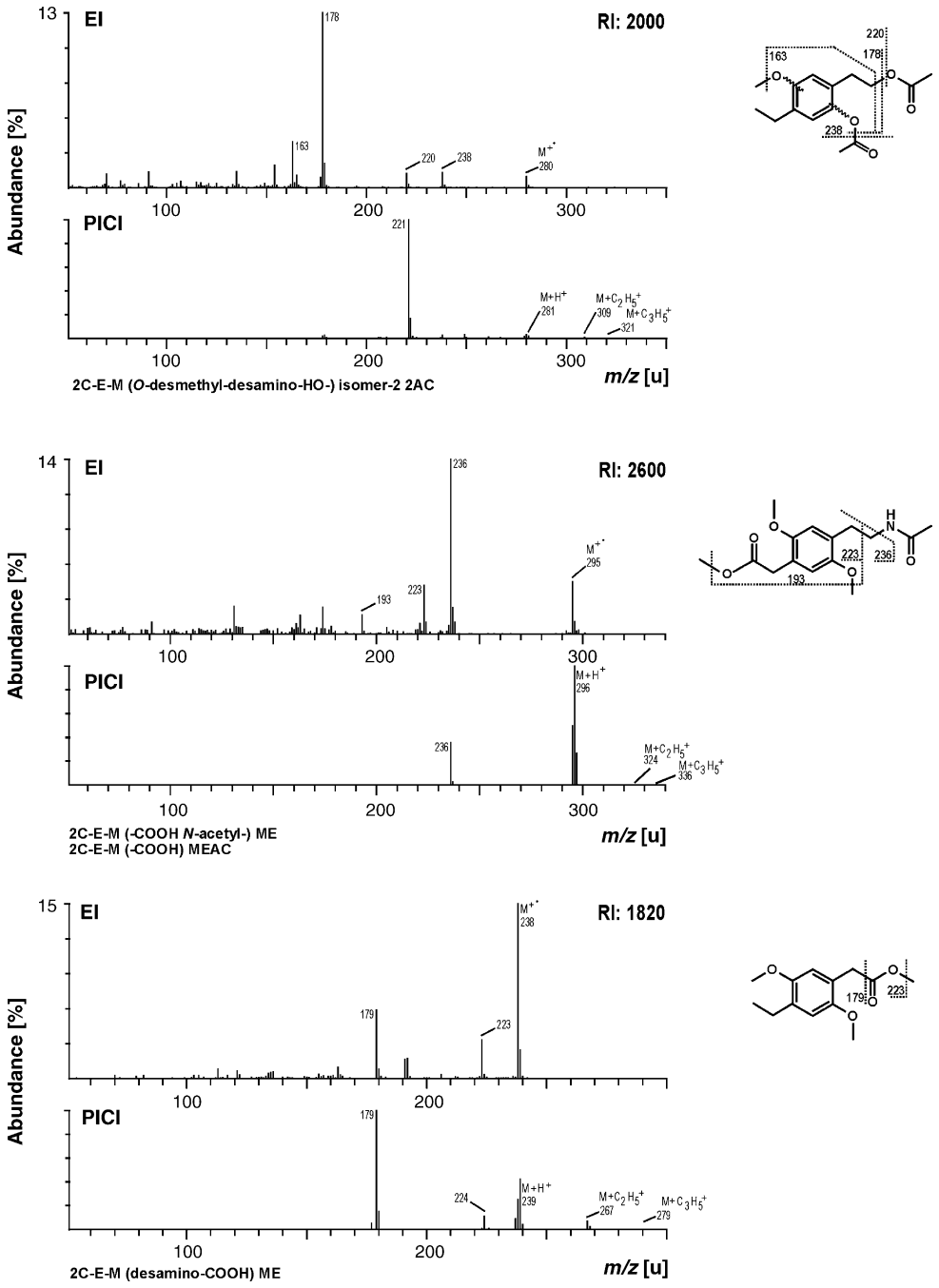

Fig. 1. (*Continued*)

1.5 ml of 10 mol/l aqueous sodium hydroxide to obtain a pH value of 8–9. Before extraction, the other aliquot of native urine was added. This mixture was extracted with 5 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3; v/v/v). After phase separation by centrifugation, the organic layer was transferred into a glass flask and evaporated to dryness. The residue was derivatized by acetylation with 100 μl of an acetic anhydride–pyridine mixture (3:2; v/v) for 5 min under microwave irradiation at about 440 W. After evaporation of the derivatization mixture, the residue was dissolved in 100 μl of methanol and 2 μl were injected into the GC–MS system.

2.5. GC–MS apparatus

The extracts were analyzed using a Hewlett Packard (Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with a HP 5989B MS Engine mass spectrometer for the metabolism studies or using a Hewlett Packard (Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with a HP 5972A MSD mass spectrometer for STA. For both apparatus, a HP MS ChemStation (DOS series) was used with HP G1034C software version C03.00. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m × 0.2 mm I.D.), cross linked methyl silicone, 330 nm

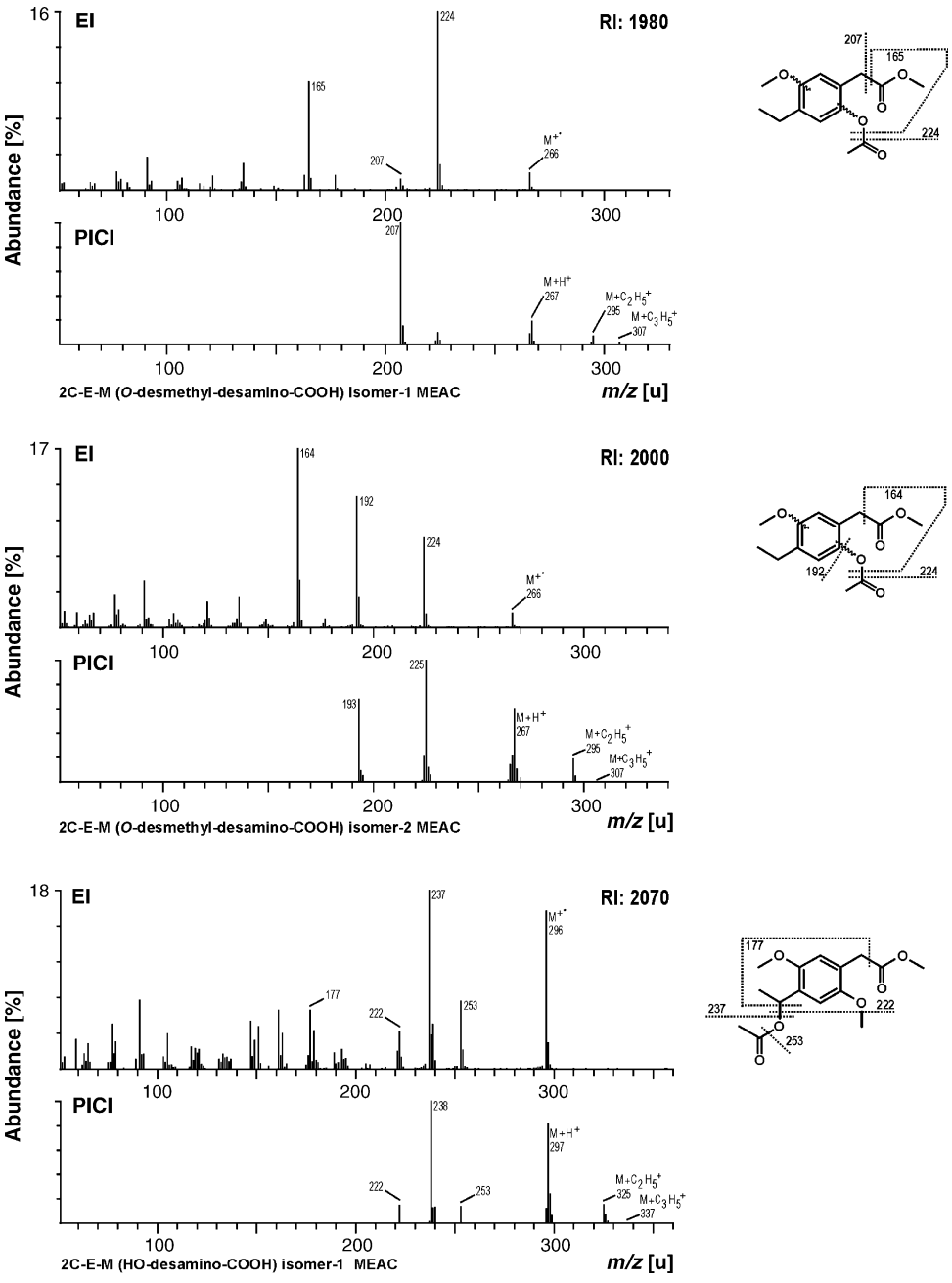

Fig. 1. (*Continued*)

film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100 to 310 °C at 30°/min, initial time 3 min, final time 8 min. The MS conditions for both apparatus were as follows: full-scan mode, *m/z* 50–800 u; EI mode, ionization energy, 70 eV, and for the HP 5989B MS Engine in the PICI mode using methane: ionization energy, 230 eV; ion source temperature, 220 °C; capillary direct interface, heated at 280 °C.

2.6. GC–MS method for STA

For toxicological detection of acetylated 2C-E and its metabolites, mass chromatography with the selected ions *m/z* 192, 251, 178 and 237 was used. The identity of the peaks in the mass chromatograms was confirmed by computerized comparison of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during this study.

3. Results and discussion

3.1. Identification of metabolites

The urinary metabolites of 2C-E were separated by GC and identified by EI and PICI MS after gentle enzymatic hydrolysis, extraction, acetylation, trifluoroacetylation, propionylation or methylation plus acetylation. Acetylation was chosen as

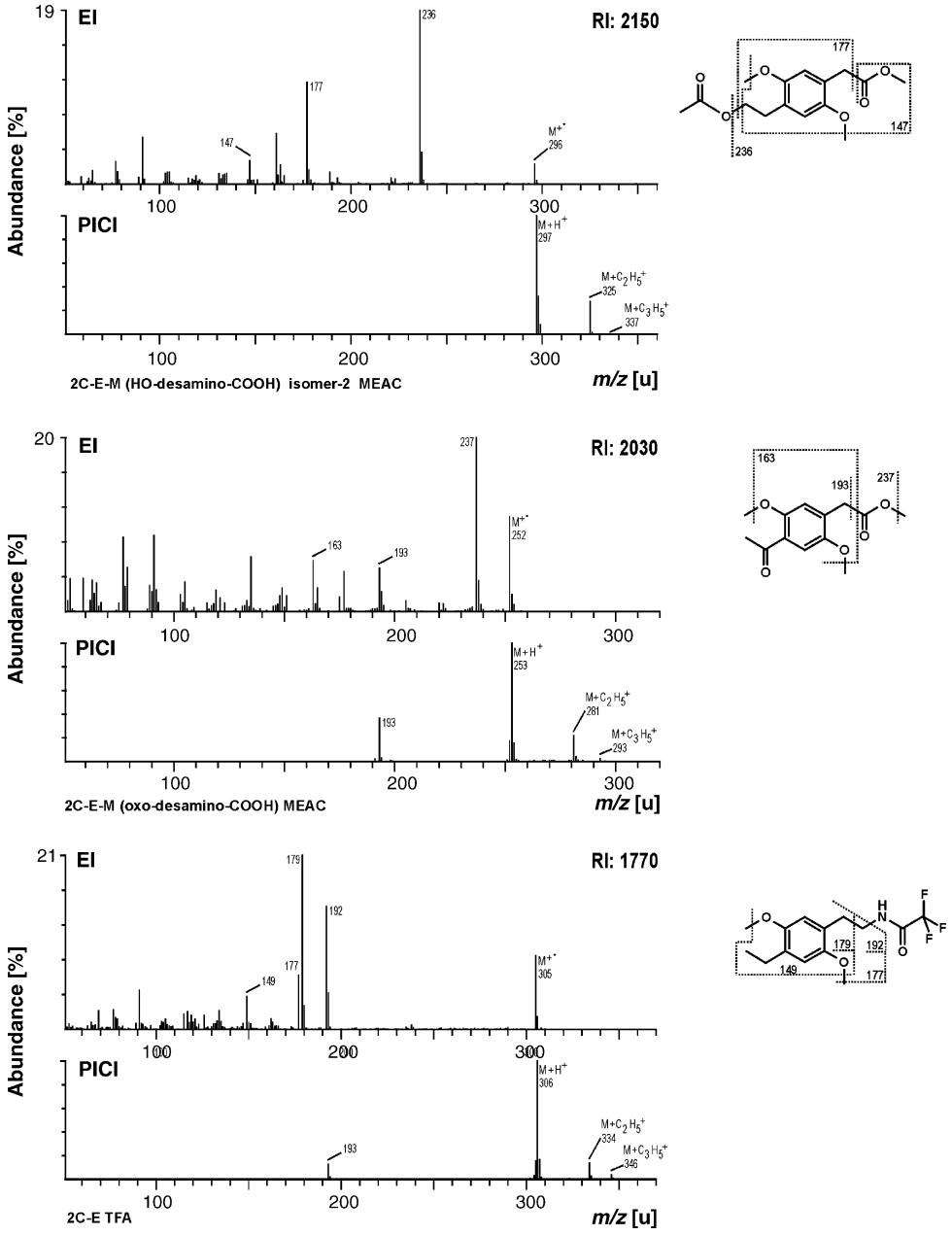

Fig. 1. (*Continued*)

derivatization step, because due to the authors' experiences it is considered as a versatile method for elucidation of the structures of metabolites [25,26,37]. Furthermore, acetylation is the standard derivatization step in the authors' STA. However, using acetylation as derivatization procedure, metabolically *N*-acetylated metabolites cannot be differentiated from acetyl derivatives. For this particular question, the presence of *N*-acetylated metabolites was confirmed in urine extracts after trifluoroacetylation. Unfortunately, the trifluoroacetyl derivatives of some compounds in question could not be detected, probably because of incomplete derivatization or hydrolysis of the trifluoroacetyl esters. Therefore, propionylation was performed to detect these compounds. For detection of acidic metabolites, the urine samples were extracted by SPE, after enzymatic cleavage of conjugates, followed by methylation and acetylation.

