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

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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-

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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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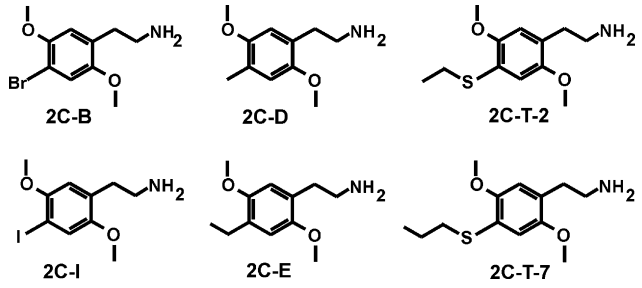

Fig. 1 – Chemical structures of the studied members of the 2C-series.

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).

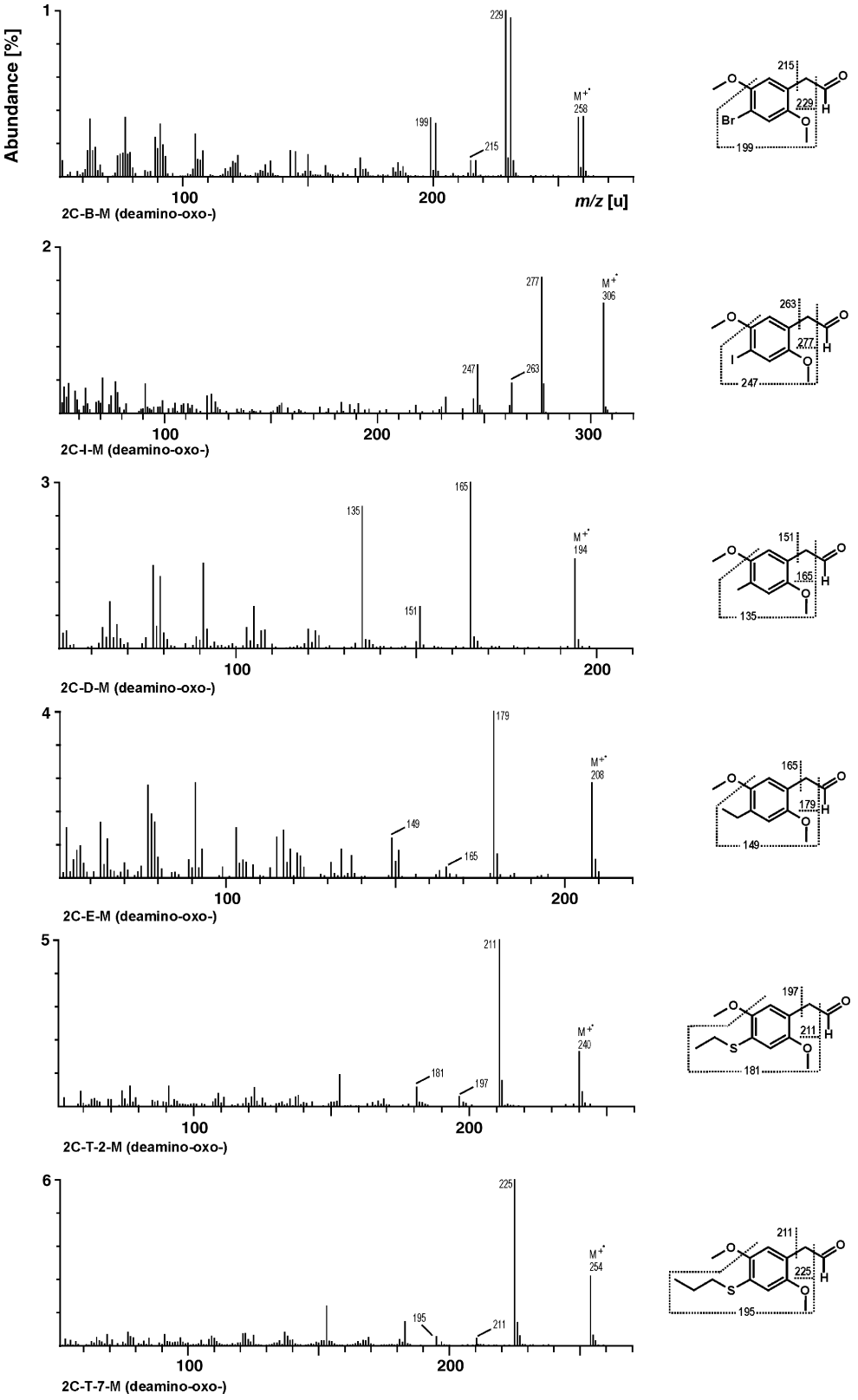

Fig. 2 – EI mass spectra, structures and predominant fragmentation patterns of the 2Cs' aldehyde metabolites.

