Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 485 (2004) 69–79
www.elsevier.com/locate/ejphar

Cytochrome *P*450 isoenzymes involved in rat liver microsomal metabolism of californine and protopine

Liane D. Paul, Dietmar Springer, Roland F. Staack, Thomas Kraemer, 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

Received 10 July 2003; received in revised form 12 November 2003; accepted 18 November 2003

Abstract

Studies are described on the cytochrome *P*450 (CYP) isoenzyme dependence of the main metabolic steps of the *Eschscholtzia californica* alkaloids californine and protopine using rat liver microsomes. Preparations of *E. californica* are in use as phytopharmaceuticals and as herbal drugs of abuse. CYP isoenyzme dependences were studied using specific chemical inhibitors for CYP1A2, CYP2D1, and CYP3A2 (α-naphthoflavone, quinine, and ketoconazole, respectively). CYP2C11 was inhibited by specific antibodies for lack of specific chemical inhibitors. Californine *N*-demethylation was mainly catalyzed by CYP3A2 and to a minor extent by CYP1A2 and CYP2D1, but not by CYP2C11. CYP2D1 and CYP2C11 were shown to be mainly involved in demethylenation of both, californine and protopine, while CYP1A2 and CYP3A2 showed only minor contribution. Kinetic parameters of the reactions were established. K_m and V_{max} values for the californine *N*-demethylation were 4.5 ± 4.7 μM and 22.9 ± 13.7 min/mg protein (high affinity) and 161.3 ± 16.7 μM and 311.8 ± 39.4 min/mg protein (low affinity), respectively. Californine demethylenation and protopine demethylenation showed substrate inhibition and K_m and V_{max} values were 5.0 ± 0.5 and 7.1 ± 0.6 μM and 83.3 ± 2.6 and 160.7 ± 4.0 min/mg protein, respectively.

Keywords: Californine; Protopine; Metabolism; Cytochrome *P*450; Liver microsome, rat

1. Introduction

Californine, (5*S*,12*S*)-5,6,12,13-tetrahydro-15-methyl-cycloocta[1,2-*f*:5,6-*f*′]bis[1,3]benzodioxol-5,12-imine, and protopine, 4,6,7,14-tetrahydro-5-methyl-bis[1,3]benzodioxolo[4,5-*c*:5′ ,6′ -g]azecin-13(5*H*)-one, are main alkaloids of *Eschscholtzia californica* CHAM. (papaveraceae) (Gertig, 1965; Guedon et al., 1990; Manske and Shin, 1965). Until today, phytopharmaceuticals based on this traditional medicinal plant are in world-wide use, especially in France and the USA. In recent years, the biological effects of *E. californica* extracts have been investigated in various animal models and in in vitro studies. From these studies, various effects were concluded, e.g. sleeping time prolongation or spasmolytic, sedative, and anxiolytic activities, which may be related to the alkaloids (Rolland et al., 1991, 2001; Vincieri et al., 1988). Protopine, widely distributed among members of various plant families, showed benzodiazepine-like activity in an in vitro study with rat brain synaptic membrane receptors (Kardos et al., 1986). According to several in vitro and in vivo studies in animals, also antiallergic, antispasmodic, anticholinergic, antiarrhythmic, antiplatelet, or antibacterial activities of protopine have been reported (Ko et al., 1989; Song et al., 2000; Ustunes et al., 1988). However, little is known about the pharmacology and toxicology of both alkaloids and of *Eschscholtzia* preparations in humans. Nevertheless, besides its use as phytopharmaceutical, *Eschscholtzia* has been described as a substitute drug for marijuana as it should evoke mild euphoria after ingestion or smoking lasting for 20–30 min (Duke, 1985; Gottlieb, 2002; Haensel et al., 1993; Siegel, 1976). Gradual increase of the dose is recommended until the desired effect occurs (Raetsch, 1998). In the 1960s, Cheney (1964) reported regular and prolonged use not to be habit-forming.

As a basis for toxicological risk assessment, the metabolism of the alkaloids californine and protopine has been investigated in a previous in vivo study in rats (Paul and Maurer, 2003). Californine was mainly metabolized by

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

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2003.11.051

N-demethylation and demethylenation. The demethylenation of either of the two methylenedioxy groups resulted in the same metabolite because of the symmetric structure of the molecule, whereas protopine was demethylenated only at the 2,3-methylenedioxy group. *N*-demethylation of protopine was not observed. The demethylenated metabolites were further altered by methylation of one of the catecholic hydroxy groups, resulting in two isomeric hydroxy methoxy metabolites.

The aim of the present study was to identify the cytochrome *P*450 (CYP) isoenzyme dependence of the main CYP-dependent metabolic steps of the alkaloids in rats: *N*-demethylation for californine and demethylenation for californine and protopine. Using rat liver microsomes, inhibition studies were performed on those isoenzymes, which have been described to be mainly involved in metabolism of xenobiotics.