The postulated structures of the (derivatized) metabolites were deduced from the fragments detected in the EI mode which were interpreted in correlation to those of the parent compound according to the rules described by, e.g. McLafferty and Turecek [39] and Smith and Busch [40]. In order to verify the molecular mass of the postulated metabolites, PICI mass spectra were recorded, because they contain abundant peaks of the protonated molecule $[M+H]^+$ with adduct ions typical for PICI using methane as reagent gas ($[M+C_2H_5]^+$ and $[M+C_3H_5]^+$). The EI and PICI mass spectra of the parent compound, the retention indices (RI), the structures and predominant fragmentation patterns of 2C-E and its metabolites after derivatization are

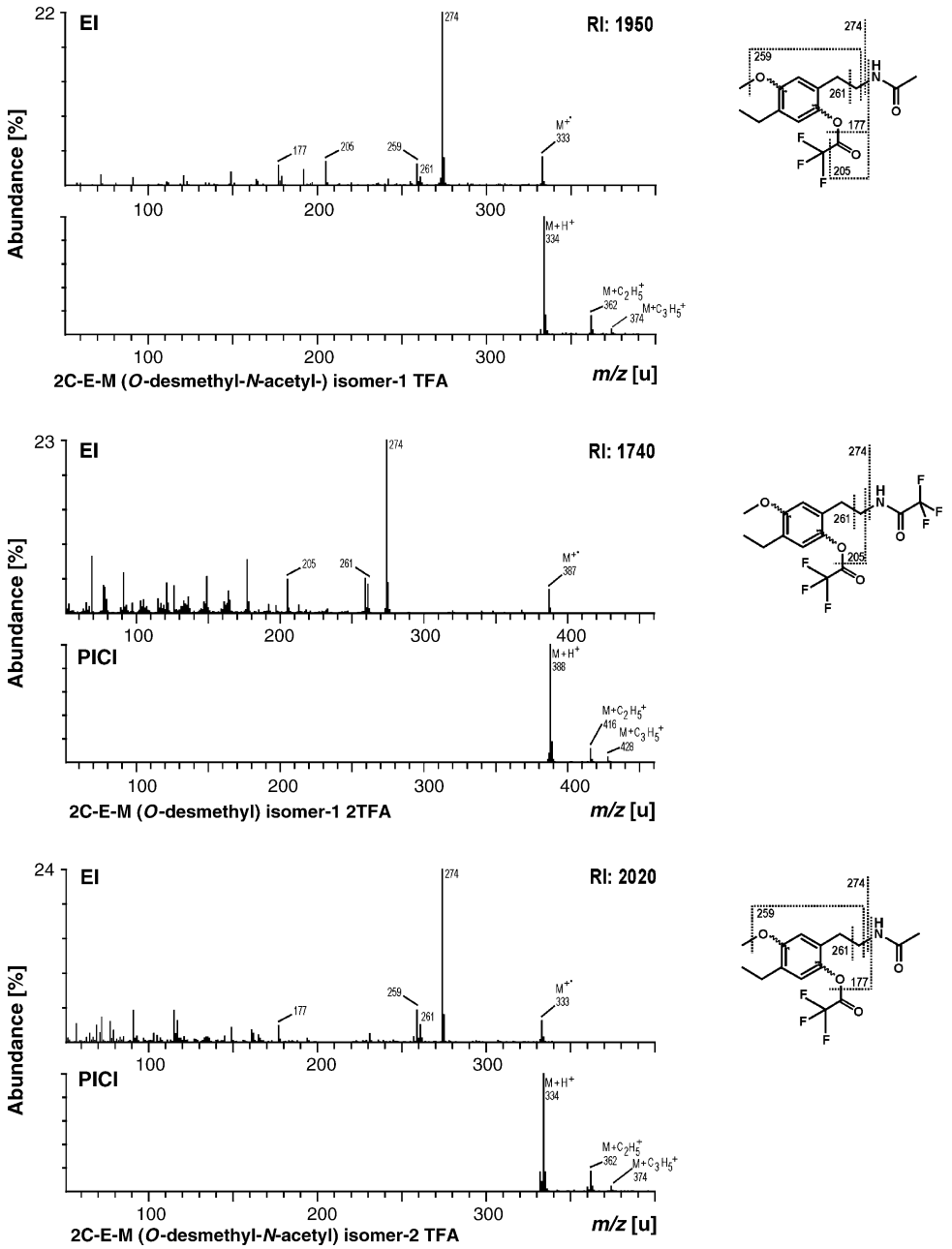

Fig. 1. (*Continued*)

shown in Fig. 1. In the following, the mass spectra numbers in Fig. 1 are given in parentheses. Besides acetylated 2C-E (1), the following metabolites could be identified in the acetylated urine sample: *N*-acetyl-acetoxy-4-ethyl-methoxy-β-phenethylamine isomer 1 (2), *N*-acetyl-acetoxy-4-ethyl-methoxy-β-phenethylamine isomer 2 (3), *N*-acetyl-4-(2′-acetoxyethyl-)-acetoxy-methoxy-β-phenethylamine isomer 1 (4), *N*-acetyl-4-(2′-acetoxyethyl-)-acetoxy-methoxy-β-phenethylamine isomer 2 (5), *N*-acetyl-4-(1′-acetoxyethyl-)-2,5-dimethoxy-β-phenethylamine (6), *N*-acetyl-4-(2′-acetoxyethyl)-2,5-dimethoxy-β-phenethylamine (7), *N*-acetyl-β-acetoxy-4-ethyl-2,5-dimethoxy-β-phenethylamine (8), *N*-acetyl-5-hydroxy-2-methoxy-4-(2′-oxoethyl)-β-phenethylamine (9), *N*-acetyl-5-acetoxy-2-methoxy-4-(2′-oxoethyl)-β-phenethylamine (10), 4-ethyl-2,5-dimethoxy-β-phenylethyl acetate (11), acetoxy-4-ethyl-methoxy-β-phenylethyl acetate isomer 1 (12), and acetoxy-4-ethyl-methoxy-β-phenylethyl acetate isomer 2 (13). In the sample worked-up by SPE, methylation and acetylation, the following compounds could be detected: *N*-acetyl-2,5-dimethoxy-4-methylcarboxymethyl-β-phenethylamine (14), methyl (4-ethyl-2,5-dimethoxyphenyl)acetate (15), methyl (acetoxy-4-ethyl-methoxyphenyl)acetate isomer 1 (16), methyl (acetoxy-4-ethyl-methoxyphenyl)acetate isomer 2 (17), methyl [4-(1′-acetoxyethyl)-2,5-dimethoxyphenyl]acetate (18), methyl [4-(2′-acetoxyethyl)-2,5-dimethoxyphenyl]acetate (19), and methyl [4-(1′-oxoethyl)-2,5-dimethoxyphenyl]acetate (20).

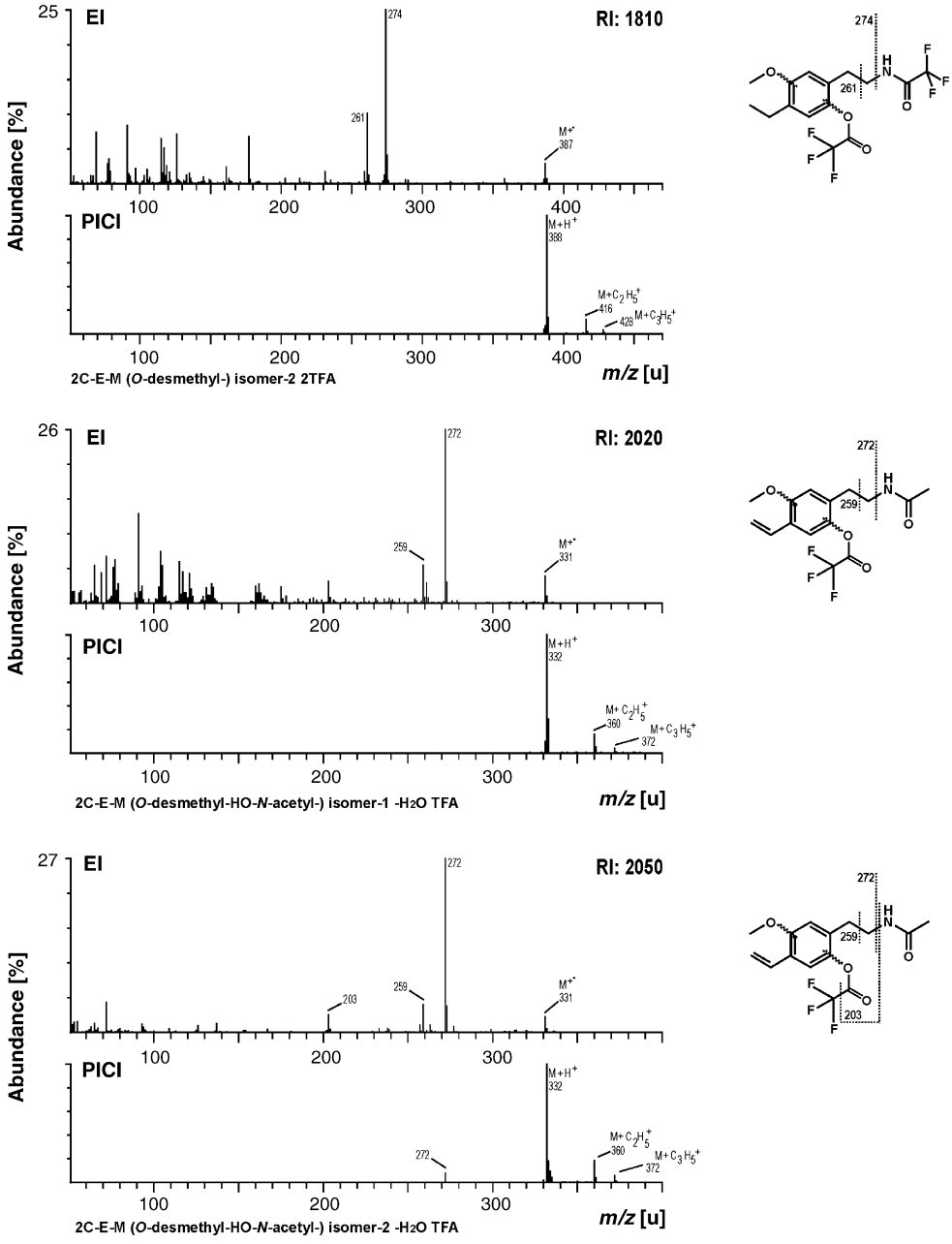

Fig. 1. (*Continued*)

For confirmation whether the *N*-acetyl derivatives were formed by metabolism or by derivatization, the urine extracts were analyzed after trifluoroacetylation. The following metabolites in question could be identified: *N*-acetyl-2C-E (1), trifluoroacetylated 2C-E (21), *N*-acetyl-4-ethyl-trifluoroacetoxy-methoxy-β-phenethylamine isomer 1 (22), *N*-trifluoroacetyl-4-ethyl-trifluoroacetoxy-methoxy-β-phenethylamine isomer 1 (23), *N*-acetyl-4-ethyl-trifluoroacetoxy-methoxy-β-phenethylamine isomer 2 (24), *N*-trifluoroacetyl-4-ethyl-trifluoroacetoxy-methoxy-β-phenethylamine isomer 2 (25), *N*-acetyl-trifluoroacetoxy-methoxy-4-vinyl-β-phenethylamine isomer 1 (26), *N*-acetyl-trifluoroacetoxy-methoxy-4-vinyl-β-phenethylamine isomer 2 (27), *N*-trifluoroacetyl-4-(2′-trifluoroacetoxyethyl-)-trifluoroacetoxy-methoxy-β-phenethylamine (28), *N*-acetyl-*N*-trifluoroacetyl-4-(2′-trifluoroacetoxyethyl-)-2,5-dimethoxy-β-phenethylamine (29), *N*-trifluoroacetyl-4-(2′-trifluoroacetoxyethyl)-2,5-dimethoxy-β-phenethylamine (30), and *N*-acetyl-5-trifluoroacetoxy-2-methoxy-4-(2′-oxoethyl)-β-phenethylamine (31). In the propionylated samples, the following compounds could be detected: *N*-acetyl-2,5-dimethoxy-4-(1′-propionyloxyethyl)-β-phenethylamine (32) and *N*-acetyl-4-ethyl-2,5-dimethoxy-β-propionyloxy-β-phenethylamine (33). Only those compounds are shown, that allowed differentiation between metabolically *N*-acetylated metabolites and the free amines.