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

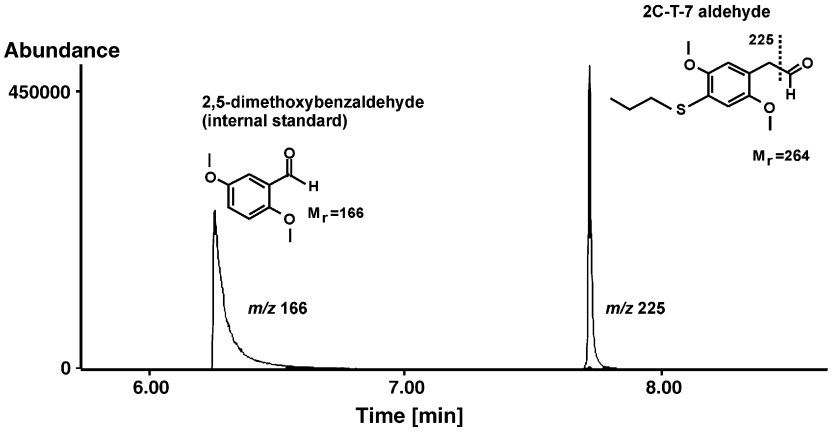

Fig. 3 – Typical mass fragmentograms of a cyclohexane extract of an incubation mixture of 250 μM 2C-T-7 with cDNA-expressed MAO-B with the following ions: *m/z* 166 or 225 for 2,5-dimethoxybenzaldehyde (IS) or 2C-T-7 aldehyde, respectively.