2. Materials and methods

2.1. Chemicals

$NADP^+$ was obtained from BIOMOL (Hamburg, Germany) and isocitrate dehydrogenase, isocitrate, EDTA, naphthoflavone, ketoconazole, phenacetin, acetaminophen, and goat nonimmune serum from Sigma-Aldrich (Deisenhofen, Germany). Ammonium formate (analytical grade) and superoxide dismutase were obtained from Fluka (Neu-Ulm, Germany). Codeine, diazepam, midazolam, α-hydroxymidazolam, morphine, morphine-d_3, acetaminophen-d_4, and quinine were obtained from Promochem (Wesel, Germany). Californine and protopine were kindly provided by Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany). Acetonitrile (HPLC grade) and all other chemicals (analytical grade) were obtained from E. Merck (Darmstadt, Germany). Selective anti-rat CYP2C11 goat serum was from Daiichi and delivered by NatuTec (Frankfurt/Main, Germany).

2.2. Preparation and characterization of rat liver microsomes

Livers of male Wistar rats were kindly provided by the Institute of Physiology, Homburg. Liver microsomes were prepared from pooled livers by fractional ultracentrifugation as described before (Kraemer et al., 2000) and resuspended in 1.15% potassium chloride buffer containing 0.1 mM EDTA. All processes were performed at 0 to 4 °C. The obtained microsomal preparation was aliquoted and stored at − 80 °C until further use. Microsomal protein concentration was determined according to Bradford (1976) by the Bio-Rad protein assay (Muenchen, Germany) using a BSA standard solution. Total CYP content was measured by the method of Omura and Sato (1964).

2.3. Microsomal incubations of californine and protopine

The incubation mixtures consisted of rat liver microsomes at a protein concentration of 0.5 mg/ml, substrate and 200 U/ml superoxide dismutase in a final volume of 50 μl of 0.1 M potassium phosphate buffer (pH 7.4). The substrates were dissolved in incubation buffer. After preincubation at 37 °C for 10 min to adjust temperature, reactions were initiated by addition of a NADPH-generating system (1.2 mM $NADP^+$, 5 mM isocitrate, 5 mM $MgCl_2$, and 2 U/ml isocitrate dehydrogenase final concentration) and carried out at 37 °C for 2 min (modified according to Maurer et al., 2000). Time period and protein content were associated with reaction linearity to assure initial rate conditions. Reactions were terminated by addition of 5 μl of perchloric acid (60%, w/w). Methanolic internal standard (californine for protopine and vice versa) was added before centrifugation in a microcentrifuge ($10\,000 \times g$, 5 min), and 5 μl of the supernatants were analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection (FLD). Controls were assayed in the same manner, except for the presence of the NADPH-generating system, which was added after termination of the reactions. Blank samples were assayed without substrates to be able to exclude analytical interferences by the matrix.

2.4. Determination of californine, protopine and their metabolites by HPLC–FLD

A Hewlett-Packard HPLC system 1050 (Agilent, AT, Waldbronn; Germany) with a LiChroCART 125-2 Superspher 60 RP-select B column (Merck) and a HP 1046 A fluorescence detector were used for analysis of the supernatants of the incubation mixtures. The mobile phase consisted of aqueous 20 mM ammonium formate buffer, adjusted to pH 3 with formic acid, and acetonitrile at a ratio of 83:17 (v/v) at a flow rate of 0.4 ml/min. The eluate was monitored using fluorescence detection at an excitation wavelength of 230 nm for californine and its metabolites, 241 nm for protopine, and 232 nm for demethylene protopine and emission wavelengths of 334, 330, and 336 nm, respectively. The column temperature was maintained at 28 °C. For analysis of the incubation mixtures with protopine, a time table was set: 0–8 min 232/336 nm, 8–14 min 241/330 nm, and 14–20 min 230/334 nm with different sensitivities (gain adjustment). Peak area ratios of the monitored metabolite and the corresponding internal standard were determined.

2.5. Confirmation of the HPLC–FLD peak identity by gas chromatography–mass spectrometry (GC/MS)

For identification of metabolites underlying the peaks in the HPLC–FLD chromatograms, incubations were

conducted under large-scale conditions (200 μM of californine or protopine, 60 min). The metabolites were isolated by collecting the corresponding fractions of several HPLC–FLD runs. The eluates were flash frozen in liquid nitrogen and lyophilized using a LYOVAG GT2 (FINN-AQUA Santasalo-Sohlberg, Muenchen, Germany). The residues were analyzed by GC/MS after acetylation and the mass spectra underlying the peaks were identified by comparison with reference spectra of the metabolites, which have been published recently (Paul and Maurer, 2003).

2.6. Kinetic assays

Linearity of metabolite formation and a sufficient metabolite production with respect to time and protein concentration was achieved with an incubation period of 2 min and a microsomal protein concentration of 0.5 mg/ml for both alkaloids (data not shown). The enzyme kinetic assays were performed with incubations of 2, 5, 10, 20, 30, 50, 100, or 200 μM of californine or of 1, 2, 5, 10, 15, 20, 40, 50, 100, 200, 300, or 500 μM of protopine dissolved in potassium phosphate buffer. The incubations were carried out in triplicate at each concentration level.