Unfortunately, PICI mass spectra of certain compound could not be recorded due to their low concentrations in the sample (nos. 11, 28 and 32). Characteristic fragmentation patterns in the

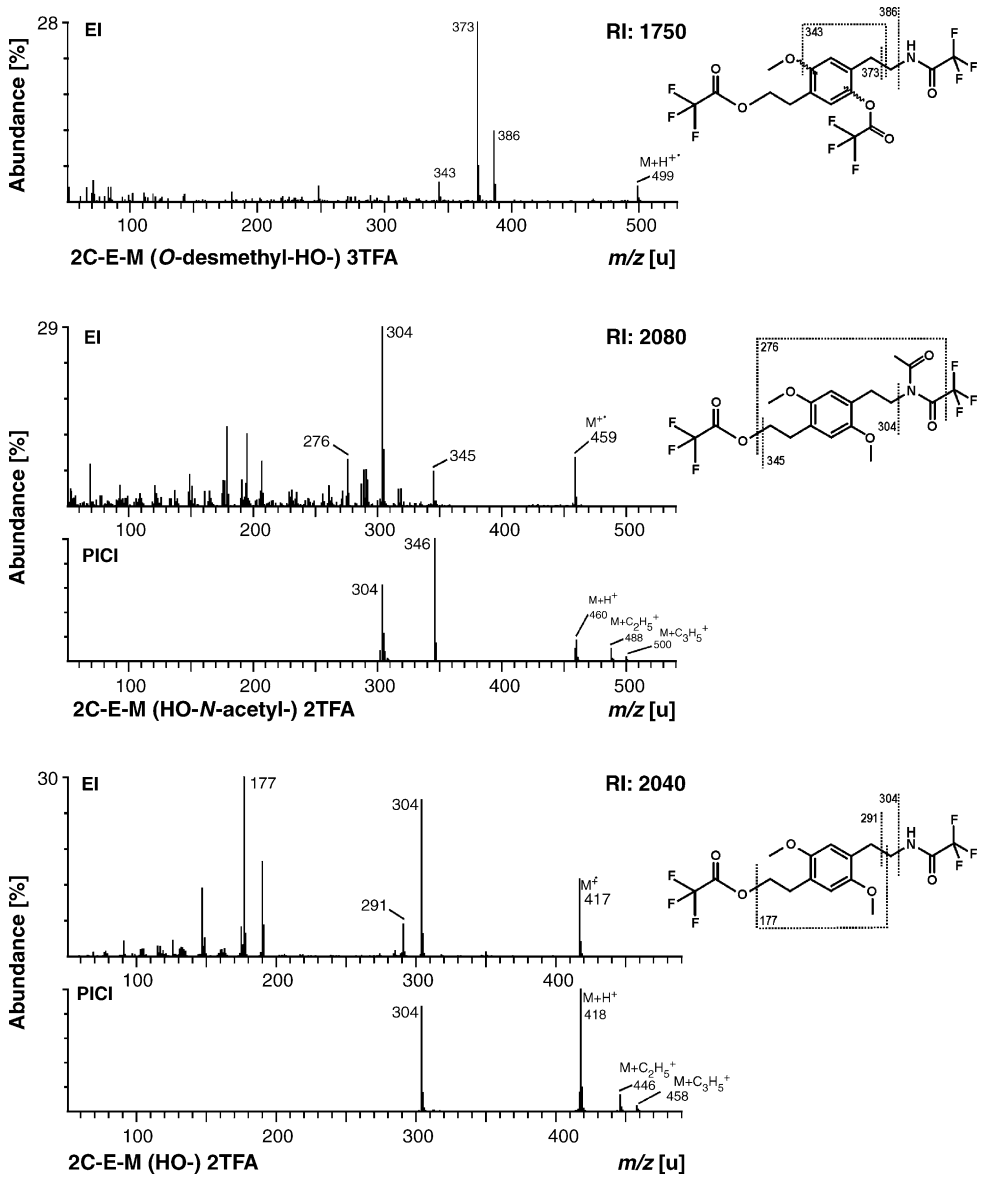

Fig. 1. (*Continued*)

EI spectra consisted of, e.g. the loss of acetamide or trifluoroacetamide, loss of the methyl moieties or the benzyl cleavage, as described for other members of the 2C-series elsewhere [25,26]. The loss of two methyl moieties can alternatively be seen as a neutral loss of CH_2O from one methoxy group [26]. To differentiate between the positions of the hydroxy group in the molecule, the fragmentation patterns were interpreted as follows. Hydroxy moiety at the position 2' of the ethyl group (mass spectra nos. 4, 5, 7, 19, 29 and 30 in Fig. 1) may lead to neutral loss of water or, due to derivatization, to loss of acetic acid (loss of *m/z* 60) or trifluoroacetic acid (loss of *m/z* 114). Location at the 1' position (mass spectra nos. 6, 18 and 32) may lead to radical loss of acetic acid (loss of *m/z* 59), trifluoroacetic acid (loss of *m/z* 113) or propionic acid (loss of *m/z* 73), because the remaining positive charge at C1′ can be stabilized by the ring system, which is not the case if the charge is located at C2′. Location of the hydroxy group at the β-position (mass spectra nos. 8 and 33) may lead to a loss of *m/z* 42 or 56, respectively, because after the loss of acetamide, a conjugated double bond system is formed, which enables the neutral or radical loss of acetic acid or propionic acid, respectively. Therefore, only the loss of an acetyl or propionyl moiety can be seen.

Under the conditions of the GC injection port, the formation of artifacts could be observed, namely the hydroxy metabolites showed the loss of acetic acid or trifluoroacetic acid, respectively (data/mass spectra not shown). Unfortunately, in the case of the metabolites nos. 26 and 27 in Fig. 1, only these artifacts could be detected, but their origin of the corresponding hydroxy metabolites is obvious.

Based on these identified metabolites, the following metabolic pathways could be postulated (Fig. 2): *O*-dealkylation of the parent compound in position 2 or 5, followed by *N*-acetylation and hydroxylation at C2′, or by deamination with oxidation to the corresponding acid or reduction to the

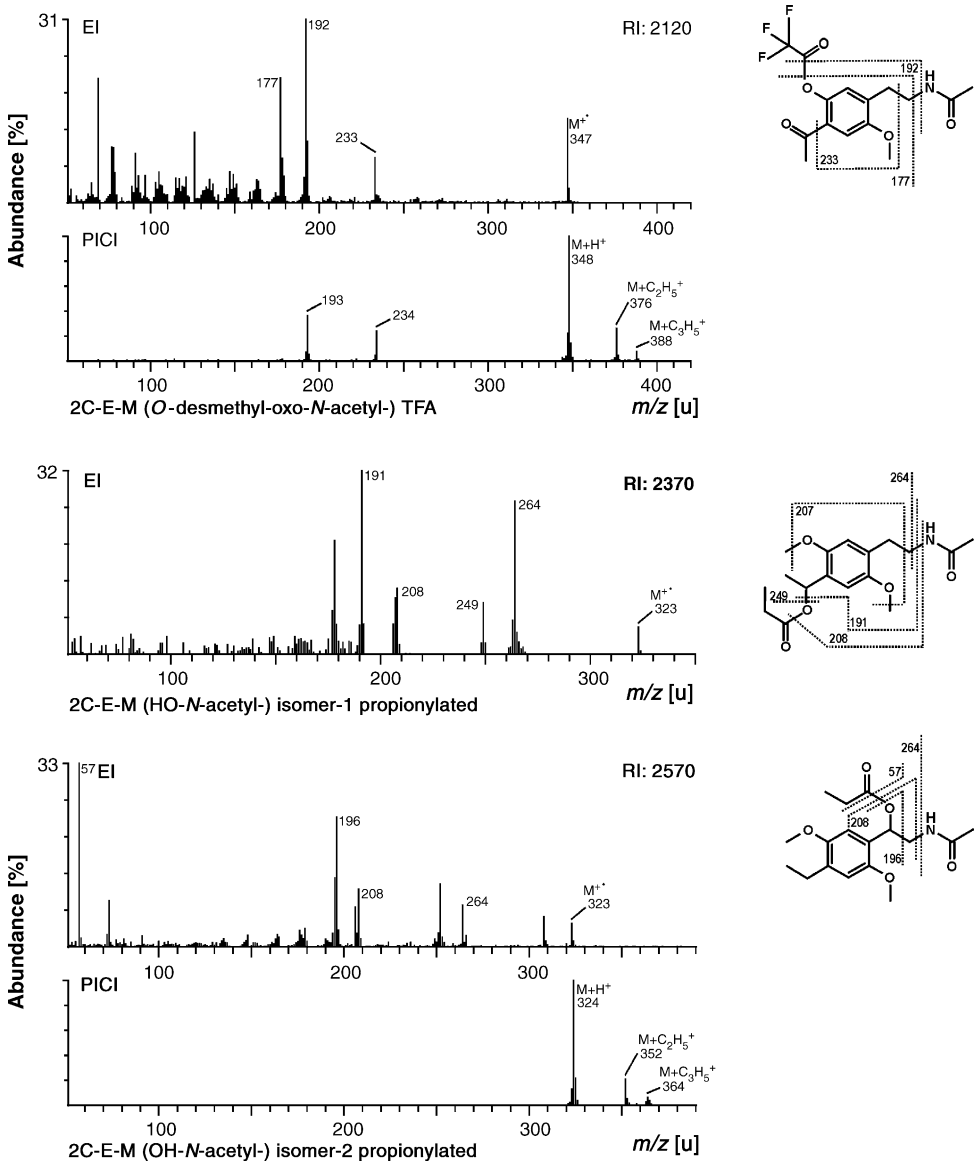

Fig. 1. (*Continued*).

corresponding alcohol. Second pathway was *N*-acetylation of the parent compound followed on the one hand by hydroxylation at position C1′ of the ethyl side chain with subsequent dealkylation and oxidation to the corresponding ketone and followed on the other hand by β-hydroxylation. A third pathway was the hydroxylation of the parent compound at position C2′ of the ethyl side chain followed by *N*-acetylation and oxidation of the hydroxy group to the corresponding acid. A further pathway was the deamination of the parent compound followed by reduction to the corresponding alcohol or by oxidation to the corresponding acid. The latter was hydroxylated at position C2′ or C1′ followed by oxidation to the corresponding ketone.

A common metabolic step was the *O*-demethylation in position 2 or 5 of the aromatic ring. However, although in the most cases two isomers were detected, the exact position of the resulting hydroxy group could not be determined by means of GC–MS. An exception was the *O*-demethyl-oxo-*N*-acetyl metabolite (mass spectra nos. 9, 10 and 31). It was obvious that this metabolite showed good chromatographic properties in the underivatized form, furthermore it showed incomplete derivatization in the acetylated and trifluoroacetylated sample. Based on these findings and the fragmentation pattern, the structure shown in the mass spectra no. 9 in Fig. 1 was postulated. This structure contains the hydroxy group in position 5 that is able to form a hydrogen bond to the oxo function forming a stable six ring. This hydroxy group should be less polar than a hydroxy function in position 2 leading to the observed chromatographic properties. Furthermore, it should not be as reactive as a hydroxy function in position 2 due to the hydrogen bond. Therefore, the underivatized metabolite was detected.

Most of the metabolites were excreted in conjugated form. Such conjugation was concluded because the peak areas of these metabolites were more abundant after glucuronidase and sulfatase hydrolysis. The metabolites that were conjugated by

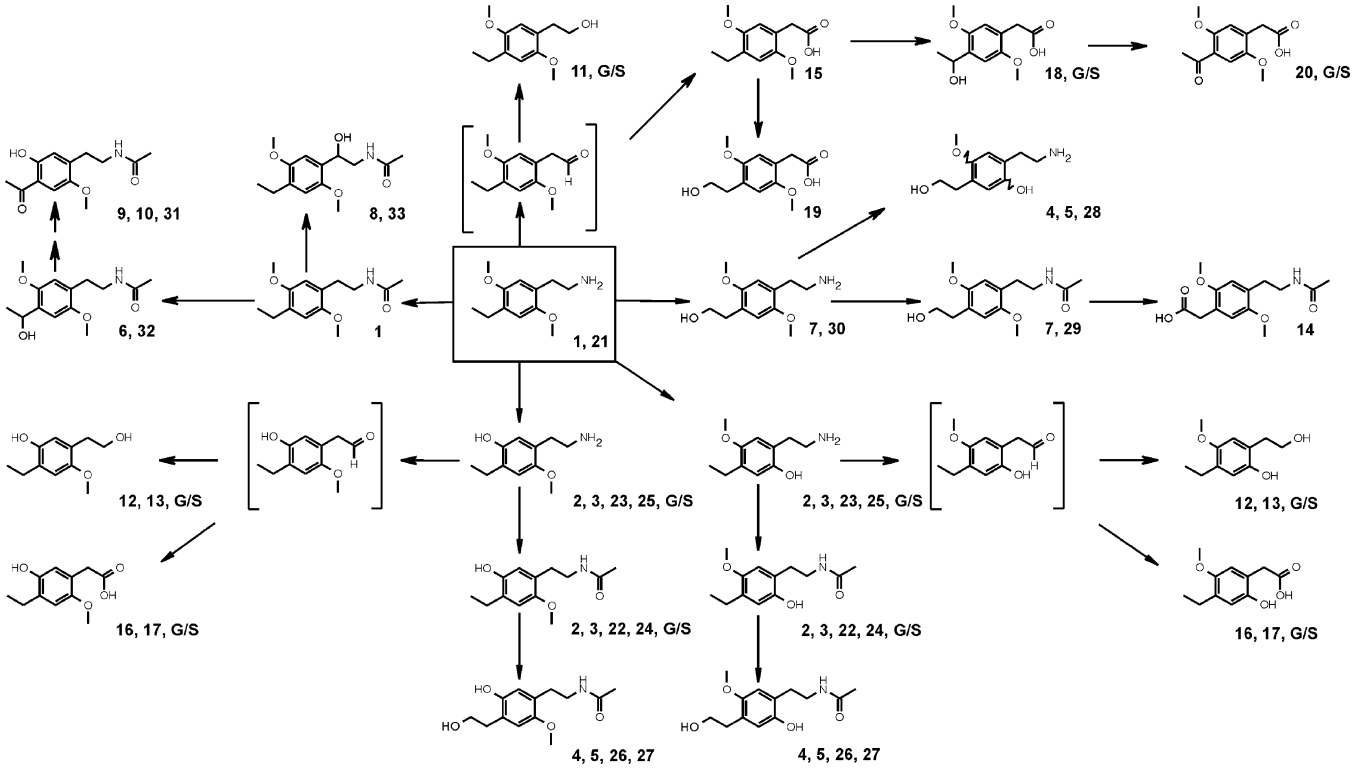

Fig. 2. Proposed scheme for metabolism of 2C-E in rats. The numbering of the compounds corresponds to that of the mass spectra in Fig. 1. The compound in parentheses are assumed intermediates. Compounds excreted as glucuronides/sulfates are marked by G/S.

glucuronidation/sulfation are indicated in Fig. 2. However, conjugation could not be examined for all metabolites, because many metabolites with an aliphatic hydroxy group formed artifacts, as mentioned above.

3.2. Detection by GC–MS within the STA

Acid hydrolysis has proven to be very efficient and fast for cleavage of conjugates [36]. However, some compounds were found to be altered or destroyed during hydrolysis [36]. Therefore, an aliquot of unhydrolyzed urine was added to the hydrolyzed aliquot before extraction. This modified sample preparation was a compromise between the necessity of a quick cleavage of conjugates and the detectability of compounds destroyed during acid hydrolysis. Although the modification of the STA procedure led to lower extract concentrations of compounds excreted in conjugated form, this modified procedure was sufficient, because of the high sensitivity of modern GC–MS apparatus [36].

The samples were extracted at pH 8–9, because metabolic formation of aromatic hydroxy groups may lead to phenolbases that are best extracted at this pH. Using a more alkaline pH for extraction leads to decreased extraction efficacies of such hydroxy metabolites which are often excreted for a longer period of time than the parent compounds [36]. Derivatization of the extracts was indispensable for sensitive detection.