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

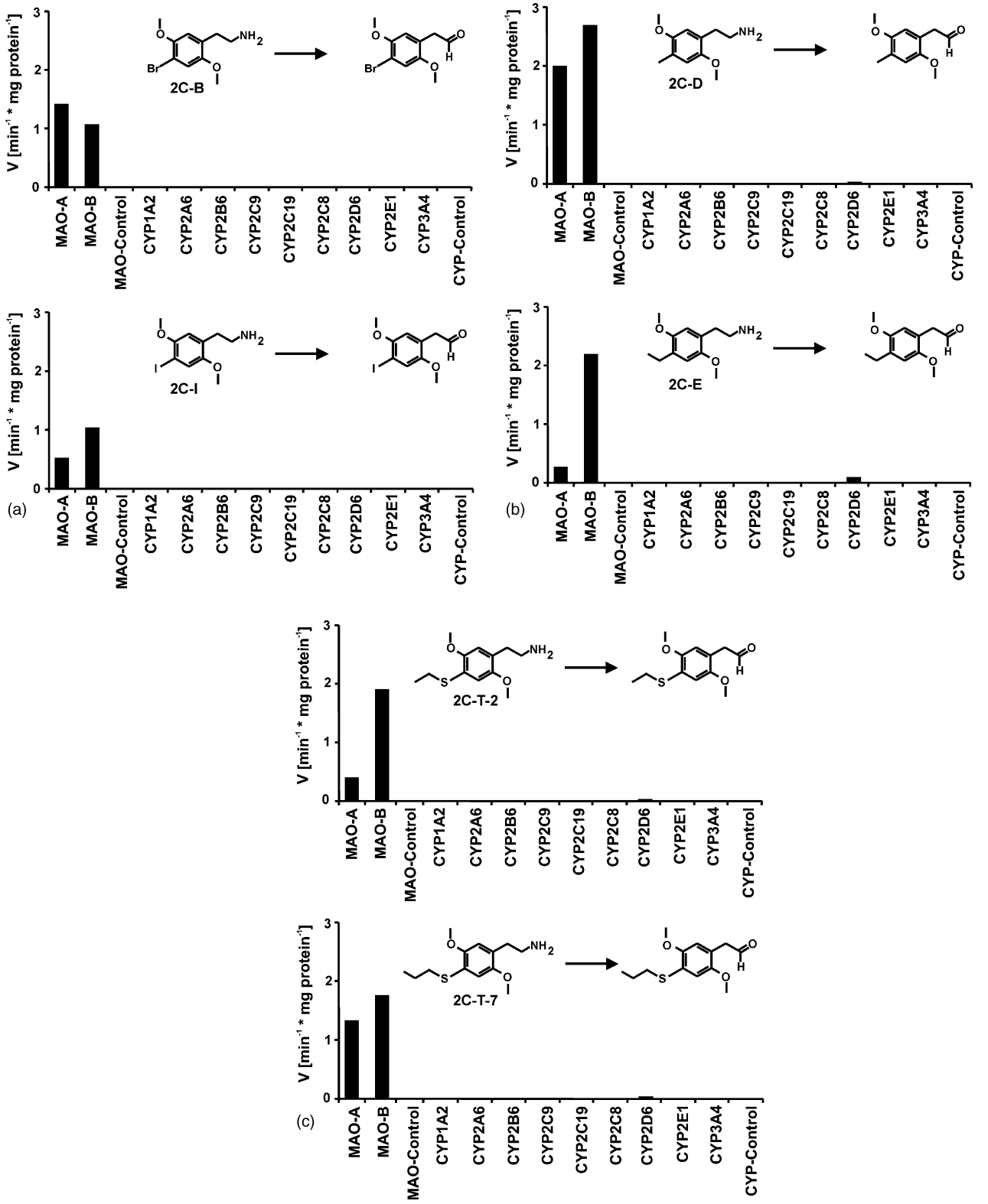

Fig. 4 – Formation rates (V) of 2C deamination (250 μM 2C compound each) with 0.2 mg/mL MAO-A or MAO-B or 50 pmol/mL of the given individual CYPs (V given as dimensionless PAR per min and mg protein) and with insect cell control microsomes.