The extraction efficacy determined for 2C-E after STA working-up was 108 ± 19.6% (mean ± S.D., $n = 5$) at 1000 ng/ml of urine.

2C-E and its metabolites were separated by GC and identified by full-scan MS. Mass chromatography with the ions *m/z* 192, 251, 178 and 237 was used to indicate the presence of the *N*-acetyl-*O*-demethyl and *N*-acetyl *O*-demethyl-oxo metabolite of 2C-E, as well as, to a minor extent, to indicate the presence of acetylated 2C-E itself. Fig. 3 shows typical reconstructed mass chromatograms with the given ions of an acetylated extract of a rat urine sample collected over 24 h after application of

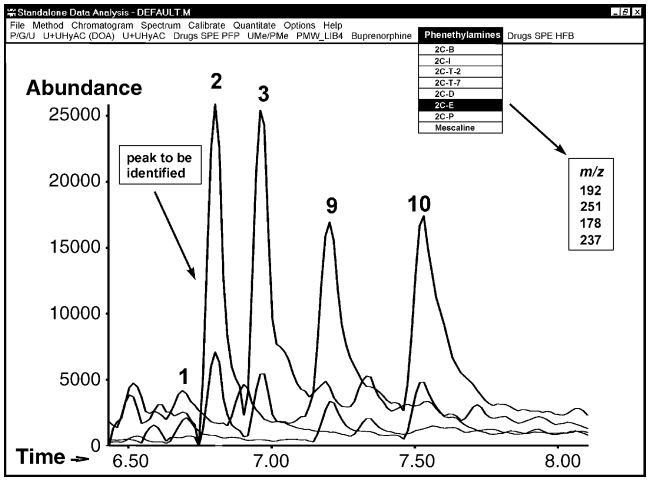

Fig. 3. Typical reconstructed mass chromatograms with the given ions of an acetylated extract of a rat urine sample collected over 24 h after intake of 0.3 mg/kg BM of 2C-E. They indicate the presence of 2C-E and its metabolites. The merged ion chromatograms can be differentiated by their colors on a color screen.

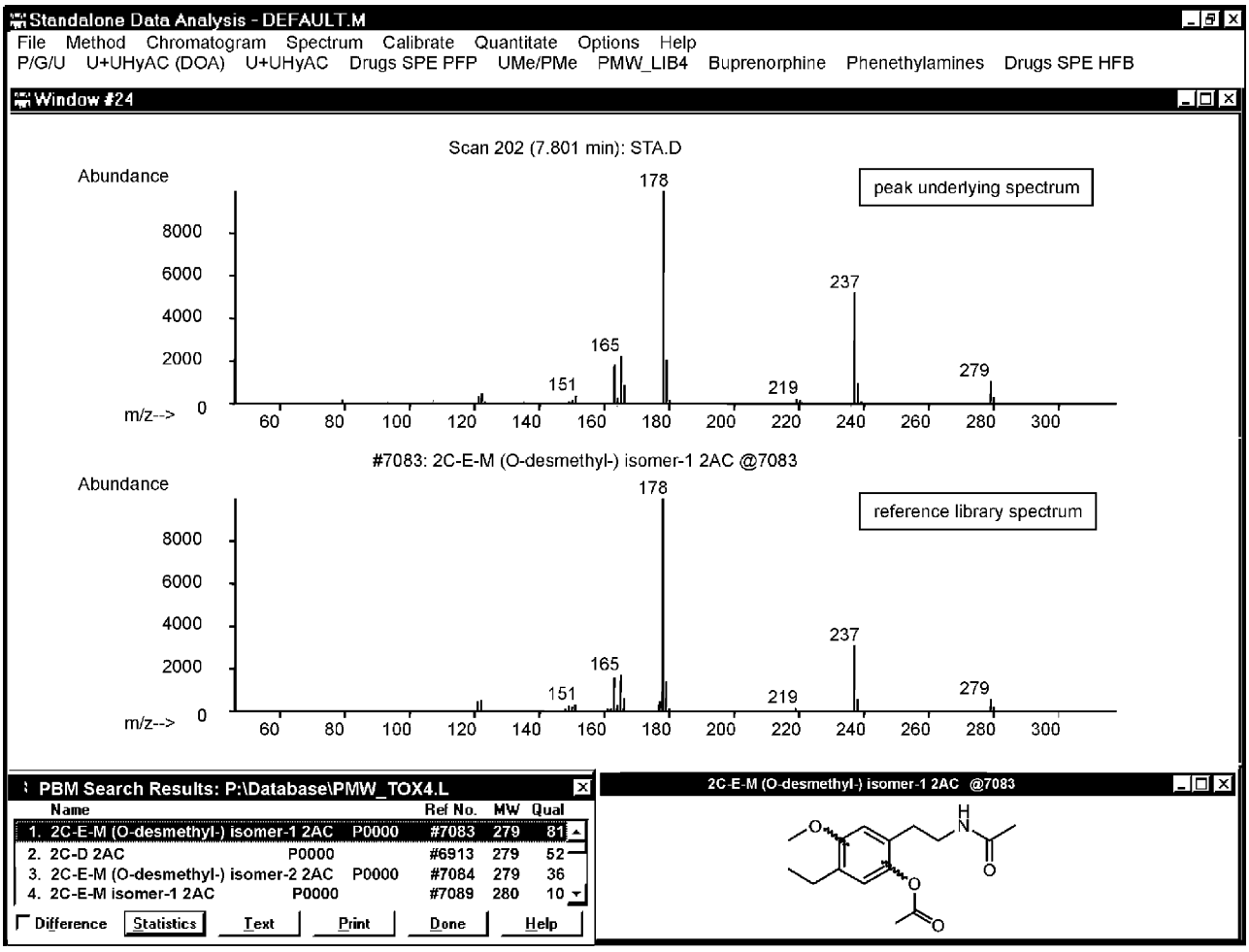

Fig. 4. Mass spectrum underlying the marked peak in Fig. 3, the reference spectrum, the structure, and the hit list found by computer library search.

0.3 mg/kg BM of 2C-E which corresponded to a common users' dose of about 10–25 mg. As exemplified in Fig. 4 for peak 2 in Fig. 3, the identity of peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study (Fig. 1). The selected ions *m/z* 192 and 251 were used for indication of acetylated 2C-E itself and detection of its *N*-acetyl-*O*-desalkyl-oxo metabolite, the ions *m/z* 178 and 237 were used for indicating the presence of the *N*-acetyl-*O*-desalkyl metabolites. Ion *m/z* 192 is a characteristic fragment resulting from loss of acetamide, ion *m/z* 251 is the molecular ion of this compound. The fragment ions *m/z* 178 and 237 result from loss of the acetyl moiety and from additional loss of acetamide, respectively. Although interferences by biomolecules or other drugs cannot be entirely excluded, they are rather unlikely, because their mass spectra and/or their RIs should be different. The RIs were recorded during the GC–MS procedure and calculated in correlation with the Kovats' indices [41] of the components of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [42]. The limit of detection of 2C-E was 10 ng/ml of urine (signal-to-noise S/N > 3). For lack of authentic human urine samples, a comparison of the metabolites found in rat and human urine after administration of 2C-E was not yet possible. However, according the earlier studies [43–47] good agreement has been reported for the metabolic pathways between rat and human.

4. Conclusions

The metabolism studies presented here showed that 2C-E was extensively metabolized. The suggested metabolic pathways were similar to those of other members of the 2C-series. *O*-Demethylation and *N*-acetylation were also main metabolic steps for 2C-B, 2C-I, 2C-T-2 and mescaline. Deamination was detected also for 2C-B, 2C-I, 2C-T-2, 2C-T-7 and mescaline. Hydroxylation of the side chain in position 4 was also detected for 2C-T-2 and 2C-T-7. The authors' STA procedure allowed the detection of an intake of a dose of 2C-E in rat urine that corresponds to a common drug users' dose. The target analytes were found to be the *N*-acetyl-*O*-demethyl metabolites of 2C-E, the *N*-acetyl-*O*-demethyl-oxo metabolite as well as acetylated 2C-E itself. As shown for many lipophilic compounds, their metabolites should become the major analytes in late phase of excretion. The authors' experience in metabolism and analytical studies on rats and humans support the assumption that the metabolites found in rat urine should also be present in human

urine. Therefore, it can be concluded that the procedure should also be applicable for human urine screening for 2C-E in clinical or forensic cases.

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2.5 STUDIES ON THE TOXICOLOGICAL DETECTION OF THE DESIGNER DRUG 4-BROMO-2,5-DIMETHOXY-β-PHENETHYLAMINE (2C-B) IN RAT URINE USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY[67]

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Short communication

Studies on the toxicological detection of the designer drug 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B) in rat urine using gas chromatography–mass spectrometry

Denis S. Theobald [a], Giselher Fritschi [b], Hans H. Maurer [a,*]

[a] *Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Saarland University, D-66421 Homburg (Saar), Germany*
[b] *Hessisches Landeskriminalamt, D-65187 Wiesbaden, Germany*

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Abstract

The phenethylamine-derived designer drug 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B) is known to be extensively metabolized in various species including humans. In rat urine, 2C-B was found to be excreted mainly via its metabolites. In the current study, the toxicological detection of these metabolites in the authors' systematic toxicological analysis (STA) procedure was examined. The STA procedure using full-scan GC–MS allowed proving an intake of a common drug abusers' dose of 2C-B by detection of the *O*-demethyl deaminohydroxy and two isomers of the *O*-demethyl metabolites in rat urine. Assuming similar metabolism, the described STA procedure should be suitable for proof of an intake of 2C-B in human urine.

Keywords: 4-Bromo-2,5-dimethoxy-β-phenethylamine; 2C-B; Designer drug; Metabolism; GC–MS

1. Introduction

4-Bromo-2,5-dimethoxy-β-phenethylamine (2C-B, Nexus, Venus, Bromo, Erox, Bees) is a hallucinogenic drug that was first synthesized in 1974 by Shulgin and Carter [1]. It belongs to the so-called 2C-series, which are phenethylamine derivatives having in common two methoxy groups in position 2 and 5 of the ring and one lipophilic substituent in position 4. The hallucinogenic properties of the 2C-drugs seem to be mediated by agonistic and/or antagonistic effects on various serotoninergic and α_1-adrenergic receptors [2–10]. In the mid 1980s, 2C-B appeared on the illicit drug market [11] and gained increasing popularity in the 1990s, when it was sold in so-called smart shops via the internet [12]. Since that time, 2C-B was identified in drugs seized in the illicit drug market all over the world [12–15]. Common drug abusers' doses for 2C-B ranged from 4 to 30 mg [14]. In 1998, it was the third most reported drug of ring substituted phenethylamines in England and Wales following MDMA and MDEA [11]. Consequently, 2C-B was scheduled in the lists of controlled substances in many countries [12]. Further evidence about its popularity among drug abusers can be found on internet web sites (http://www.erowid.org/, http://www.lycaeum.org/; June 2006) where experience reports and descriptions of 2C-B have been published.

The metabolism of 2C-B has been extensively studied in rats [16–18], mice [19], and in hepatocytes from various species including humans [20,21]. Preliminary data are also available on excretion of 2C-B metabolites in human urine [22]. All these metabolism studies showed that major metabolic steps of 2C-B were *O*-demethylation of the parent compound, *N*-acetylation, and deamination with oxidation to the corresponding acids or reduction to the corresponding alcohols and combinations of these steps. Theobald et al. could further identify a deaminohydroxy-side chain hydroxy, a *O*-demethyl deaminohydroxy-side chain hydroxy, and a *O*-demethyl deaminohydroxy-side chain oxo metabolite [23], which is in accordance with the data found for the iodo analogue 2C-I [16].

In clinical and forensic toxicology, such drugs of abuse must be analyzed for monitoring an abuse or a poisoning. Some stud-

* Corresponding author. Tel.: +49 68411626050; fax: +49 68411626051.
E-mail address: hans.maurer@uniklinikum-saarland.de (H.H. Maurer).

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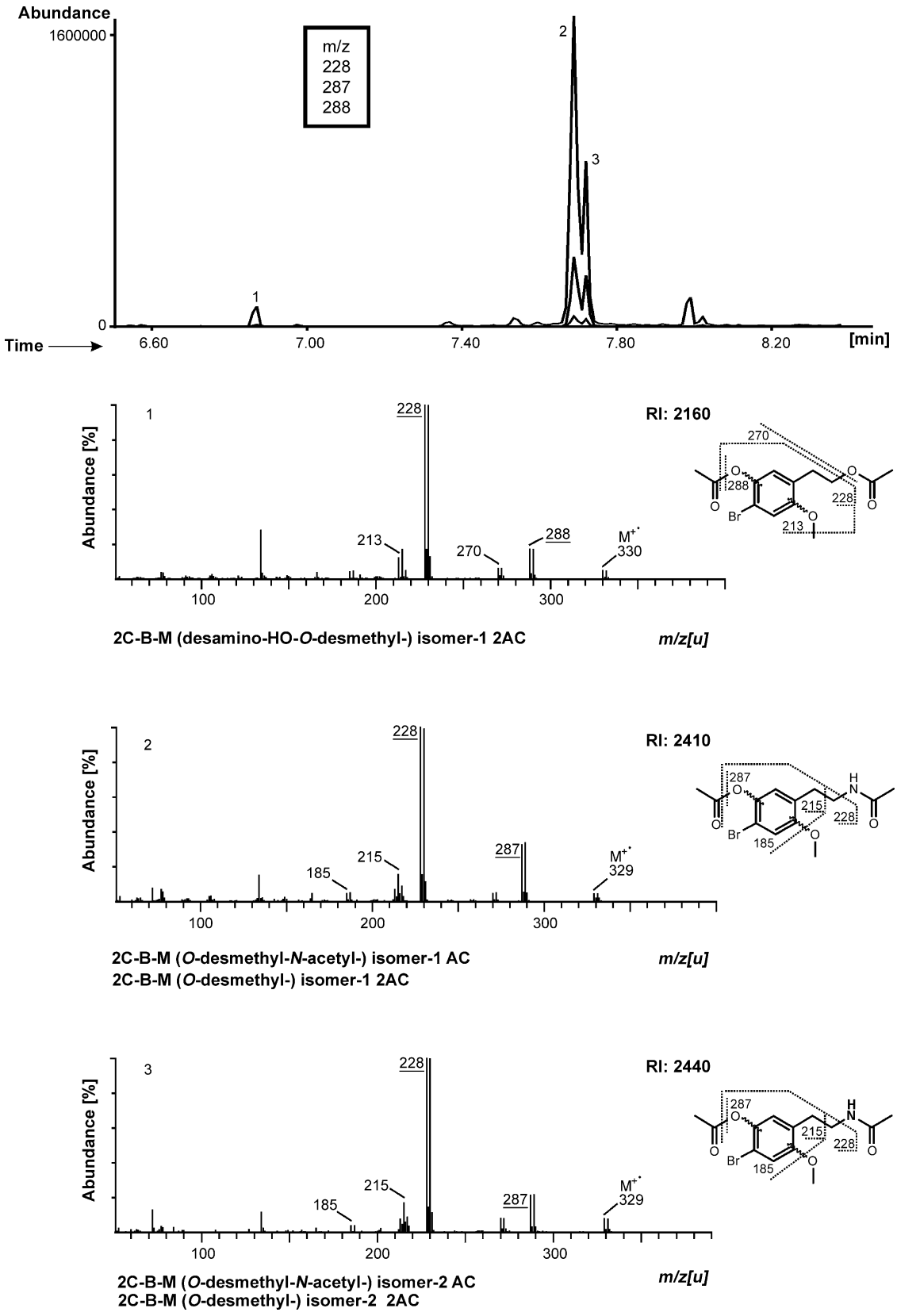

Fig. 1. Typical reconstructed mass chromatograms with the given ions of an acetylated extract of a rat urine sample collected over 24 h after intake of 0.3 mg/kg BM of 2C-B (upper part). They indicate the presence of 2C-B metabolites. EI mass spectra, RIs, structures and predominant fragmentation patterns of 2C-B metabolites included in the STA procedure after acetylation (lower part). The numbers of the spectra correspond to those of the peaks in the upper part. Selected ions are underlined.

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ies have been published on the detection of 2C-B itself in blood and/or urine [24–27]. However, as shown by the metabolism studies, 2C-B itself was excreted into urine only to a very small extent, whereas the metabolites played the main role in the excretion process. Ingestion of other compounds of the 2C-series could be screened for by the authors' systematic toxicological analysis (STA) procedure in urine by GC–MS and this STA procedure allows simultaneous detection of about 2000 other drugs, poisons and/or their metabolites [16,23,28–33]. Therefore, the aim of this study was to investigate the detectability of these major 2C-B metabolites as target analytes within the authors' STA procedure.

2. Experimental

2.1. Chemicals and reagents

2C-B tartrate was provided by Hessisches Landeskriminalamt (Wiesbaden, Germany) for research purposes. All chemicals and biochemicals were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

2.2. Urine samples

The investigations were performed using urine of male Wistar rats (about one year old and 400 g body mass (BM), Ch. River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 0.3 mg/kg BM dose for the STA study in aqueous suspension by gastric intubation ($n = 2$). Urine was collected separately from the faeces over a 24-h period. The samples were directly analyzed. Blank rat urine samples were collected before drug administration to check whether they were free of interfering compounds.

2.3. Sample preparation for toxicological analysis

A 5-ml portion of urine was worked-up as previously described for 2C-E [32]. After acidic hydrolysis, the liquid–liquid extract was derivatized by acetylation. Aliquots (2 μl) were injected into the GC/MS system.

2.4. GC–MS apparatus and method

A Hewlett Packard (Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with a HP 5972A MSD mass spectrometer was used under the condition described for 2C-E [32]. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m × 0.2 mm I.D.), cross linked methyl silicone, 330 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100 to 310 °C at 30°/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full-scan mode, *m/z* 50–800 u; EI mode, ionization energy, 70 eV; ion source temperature, 220 °C; capillary direct interface, heated at 280 °C.

For toxicological detection of acetylated 2C-B metabolites, mass chromatography with the selected ions *m/z* 228, 287, and 288 was used. The identity of the peaks in the mass chromatograms was confirmed by computerized comparison of the mass spectra underlying the peaks (after background subtraction) with reference spectra recorded during the previous metabolism study [23,34].

3. Results and discussion

Using the STA procedure, the most abundant isomer of bisacetylated deamino hydroxy *O*-demethyl 2C-B as well as the bisacetylated two isomers of *O*-demethyl 2C-B were found to be the target analytes. The latter could also be formed by monoacetylation of the corresponding *N*-acetyl conjugates [17,23]. They could be detected by mass chromatography with the ions *m/z* 228, 287, and 288. Fig. 1 (upper part) shows typical reconstructed mass chromatograms with the given ions of an acetylated extract of a rat urine sample collected over 24 h after application of 0.3 mg/kg BM of 2C-B which corresponded to a common abusers' dose of about 20 mg. The lower part of Fig. 1 shows the EI mass spectra, the retention indices (RI), the structures and the predominant fragmentation patterns of the three target analytes. In poisoning cases, 2C-B and most of the other metabolites should also be detectable using the reference spectra published elsewhere [23,34], because they were detected in rat urine after a 20-fold higher dose.

Although interferences by biomolecules or other drugs cannot be entirely excluded, they are rather unlikely, because their mass spectra and/or their RIs should be different. In addition, the characteristic bromine isotope clusters in the EI mass spectra of 2C-B facilitate its unambiguous identification.

For lack of authentic human urine samples, a comparison of the metabolites found in rat and human urine after administration of 2C-B was not yet possible. However, in other studies good correspondence has been reported for the metabolic pathways as well as for the detectability between rats and humans [35–39]. Finally, de Boer et al. [22] have reported detection of *O*-demethyl 2C-B in human urine.

4. Conclusions

The authors' STA procedure allowed proving an intake of a common drug abusers' dose of 2C-B in rat urine by detection of its major metabolites. Earlier studies and the authors' experience in metabolism and analytical studies on rats and humans support the assumption that the metabolites found in rat urine should also be present in human urine. Therefore, it can be concluded that the procedure should also be applicable for human urine screening for 2C-B in clinical or forensic toxicology.

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2.6 STUDIES ON THE METABOLISM AND TOXICOLOGICAL DETECTION OF THE DESIGNER DRUG 2,5-DIMETHOXY-4-METHYL-β-PHENETHYLAMINE (2C-D) IN RAT URINE USING GASCHROMATOGRAPHIC-MASSSPECTROMETRIC TECHNIQUES[68]

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2.7 Identification Of Monoamine Oxidase and Cytochrome P450 Isoenzymes Involved In The Deamination Of Phenethylamine-Derived Designer Drugs (2C-series)[69]

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Identification of monoamine oxidase and cytochrome P450 isoenzymes involved in the deamination of phenethylamine-derived designer drugs (2C-series)

Denis S. Theobald, Hans H. Maurer *

Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Saarland, D-66421 Homburg (Saar), Germany

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ABSTRACT

In recent years, several compounds of the phenethylamine-type (2C-series) have entered the illicit drug market as designer drugs. In former studies, the qualitative metabolism of frequently abused 2Cs (2C-B, 2C-I, 2C-D, 2C-E, 2C-T-2, 2C-T-7) was studied using a rat model. Major phase I metabolic steps were deamination and *O*-demethylation. Deamination to the corresponding aldehyde was the reaction, which was observed for all studied compounds. Such reactions could in principal be catalyzed by two enzyme systems: monoamine oxidase (MAO) and cytochrome P450 (CYP). The aim of this study was to determine the human MAO and CYP isoenzymes involved in this major metabolic step and to measure the Michaelis–Menten kinetics of the deamination reactions. For these studies, cDNA-expressed CYPs and MAOs were used. The formation of the aldehyde metabolite was measured using GC–MS after extraction. For all compounds studied, MAO-A and MAO-B were the major enzymes involved in the deamination. For 2C-D, 2C-E, 2C-T-2 and 2C-T-7, CYP2D6 was also involved, but only to a very small extent. Because of the isoenzymes involved, the 2Cs are likely to be susceptible for drug–drug interactions with MAO inhibitors.

1. Introduction

The members of the so-called 2C-series belong to a class of designer drugs that are all phenethylamine derivatives. Their chemical structures comprise a primary amine functionality separated from the phenyl ring by two carbon atoms (“2C”), the presence of methoxy groups in positions 2 and 5 of the aromatic ring, and a lipophilic substituent in position 4 of the aromatic ring (alkyl, halogen, alkylthio, etc.) [1]. Typical 2Cs are 4-bromo-2,5-dimethoxy-β-phenethylamine (2C-B), 4-iodo-2,5-dimethoxy-β-phenethylamine (2C-I), 2,5-dimethoxy-4-methyl-β-phenethylamine (2C-D), 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E), 4-ethylthio-2,5-dimethoxy-β-phenethylamine (2C-T-2), and 2,5-dimethoxy-4-propylthio-β-

* *Corresponding author*. Tel.: +49 6841 1626050; fax: +49 6841 1626051.
E-mail address: hans.maurer@uniklinikum-saarland.de (H.H. Maurer).

Abbreviations: *2C-B, 4-bromo-2,5-dimethoxy-β-phenethylamine; 2C-I, 4-iodo-2,5-dimethoxy-β-phenethylamine; 2C-D, 2,5-dimethoxy-4-methyl-β-phenethylamine; 2C-E, 4-ethyl-2,5-dimethoxy-β-phenethylamine; 2C-T-2, 4-ethylthio-2,5-dimethoxy-β-phenethylamine; 2C-T-7, 2,5-dimethoxy-4-propylthio-β-phenethylamine; 5-HT, 5-hydroxy tryptamine (serotonin); MAO, monoamine oxidase; CYP, cytochrome P450; K_m, Michaelis–Menten constant; V_{max}, maximal turnover rate; PAR, peak area ratio; SIM, selected-ion monitoring; EI, electron ionization; IS, internal standard; LC–MS, liquid chromatography–mass spectrometry; APCI, atmospheric pressure chemical ionization; HPLC-UV, high performance liquid chromatography ultra violet detection*

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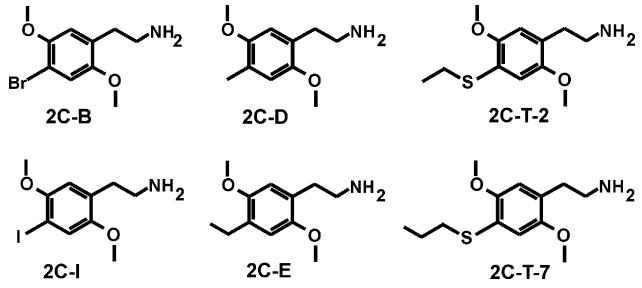

Fig. 1 – Chemical structures of the studied members of the 2C-series.

phenethylamine (2C-T-7) [2–5]. Their chemical structures are depicted in Fig. 1.

Most of known members of the 2C-series were synthesized and described by Shulgin during the 1970s and 1980s [1]. Since the 1990s, they have entered the illicit drug market as recreational drugs [3]. Later the 2Cs were sold in so-called ''smart shops'' and were mentioned in scene books and on so-called drug information web sites (http://www.erowid.org, http://www.lycaeum.org June 2006) [3]. Furthermore, seizures by the police of tablets containing 2Cs or combinations of them with other drugs were reported in recent years [6–11]. As a consequence, several 2Cs have been scheduled in many countries [12–14].

Only little information is available on pharmacological properties of the 2Cs, but it is known, that the compounds of the 2C-series show affinity to 5-HT_2 receptors, acting as agonists or antagonists at different receptor subtypes [15–23]. For 2C-B, partial agonism at the α_1-adrenergic receptor was described [24,25]. Little is known about the toxicology of these compounds, but at least for 2C-T-7 fatal intoxications have been reported during 2000/2001 [4,12,26].

In recent studies, the metabolism of several 2Cs was studied mainly in rats [27–33], but also in humans [34], mice [35], and hepatocytes of different species [36,37]. One major metabolic step was the deamination of the parent compound to the corresponding aldehyde. These aldehydes could not be detected in urine, most probably because they were rapidly reduced or oxidized to the respective alcohols and carboxylic acids, which were present in urine.

The involvement of particular isoenzymes in the biotransformation of a new therapeutic drug has to be thoroughly investigated before it can be marketed. Such investigations allow to predict possible drug–drug-interactions, inter-individual variations in pharmacokinetic profiles and increased appearance of side effects and serious poisonings [38]. Such risk assessment is typically performed for substances intended for therapeutic use, but not for drugs of the illicit market. In addition, there is good evidence that genetic variations in drug metabolism have important behavioral consequences that can alter the risk of drug abuse and dependence [39].

Regarding the above mentioned deamination reaction, isoenzymes of the monoamine oxidase (MAO) and cytochrome P450 (CYP) type might be able to catalyze this reaction. MAO enzymes A and B are outer mitochondrial membrane-bound flavoenzymes that can be found mainly in neuronal and glia cells, but also in the liver. They catalyze the oxidation of primary, secondary, and some tertiary amines to their corresponding protonated imines with further non-enzymatic hydrolysis of the imine products to the corresponding aldehyde [40]. Their physiological substrates are neurotransmitters such as dopamine or noradrenaline, which show structural similarity to the 2Cs [41]. Consistently, phenethylamine is a specific substrate for MAO-B [41]. CYP enzymes are located in membranes, mainly the endoplasmic reticulum, and can be found mainly in the liver. They are also able to catalyze deamination via oxidation of the α-carbon atom next to the nitrogen [42].

Therefore, isoenzymes of the MAO- and CYP-type were studied concerning their ability to catalyze deamination of the 2Cs. Furthermore, the enzyme kinetics of these reactions was measured and the kinetic data like Michaelis–Menten constants (K_m) and the maximal turnover rates (V_{max}) were determined.

2. Materials and methods

2.1. Materials

For research purposes, hydrochlorides of 2C-D and 2C-E were provided by Dejachem (Schwendi, Germany), 2C-B tartrate by Hessisches Landeskriminalamt (Wiesbaden, Germany), 2C-I hydrochloride by Landeskriminalamt Baden-Württemberg (Stuttgart, Germany), 2C-T-2 hydrochloride by Bundeskriminalamt (Wiesbaden, Germany), and 2C-T-7 hydrochloride by Bayerisches Landeskriminalamt (Munich, Germany).