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

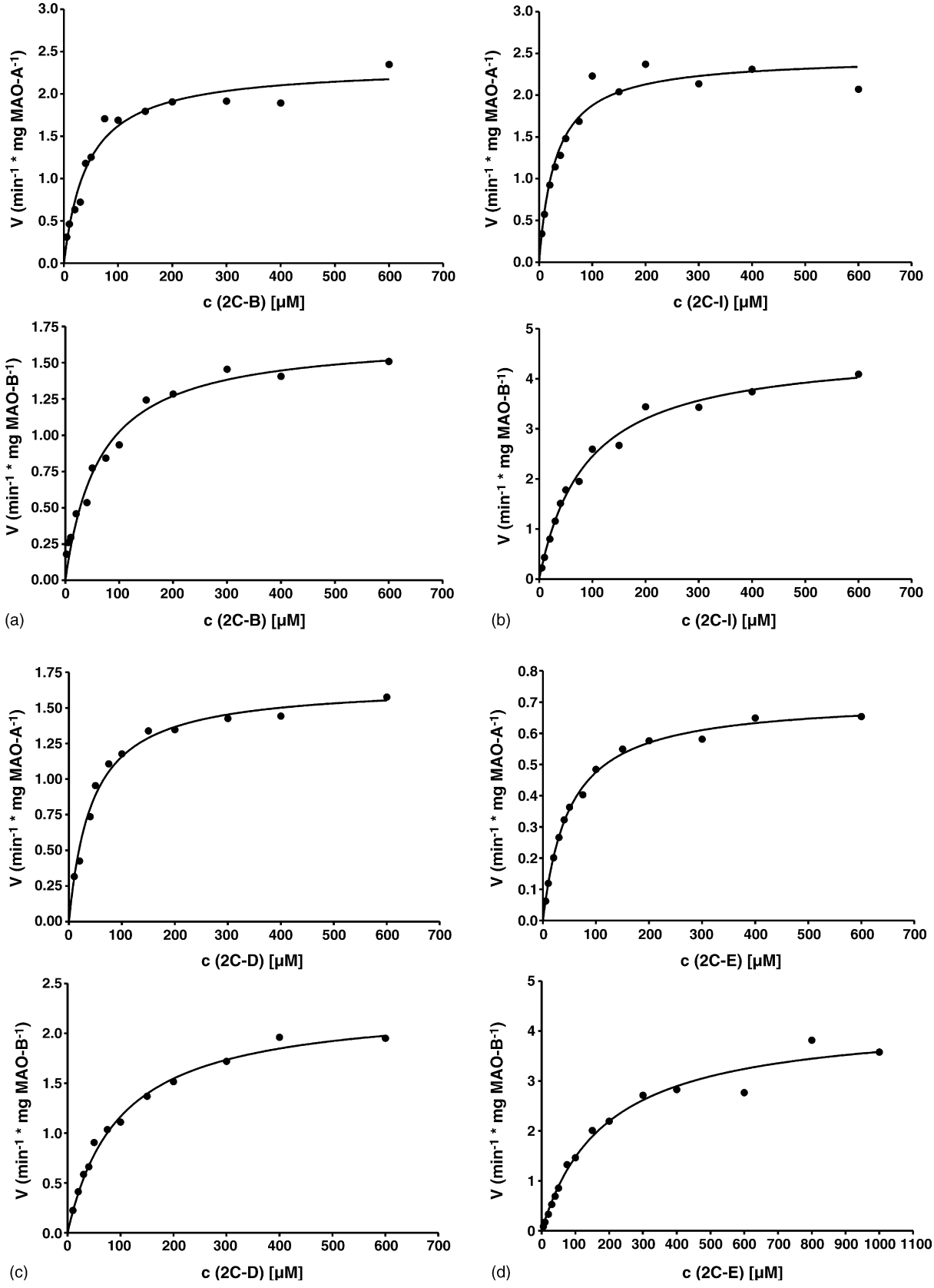

Fig. 5 – Michaelis–Menten plots for 2C deamination catalyzed by MAO-A or MAO-B. Values represent the mean of duplicate incubations. V given as dimensionless PAR per min and mg protein. Curves were calculated by nonlinear curve fit according to Eq. (1) (one-site binding model).

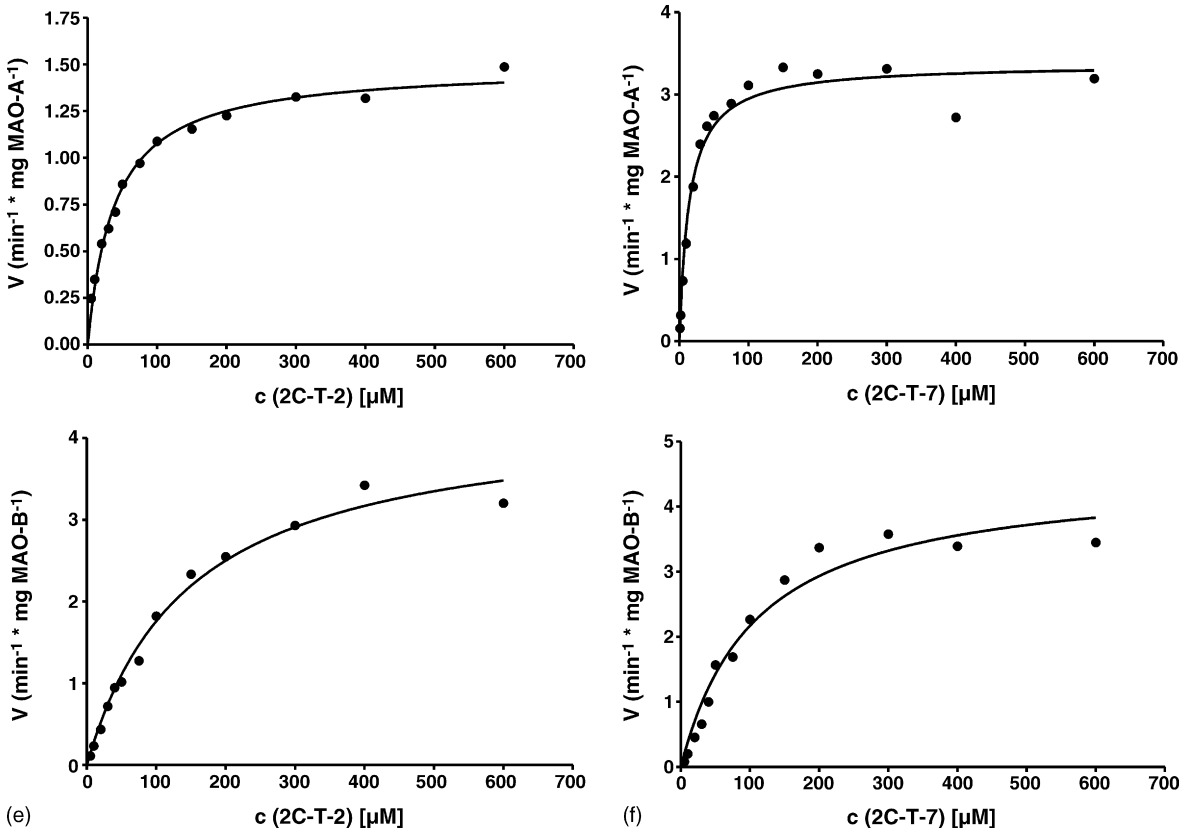

Fig. 5. (Continued).

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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