$NADP^+$ was obtained from Biomol, isocitrate and isocitrate dehydrogenase from Sigma, all other chemicals and reagents from Merck. The following microsomes were from Gentest and delivered by NatuTec: baculovirus-infected insect cell microsomes containing 1 nmol/mL human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 (supersomes), baculovirus-infected insect cell microsomes containing 5 mg/mL human cDNA-expressed MAO-A or MAO-B (supersomes), wild-type baculovirus-infected insect cell microsomes (control supersomes). After delivery, the microsomes were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Microsomal incubations

For the CYP enzymes, typical incubation mixtures (final volume: 50 μL) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM Mg^{2+}, 5 mM isocitrate, 1.2 mM $NADP^+$, 2 U/mL isocitrate dehydrogenase, 200 U/mL superoxide dismutase, and various concentrations of substrate at 37 °C. For the MAO enzymes, typical incubation mixtures (final volume: 50 μL) consisted of 100 mM phosphate buffer (pH 7.4), and various concentrations of substrate at 37 °C. The substrate was added after dilution of a 25 mM aqueous stock solution in buffer. Reactions were started by addition of the ice-cold microsomes and terminated with 5 μL of perchloric acid 60% (w/w).

2.3. Initial screening studies

In order to investigate the involvement of particular MAOs or CYPs in metabolism of the 2Cs, 250 μM of the respective 2C compound (2C-B, 2C-I, 2C-D, 2C-E, 2C-T-2, or 2C-T-7) and 50 pmol/mL CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, 0.2 mg/mL MAO-A, or 0.2 mg/mL MAO-B were incubated for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM or 90 mM Tris buffer, according to the Gentest manuals.

2.4. Enzyme kinetic studies

Duration of and protein content for all incubations were in the linear range of metabolite formation (data not shown). Kinetic constants were derived from incubations ($n = 2$ each) with the following 2C concentration ranges, incubation times and protein concentrations: 5–600 μM 2C-B with 0.05 mg MAO-A/mL for 30 min, 2–600 μM 2C-B with 0.05 mg MAO-B/mL for 30 min, 5–600 μM 2C-I with 0.05 mg MAO-A/mL for 30 min, 5–600 μM 2C-I with 0.05 mg MAO-B/mL for 30 min, 10–600 μM 2C-D with 0.05 mg MAO-A/mL for 30 min, 10–600 μM 2C-D with 0.05 mg MAO-B/mL for 30 min, 5–600 μM 2C-E with 0.1 mg MAO-A/mL for 25 min, 5–1000 μM 2C-E with 0.05 mg MAO-B/mL for 30 min, 5–600 μM 2C-T-2 with 0.05 mg MAO-A/mL for 30 min, 5–600 μM 2C-T-2 with 0.05 mg MAO-B/mL for 30 min, 1–600 μM 2C-T-7 with 0.05 mg MAO-A/mL for 30 min, 5–600 μM 2C-T-7 with 0.03 mg MAO-B/mL for 30 min.

Apparent K_m and V_{max} values for single isoenzymes were estimated by nonlinear curve fit according to the Michaelis–Menten equation:

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

Unfortunately, no reference substances of the metabolites were available. Therefore, only relative estimations of V_{max} values, expressed as dimensionless peak area ratios (PAR) per minute and mg protein could be obtained.

2.5. Extraction of the metabolites

After termination of the incubation, the samples were extracted with 50 μL cyclohexane containing 0.01 mM 2,5-dimethoxybenzaldehyde as internal standard. The samples were shaken for 2 min on a rotary shaker and centrifuged for 1 min. After centrifugation, the organic phases were transferred to autosampler vials. A 1 μL aliquot was directly injected into the GC–MS apparatus and analyzed in the full scan and selected-ion monitoring (SIM) mode.

2.6. Identification of the metabolites

The extracted aldehyde metabolites of the respective 2C compounds were separated by GC and identified by electron ionization (EI) mass spectrometry in the full scan mode by their recorded mass spectra. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode, which were interpreted in correlation to those of other metabolites detected in previous studies [27,29–31,43,44]. The interpretations were according to the rules described by, e.g. McLafferty and Turecek [45] and Smith and Busch [46].

2.7. Statistical analysis

All statistics were calculated using GraphPad Prism 3.02 software (San Diego, CA) designed for nonlinear curve fit analysis. The Michaelis–Menten parameters K_m and V_{max} were calculated by fitting kinetic data to a one-site binding model.

2.8. GC–MS conditions and quantification in microsomal incubation extracts

2.8.1. Apparatus

The samples were analyzed using a Hewlett Packard (Agilent) HP 6890 Series GC system combined with an HP 5972 Series mass selective detector, an HP 6890 Series injector and an HP Chem Station software G1701AA Version A.03.00.

2.8.2. GC–MS conditions

GC conditions were as follows: splitless injection mode; column, HP-5MS capillary (30 m × 0.25 mm i.d.), 5% phenyl methyl siloxane, 250 nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow rate, 0.6 mL/min; column temperature, 50 °C for 3 min, then increased to 310 °C at 40 °C/min and was held at this temperature for 1 min. MS conditions were as follows: transfer line heater, 280 °C; source temperature, 140 °C; EI mode; ionization energy, 70 eV; selected-ion monitoring with the following program: solvent delay, 4 min; *m/z* 166 for the internal standard 2,5-dimethoxybenzaldehyde, *m/z* 229 for 2C-B aldehyde, *m/z* 277 for 2C-I aldehyde, *m/z* 165 for 2C-D aldehyde, *m/z* 179 for 2C-E aldehyde, *m/z* 211 for 2C-T-2 aldehyde and *m/z* 225 for 2C-T-7 aldehyde. For full-scan mode a range of *m/z* 50–800 was detected. The PARs between the respective 2C compound and 2,5-dimethoxybenzaldehyde (IS) were determined.

3. Results

3.1. GC–MS procedures

The aldehyde metabolites were identified by their MS fragmentation pattern in the full-scan mode. The EI mass spectra, the structures and predominant fragmentation patterns of them are shown in Fig. 2. As observed for many other metabolites of the 2Cs [27–31], the benzyl cleavage was the major fragmentation step, and the resulting *m/z* value was chosen as target ion in the SIM procedure. Since the extraction was done at acidic pH, the parent compounds were not extracted and are therefore not present in the GC–MS runs.

The applied GC–MS conditions provided baseline separation of all aldehydes and the internal standard. The mass fragmentograms in Fig. 3 show exemplarily the separation for 2C-T-7. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and IS; data not shown).

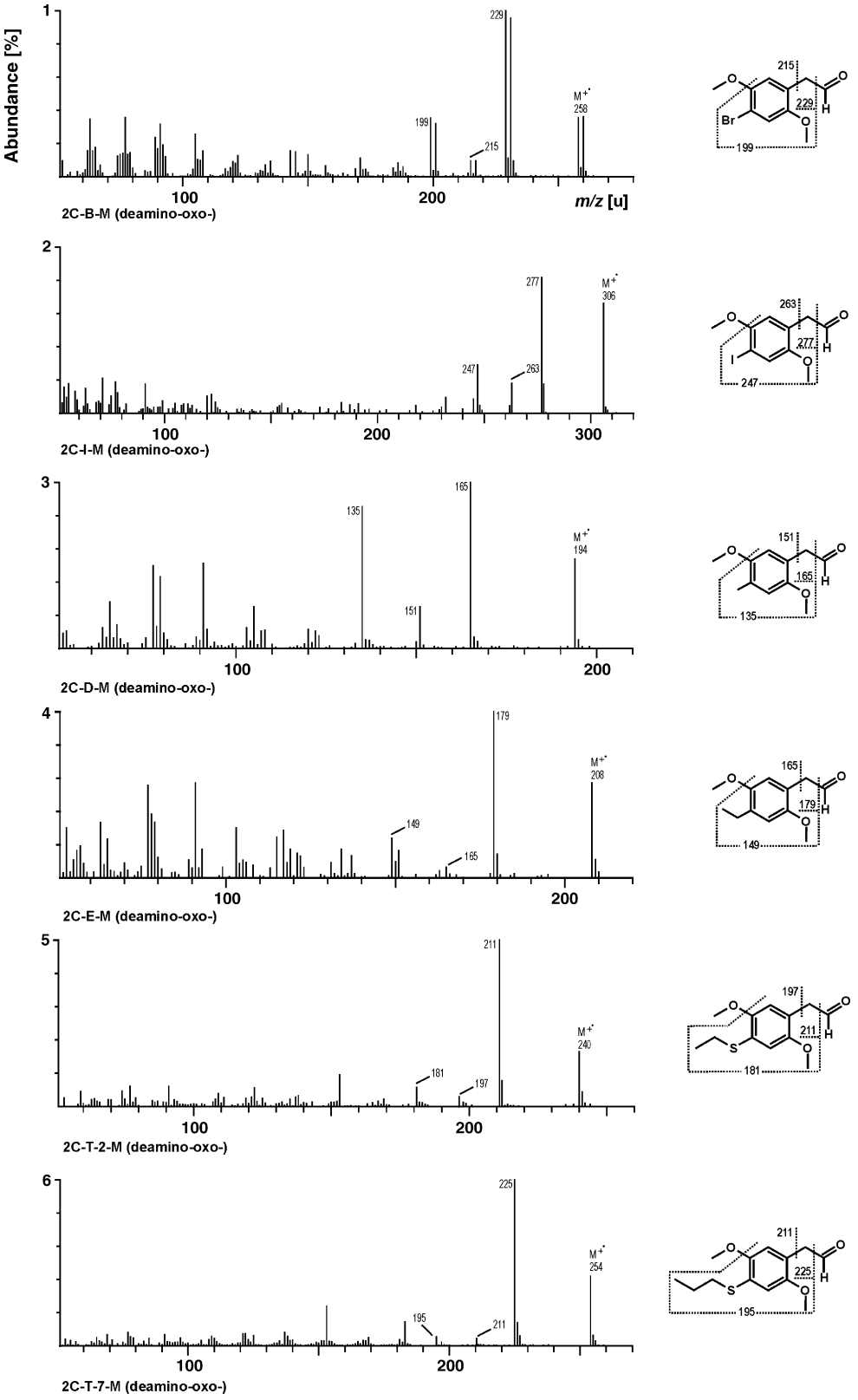

Fig. 2 – EI mass spectra, structures and predominant fragmentation patterns of the 2Cs' aldehyde metabolites.

The ion *m*/z 166 for the internal standard was the molecular ion of this compound, whereas the chosen target ion for the respective aldehyde metabolite resulted from benzyl cleavage of this compound.

3.2. *Initial screening studies*

The formation rates depicted in Fig. 4 show that among the 11 tested enzymes, MAO-A and B were the major enzymes

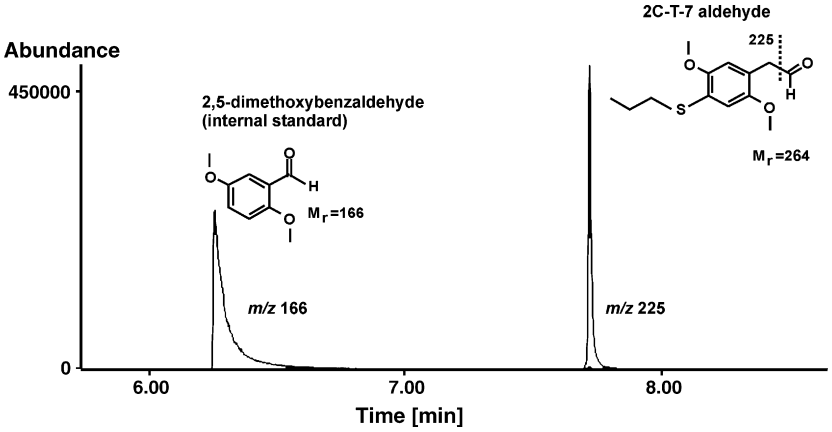

Fig. 3 – Typical mass fragmentograms of a cyclohexane extract of an incubation mixture of 250 μM 2C-T-7 with cDNA-expressed MAO-B with the following ions: *m/z* 166 or 225 for 2,5-dimethoxybenzaldehyde (IS) or 2C-T-7 aldehyde, respectively.

involved in the deamination of the 2Cs. For 2C-D, 2C-E, 2C-T-2 and 2C-T-7, CYP2D6 was also involved, but only to a small extent. The respective 2C aldehydes were not detectable in incubations with the other cDNA-expressed CYPs or with insect cell control microsomes.

3.3. *Kinetic studies*

All incubations were carried out at initial rate conditions, a prerequisite for Michaelis–Menten kinetics. All of the kinetics of the investigated reactions with single cDNA-expressed MAOs showed a typical hyperbolic profile, as shown in Fig. 5. The kinetic parameters (apparent K_m and V_{max}) for these reactions are listed in Table 1. They were estimated using Michaelis–Menten Eq. (1).

In general, V_{max} values could only be expressed as arbitrary units, because the metabolites could not be quantified without reference substance. The V_{max} values in Table 1 are expressed as dimensionless PAR per min and mg MAO.

4. Discussion

In the current study, the isoenzyme dependency of one of the major metabolic steps in the metabolism of six compounds of the 2C-series was studied. The deamination reaction might principally be catalyzed by MAO or CYP isoenzymes. Therefore, MAO-A and MAO-B, as well as the most important CYPs involved in drug metabolism were tested for their capability to catalyze this reaction. The incubation procedure for the CYPs was a well established and published method, which was already used to study enzyme kinetics of other designer drugs [47–54]. In the described assays, superoxide dismutase was added to suppress the formation of reactive oxygen species. The incubation procedure for the MAOs was close to the manufacturers guidelines and to a published procedure [55], but the final volume was reduced following the CYP procedure. The best way to analyze the terminated incubation mixture without loss of metabolites would be to inject it directly into a liquid chromatography–mass spectrometry (LC–MS) system [48–53]. However, preliminary studies with the model substances phenyl acetaldehyde and 2,5-dimethoxybenzaldehyde showed that the sensitivity of the LC–MS system described in Refs. [48–53] was not that of the GC–MS, perhaps because of incomplete ionization of the aldehydes. This was shown in a dilution experiment, where low concentrations of the aldehydes could not be detected with LC–MS but with GC–MS. Furthermore, after incubation of the 2Cs with MAO, no metabolites could be detected with LC–MS but with GC–MS (data not shown). Therefore, GC–MS was chosen for detection of the metabolites. Since it was not possible for GC–MS to inject the terminated incubation mixture directly, an extraction step had to be added. For the choice of the best extracting agent, several solvents were tested for their ability to extract the aldehyde metabolites. Furthermore, solutions of the model substances phenyl acetaldehyde and 2,5-dimethoxybenzaldehyde were extracted with several extracting agents. Cyclohexane showed a recovery of nearly 100% for extraction of the two model substances at acidic pH and was therefore chosen for the extraction of the aldehyde metabolites. At more basic pH, no aldehyde could be extracted perhaps due to the formation of hydrates.

Another problem was the lack of reference standards for the aldehyde metabolites. In extensive preliminary experiments, it was tried to synthesize these reference standards. But neither the Dess Martin oxidation of the parent compounds at low temperature, nor other trials led to a satisfying result. As no reference substances of the monitored metabolites were available for their exact quantification, only PARs could be determined instead of absolute metabolite concentrations. However, this did not affect the conclusions drawn from the kinetic estimations [48–50,52]. Linearity of the mass spectrometer response over the estimated concentration range could be shown for phenyl acetaldehyde and 2,5-dimethoxybenzaldehyde, which are structurally closely related to the monitored metabolites (data not shown), so one might reasonably assume linearity of the mass spectrometer response of the 2Cs' aldehyde metabolites.

The initial screening studies with the two human hepatic MAOs and nine most abundant human hepatic CYPs were

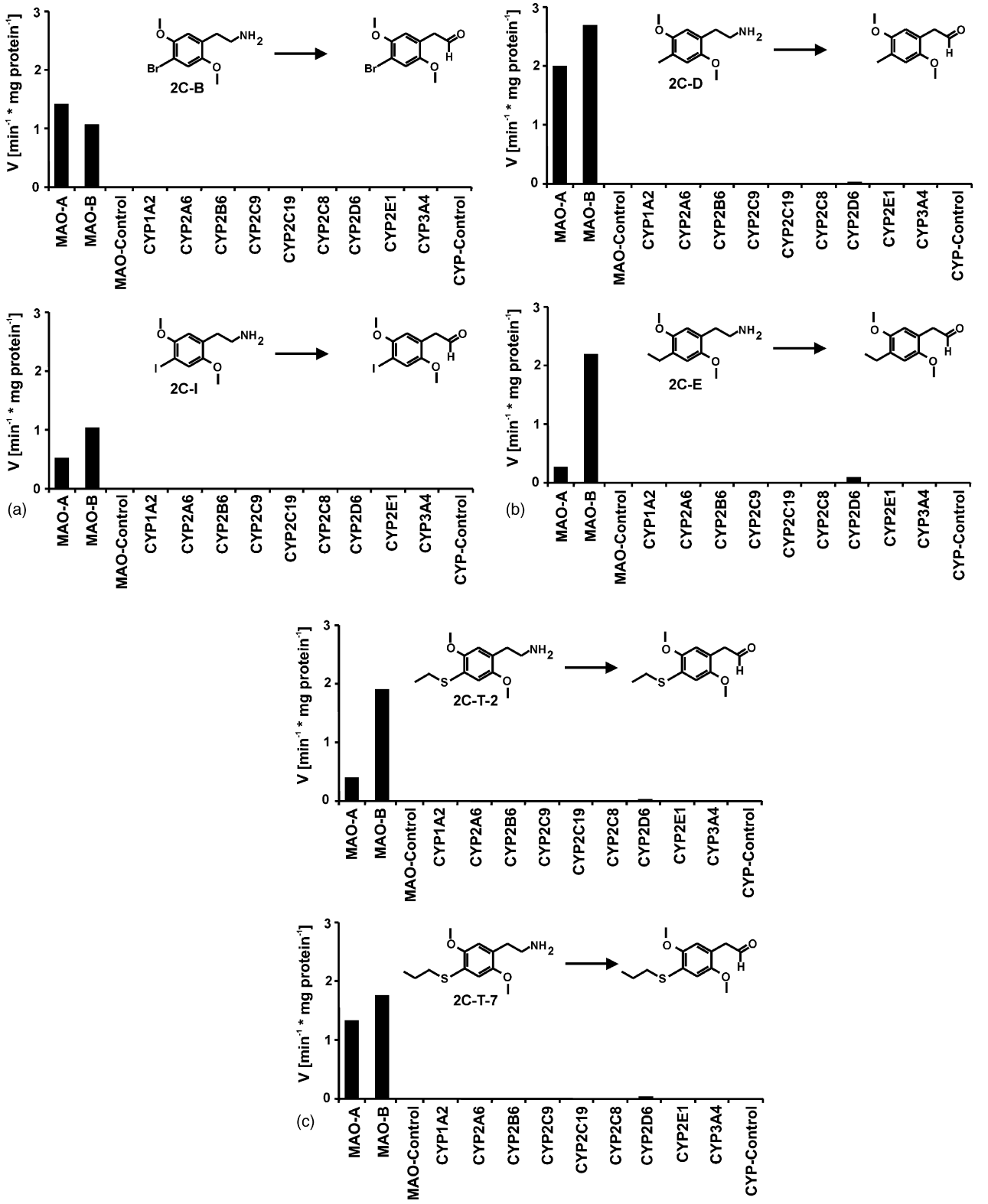

Fig. 4 – Formation rates (V) of 2C deamination (250 μM 2C compound each) with 0.2 mg/mL MAO-A or MAO-B or 50 pmol/mL of the given individual CYPs (V given as dimensionless PAR per min and mg protein) and with insect cell control microsomes.

performed to identify their possible role in 2C deamination. According to the supplier's recommendations, the incubation conditions chosen were adequate to make a statement on a general involvement of a particular MAO or CYP. The data revealed that MAO-A and MAO-B were capable of catalyzing the monitored reaction. For 2C-D, 2C-E, 2C-T-2 and 2C-T-7, also CYP2D6 was involved, but with low formation rate. Only the kinetic profiles of the reactions by MAO-A and MAO-B were

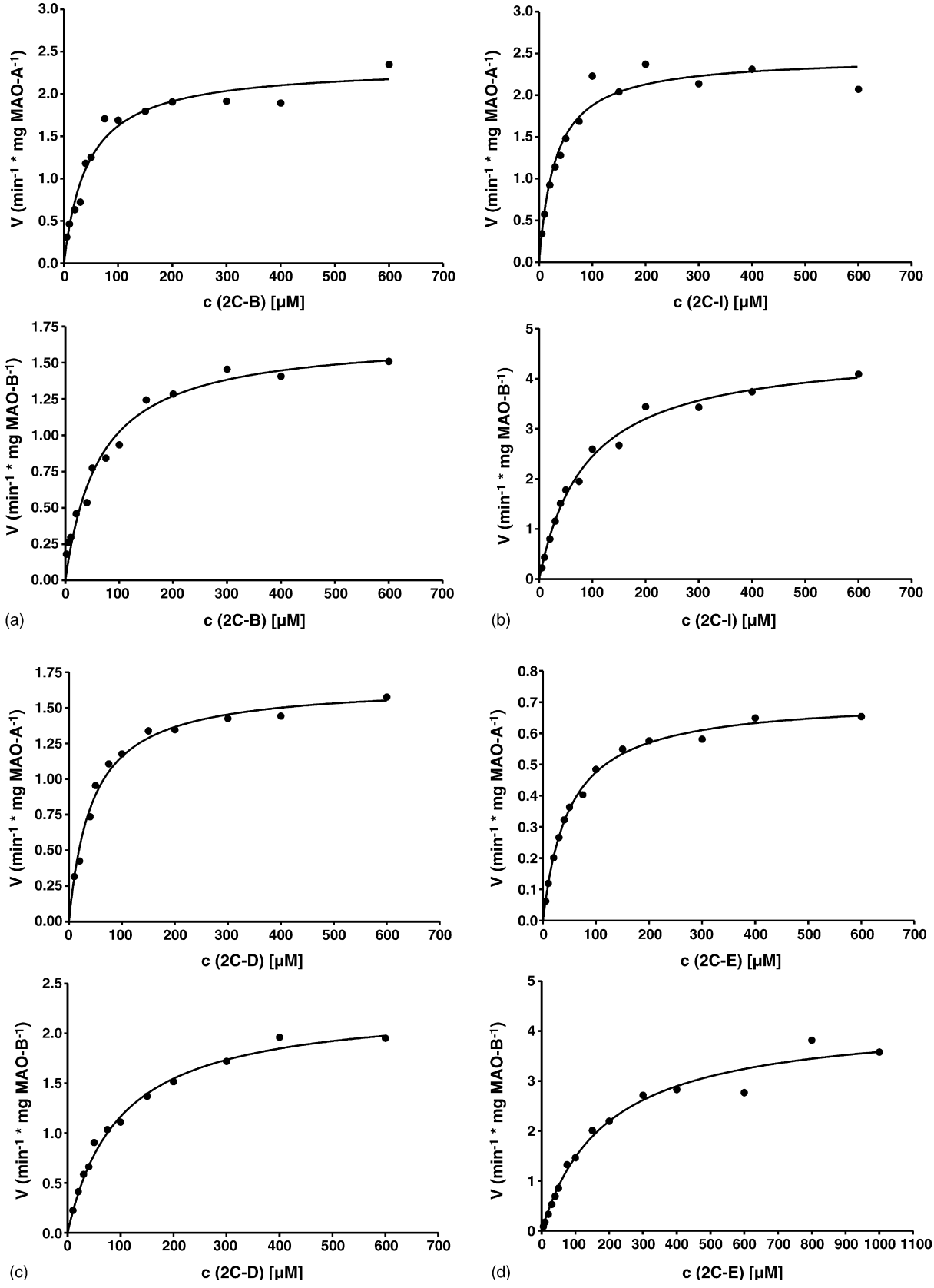

Fig. 5 – Michaelis–Menten plots for 2C deamination catalyzed by MAO-A or MAO-B. Values represent the mean of duplicate incubations. V given as dimensionless PAR per min and mg protein. Curves were calculated by nonlinear curve fit according to Eq. (1) (one-site binding model).

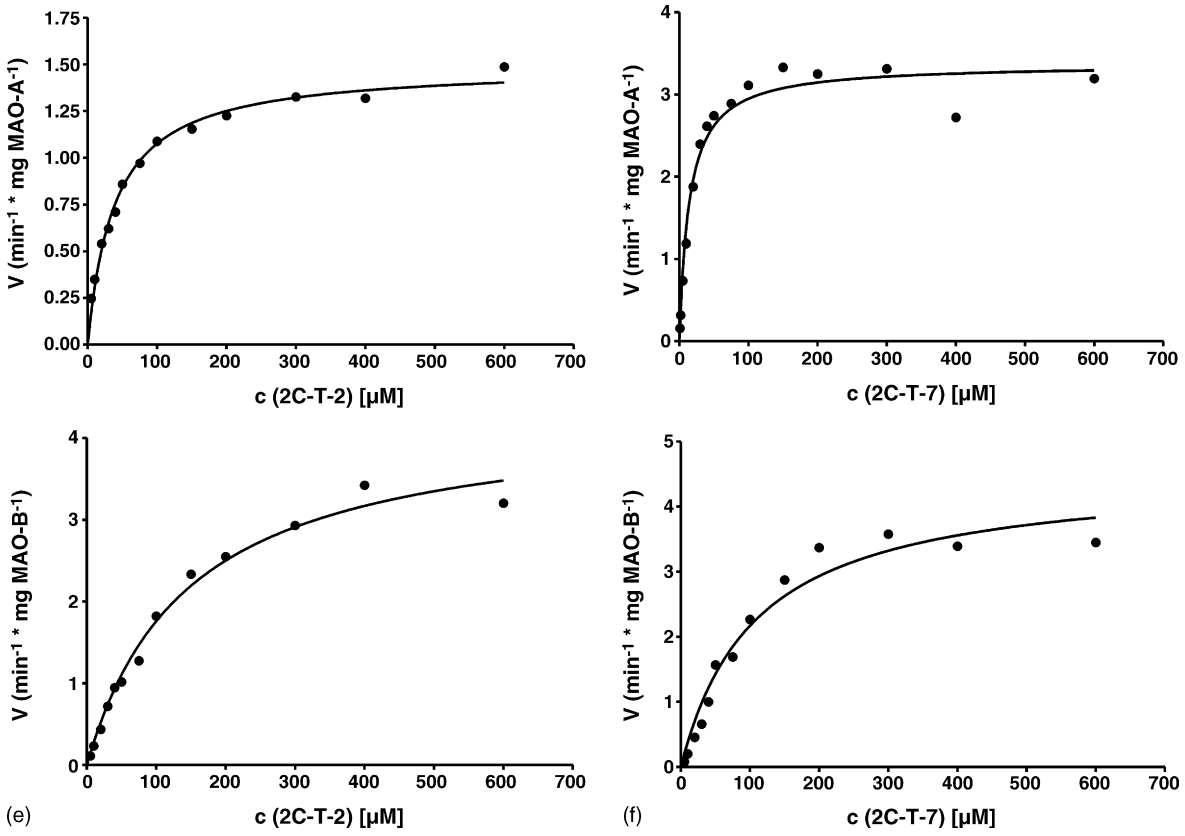

Fig. 5. (Continued).

further investigated. Kinetic assays with these enzymes were performed under initial rate conditions, a prerequisite for Michaelis–Menten kinetics [56]. These conditions were chosen according to previous experiments concerning the enzyme concentration and time linearity. Furthermore, less than 20% of substrate was metabolized in all incubations, as determined with HPLC-UV after direct injection of the incubation mixtures (data not shown). This method was used since the parent compounds were not extracted at the acidic pH of the incubation mixture and therefore were not present in the GC–MS runs.

As expected, classical hyperbolic Michaelis–Menten plots (Fig. 5) were found using cDNA-expressed MAOs. The apparent K_m and V_{max} values of the investigated MAOs were calculated by nonlinear curve fit according to Eq. (1). The apparent K_m values listed in Table 1 show that all studied 2Cs have a slightly higher affinity for MAO-A than for MAO-B. Furthermore, the differences of the K_m values between MAO-A and B are getting greater by an increasing 4-substituent size. These facts might be explained by the size of the binding pockets of both, MAO-A and B. Miller et al. reported for several 4-substituted benzylamines, that increasing the 4-substituent size resulted in tighter binding to MAO-A [40]. For 4-substituted phenethylamines, Nandigama et al. reported similar results [57]. The reason for this might be a large binding pocket for 4-substituents in the case of MAO-A,

Table 1 – Kinetic data of 2C deamination catalyzed by MAO-A and MAO-B

	Apparent K_m (best fit value ± standard error) for MAO-A	Apparent K_m (best fit value ± standard error) for MAO-B	V_{max} (best fit value ± standard error) for MAO-A	V_{max} (best fit value ± standard error) for MAO-B
2C-B	43.8 ± 8.7	63.8 ± 7.7	2.3 ± 0.1	1.7 ± 0.1
2C-I	31.1 ± 4.1	88.3 ± 7.2	2.5 ± 0.1	4.6 ± 0.1
2C-D	41.3 ± 3.6	96.9 ± 9.7	1.7 ± 0.04	2.3 ± 0.1
2C-E	49.6 ± 3.3	187.8 ± 19.1	0.7 ± 0.01	4.3 ± 0.2
2C-T-2	38.8 ± 2.7	146.0 ± 13.0	1.5 ± 0.03	4.3 ± 0.2
2C-T-7	14.4 ± 2.1	108.5 ± 19.2	3.4 ± 0.1	4.5 ± 0.3

Units are: apparent K_m in µM, V_{max} in dimensionless PAR/min and mg protein.

whereas MAO-B should contain a small hydrophobic binding pocket for 4-substituents [58]. Furthermore, MAO-B showed in general with exception of 2C-B, increased V_{max} value compared to MAO-A for a single 2C compound. One might speculate, that MAO-A has a higher affinity for the 2Cs than MAO-B, but MAO-B has the higher capacity for the 2Cs concerning the deamination reaction. However, statements concerning the measured V_{max} values are difficult, because quantification of metabolites was not possible, as mentioned before. As MAO-A and MAO-B are involved in one of the major metabolic steps of the 2Cs, the 2Cs might be susceptible for drug-drug interactions with MAO inhibitors possibly leading to elevated plasma concentrations of the 2Cs, and therefore increasing the probability of toxic side effects. Such inhibitors are used as antidepressants such as tranylcypromine and moclobemide or as antiparkinsonians such as selegiline. Amphetamine derivatives, which are often abused together with the 2Cs are also known to be potent MAO inhibitors [59,60]. Beside this, due to the relatively high apparent K_m values of the 2Cs, further studies on their MAO inhibitory potential are required. Such inhibition would lead to further interactions for example with indirect sympathomimetics such as cocaine, or with food ingredients such as tyramine. However, the question whether drug interactions are of relevance for 2C pharmacokinetics and/or clinical outcome of intoxications cannot be answered at the moment due to lack of sufficient authentic human data.

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3 CONCLUSIONS

The studies presented here showed that the phenethylamine-derived designer drugs 2C-B, 2C-I, 2C-D, 2C-E, 2C-T-2 and 2C-T-7 were metabolized mainly by *O*-demethylation and deamination with subsequent oxidation to the corresponding acid or reduction to the corresponding alcohol. Further steps were the side chain hydroxylation and in the case of the sulfur containing 2Cs, also the sulfoxidation. As metabolic phase II reactions partial glucuronidation or sulfatation and *N*-acetylation were observed. Furthermore, combinations of these steps as well as minor metabolites were also detected.[63-68]

The developed screening procedures allowed the detection of the studied 2Cs in rat urine after administration of common drug abusers' doses mainly via their metabolites.

In vitro studies showed that MAO-A and MAO-B were the major isoforms catalyzing the deamination of the 2Cs, although in some cases CYP2D6 was also involved for a very small amount. All studied 2Cs have a slightly higher affinity for MAO-A than for MAO-B, which can be explained by the size of the binding pocket of the enzyme for the 4-substituent of the 2Cs.[69]

This detailed knowledge of the metabolic steps of designer drugs is an important prerequisite for assessing possible interaction with other drugs or food ingredients as well as inter-individual pharmacogenetic differences. MAO-A and MAO-B are the major enzymes catalyzing the studied metabolic step, and these enzymes are targets for frequently used drugs such as antidepressants or antiparkinsonians. Therefore, it can be expected that drug-drug interactions with these compounds would be possible. Whether these are of clinical relevance can not be concluded at the moment. Further studies on the pharmacology and toxicology of the metabolites together with well documented clinical data will be necessary. Furthermore, studies on the isoenzymes dependency of other metabolic steps, especially the *O*-demethylation are required.

4 SUMMARY

In the presented studies, metabolism and toxicological analysis in urine of the phenethylamine-derived designer drugs 2C-B, 2C-I, 2C-D, 2C-E, 2C-T-2 and 2C-T-7 were investigated. Furthermore, MAO and CYP isoform dependence of one of the major metabolic steps was elucidated to predict possible drug-drug interactions and influence of genetic polymorphisms.[63-69]

The qualitative **metabolism** was studied in Wistar rats, that were administered a high dose of the corresponding 2C compound. Urine was collected over 24 hours. After enzymatic cleavage of conjugates, acidic and basic liquid-liquid extraction as well as solid-phase extraction and derivatization of the extracts, metabolites could be identified by means of GC-MS.

The 2Cs were mainly metabolized by O-demethylation in position 2 and 5 of the ring, respectively, by deamination followed by oxidation to the corresponding acid or reduction to the corresponding alcohol. Further metabolic steps were side chain hydroxylation and in the case of sulfur containing 2Cs, sulfoxidation. Metabolic phase II reactions were partial glucuronidation or sulfatation and *N*-acetylation. Combinations of these steps and minor metabolites could also be detected.

The **toxicological analysis** was based on the results of the metabolism studies and focused on the detection of the parent compound and corresponding major metabolites in urine. The rats were administered the drug at a dose corresponding to a common drug abusers' dose. Sample preparation included acid hydrolysis for rapid cleavage of conjugates, liquid-liquid extraction and microwave-assisted acetylation. The analytes were separated by GC and the mass spectra, recorded in the full-scan mode, were electronically filed. The files allowed to trace certain mass fragments by extracting single ions from the full-scan chromatogram. If a peak for a fragment mass appeared, that was indicative for the presence of the analyte targeted, the full mass spectrum underlying this peak could be inspected. Comparison of this mass spectrum with a reference spectrum allowed to draw conclusions on the presence of the suspected analyte.

The knowledge of the involvement of particular **monoamine oxidase** (MAO) or **cytochrome P450** (CYP) in the biotransformation of a new drug allows to predict possible drug-drug interactions, inter-individual variations in pharmacokinetic profiles

and increased appearance of side effects and serious poisonings.[61] In addition, there is good evidence that genetic variations in drug metabolism have important behavioral consequences that can alter the risk of drug abuse and dependence.[62] Therefore, as a basis for further studies, those MAO and CYP isoforms were identified that were involved in the one of the major metabolic steps, namely the deamination reaction. For identification of the human MAO and CYP enzymes, *in vitro* experiments with microsomes of insect cells infected with baculoviruses were performed. The transfected DNA coded for individual human hepatic CYP isoforms. Maximal turnover rates of the substrates and K_m values were determined from the cDNA-expressed MAO isoforms.

MAO-A and MAO-B were the major enzymes involved in the deamination reaction. For 2C-D, 2C-E, 2C-T-2 and 2C-T-7, CYP2D6 was also involved, but only to a small extent. All studied 2Cs have a slightly higher affinity for MAO-A than for MAO-B, which can be explained by the size of the binding pocket of the enzyme for the 4-substituent of the 2Cs.

As MAO-A and MAO-B are involved in one of the major metabolic steps of the 2Cs, the 2Cs might be susceptible for drug-drug interactions with MAO inhibitors possibly leading to elevated plasma concentrations of the 2Cs, and therefore increasing the probability of toxic side effects. Such inhibitors are used as antidepressants or as antiparkinsonians. However, the question whether drug interactions are of relevance for 2C pharmacokinetics and/or clinical outcome of intoxications cannot be answered at the moment due to lack of sufficient authentic human data.

Further studies on the pharmacology and toxicology of the metabolites together with well documented clinical data will be necessary.

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6 ABBREVIATIONS

2C-B	4-bromo-2,5-dimethoxy-β-phenethylamine
2C-I	4-iodo-2,5-dimethoxy-β-phenethylamine
2C-D	2,5-dimethoxy-4-methyl-β-phenethylamine
2C-E	4-ethyl-2,5-dimethoxy-β-phenethylamine
CYP	cytochrome P450
2C-T-2	4-ethylthio-2,5-dimethoxy-β-phenethylamine
2C-T-7	2,5-dimethoxy-4-propylthio-β-phenethylamine
GC-MS	gas chromatography-mass spectrometry
MAO	monoamine oxidase
FAD	flavin adenine dinucleotide
L-DOPA	L-dihydroxy phenylalanine
5-HT	5-hydroxytryptamine (serotonin)
cDNA	copy deoxyribonucleic acid
K_m	substrate concentration at half of the maximal turnover rate
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine

OR	oxidoreductase
PMA	para-Methoxyamphetamine
PMMA	para-Methoxymethamphetamine
V_{max}	maximal turnover rate

7 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurden der Metabolismus und die Nachweisbarkeit der neuen Designerdrogen des Phenethylamin-Typs 2C-B, 2C-I, 2C-D, 2C-E, 2C-T-2 und 2C-T-7 im Urin untersucht. Darüber hinaus wurde die Monoaminoxidase und Cytochrom P450 Isoformenabhängigkeit eines Hauptmetabolismusschrittes untersucht.

Die qualitativen **Metabolismusuntersuchungen** erfolgten an Wistar-Ratten, denen eine hohe Dosis der jeweiligen Substanz verabreicht wurde. Der 24-Stunden-Sammelurin diente als Untersuchungsmaterial. Nach enzymatischer Konjugatspaltung, saurer und basischer flüssig-flüssig Extraktion bzw. Festphasenextraktion des Urins und anschließender Derivatisierung des Extraktes konnten Metaboliten mittels GC-MS identifiziert werden.

Die 2Cs wurden hauptsächlich durch O-Demethylierung in Position 2 bzw. 5 des Ringes oder durch Deaminierung gefolgt von Oxidation zur entsprechenden Säure oder Reduktion zum entsprechenden Alkohol metabolisiert. Weitere Metabolismusschritte waren die Seitenkettenhydroxylierung und im Falle der Schwefel enthaltenden 2Cs, Sulfoxidation. Als Phase-II-Reaktionen konnten partielle Glucuronidierung oder Sulfatierung und *N*-Acetylierung gefunden werden. Kombinationen dieser Schritte konnten ebenso detektiert werden wie auch niedrig konzentrierte Metaboliten.

Das **toxikologische Nachweisverfahren** basierte auf den Ergebnissen der Metabolismusuntersuchungen und konzentrierte sich auf die Detektion der Muttersubstanzen und/oder der jeweiligen Hauptmetaboliten im Urin. Dazu erhielten die Ratten eine Dosis der jeweiligen Substanz, die einer üblichen Dosierung bei Drogenkonsumenten entsprach. Die Probenvorbereitung umfasste eine saure Hydrolyse zur raschen Konjugatspaltung, Flüssig-Flüssig-Extraktion und mikrowellenunterstütze Acetylierung. Die Probe wurde gaschromatographisch aufgetrennt und die entsprechenden Massenspektren im full-scan Modus aufgezeichnet. Die elektronische Aufzeichnung ermöglichte es, den Verlauf bestimmter Massenfragmente nachträglich separat zu verfolgen. Trat ein Peak der für den Analyten charakteristischen Fragmentmasse auf, konnte anschließend das dem Peak unterliegende Massenspektrum mit dem Referenzmassenspektrum verglichen werden und somit eine eindeutige Aussage über die Präsenz des jeweiligen Metaboliten getroffen werden.

Die Kenntnis, welches der **Monoaminoxidase** oder der **Cytochrom P450** Isoenzyme an der Biotransformation von Arzneistoffen oder Missbrauchsdrogen beteiligt ist, ermöglicht die Vorhersage potentieller Interaktionen mit anderen Arzneistoffen, pharmakogenetischer Unterschiede der Pharmakokinetik sowie von Nebenwirkungen und möglichen Vergiftungen. Darüber hinaus gibt es Hinweise dafür, dass ein inter-individuell unterschiedlicher Metabolismus Einfluß auf Drogenmissbrauch sowie Drogenabhängigkeit hat. Als Basis für weitergehende Untersuchungen wurden die MAO und CYP-Isoformen identifiziert, die einen der wichtigsten Metabolismusschrittes katalysieren, der Deaminierung. Zur Identifizierung der humanen CYP-Enzyme wurden *in vitro* Versuche mit Mikrosomen von mit Viren infizierten Insektenzellen durchgeführt. Die transfizierte DNA codierte für einzelne humane hepatische CYP-Isoformen. Maximale Umsatzraten der Substrate (V_{max}) und K_m-Werte wurden von den cDNA-exprimierten MAO-Isoformen bestimmt.

Es stellte sich heraus, dass MAO-A und B die Enzyme waren, die hauptsächlich an der Deaminierung der 2Cs beteiligt sind. Bei 2C-D, 2C-E, 2C-T-2 und 2C-T-7 war auch CYP2D6 beteiligt, allerdings zu einem sehr geringen Anteil. Alle untersuchten 2Cs zeigen eine leicht höhere Affinität zu MAO-A als zu MAO-B. Dies kann mit der unterschiedlichen Größe der Bindungstasche der beiden Enzyme für den 4-Substituenten der 2Cs erklärt werden.

Da MAO-A und B an einem der Hauptmetabolismusschritte der 2Cs beteiligt sind, könnten die 2Cs Wechselwirkungen mit MAO-Hemmern zeigen, was möglicherweise zu erhöhten Plasmakonzentrationen der 2Cs und damit zu einer erhöhten Wahrscheinlichkeit toxischer Wirkungen führen könnte. Solche MAO-Hemmer werden therapeutisch als Antidepressiva oder auch Antiparkinsonmittel eingesetzt. Allerdings kann die Frage, ob Interaktionen relevant für die Pharmakokinetik und/oder das klinische Ergebnis von Intoxikationen sind, zur Zeit aufgrund fehlender Humandaten nicht beantwortet werden.

Hierfür sind weitere klinische Studien zur Pharmakologie und Toxikologie der Metaboliten sowie gut dokumentierte klinische Falldaten notwendig.