Initially, Eadie–Hofstee plots were applied for visual inspection of the data, since deviations from simple Michaelis–Menten enzyme kinetics could be readily seen. If the corresponding Eadie–Hofstee plot was linear indicating simple Michaelis–Menten kinetics, Eq. (1) was applied for calculating the kinetic values K_m and V_{max} using nonlinear regression analysis. In case of a concave Eadie–Hofstee plot indicating biphasic kinetics, Eq. (2) was applied for calculating the kinetic values (V, velocity; V_{max}, maximal velocity; K_m, substrate concentration at half-maximal velocity; [S], substrate concentration).

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

$$V = \frac{V_{max,1} \times [S]}{K_{m,1} + [S]} + \frac{V_{max,2} \times [S]}{K_{m,2} + [S]} \quad (2)$$

If substrate inhibition occurred, data were fitted to Eq. (3) to determine kinetic parameters, using nonlinear regression analysis.

$$V = \frac{V_{max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} \quad (3)$$

2.7. Determination of K_i values of specific chemical inhibitors

CYP isoenzyme-specific inhibitors and substrates were used to study the main metabolic steps of californine and protopine in pooled rat liver microsomes. The K_m values of isoenzyme specific probe substrates for the selected isoenzymes were determined according to the incubation conditions used for californine and protopine except for incubation times and substrate concentrations. Reaction rates were shown to be linear under the conditions used (data not shown). K_i determinations of isoenzyme specific chemical inhibitors were carried out as mentioned above by incubating probe substrates at concentrations near the respective K_m values and different concentrations of corresponding inhibitors. After preincubation of microsomes with probe substrate and inhibitor at 37 °C for 10 min, the reaction was started with NADPH-generating system. As the solutions of chemical inhibitors were prepared in methanol, pure methanol was included in the control incubations (without inhibitors) to correct for any effects of the solvent on the microsomal activity. The maximal concentration of methanol did not exceed 1%. K_i values were calculated according to Cheng and Prusoff (1973), using GraphPad Prism 3.02 software (GraphPad Software, San Diego, CA). Use of this equation was possible, as all chosen inhibitors acted competitively (Boobis et al., 1990; Kotegawa et al., 2002). The immunoinhibition of CYP2C11 was examined by preincubating rat liver microsomes with various amounts of CYP2C11 selective antiserum at room temperature for 30 min, before the other components of the incubation medium were added and the reaction was carried out as described above. In the control experiments, goat nonimmune serum was added instead of CYP2C11 antiserum. The amount of the CYP2C11 selective antibodies used was estimated according to data sheets of Gentest. Antiserum was diluted 1:2.5 with buffer and 3.13 μl of the dilution was added to the incubation mixture (5 μl antiserum/100 μg microsomal protein).

2.8. CYP1A2 inhibition assay

A phenacetin *O*-deethylase assay (Butler et al., 1989; Sesardic et al., 1988; Shimada et al., 1997) was used for K_i determination of α-naphthoflavone (Clarke, 1998; Nakajima et al., 1999; Ono et al., 1996). The concentration range of phenacetin for K_m determination was 2–500 and 0.1–100 μM of α-naphthoflavone for K_i determination. In this assay, the incubations were carried out as described above for 10 min and terminated by addition of 50 μl of ethyl acetate. After addition of acetaminophen-d_4 as internal standard, shaking and centrifugation, 2 μl of the organic layer (supernatant) were injected into the liquid chromatography–mass spectrometry (LC/MS) apparatus for separation and determination. An AT 1100 atmospheric pressure chemical ionization electrospray (APCI) LC/MS, SL version, including an AT 1100 Series HPLC system which consisted of a degasser, a binary pump and an autosampler with the A.08.03 software was used. The liquid chromatographic conditions were as

follows: gradient elution was achieved on a Merck LiChroCART column (125 × 2-mm internal diameter) with Superspher 60 RP Select B as stationary phase and a LiChroCART 10-2 Superspher 60 RP Select B guard column. The mobile phase consisted of aqueous ammonium formate (5 mM, adjusted to pH 3 with formic acid, eluent A) and acetonitrile (eluent B). Until the beginning of the analysis, the HPLC system was flushed with a 60:40 mixture of the two eluents. The gradient and the flow rate were programmed as follows: 0–4 min 40% B (flow: 0.4 ml/min), 4–7 min 90% B (flow: 0.6 ml/min), 7–10 min 40% B (flow: 0.4 ml/min). The following APCI inlet conditions were applied: drying gas, nitrogen (7 l/min, 300 °C), and nebulizer gas, nitrogen (172.5 kPa); capillary voltage, 4000 V; vaporizer temperature, 400 °C; corona current, 5.0 μA; positive selected-ion monitoring (SIM) mode for quantification, fragmentor voltages 100 and 200 V. The following target and qualifier ions were selected from the full mass spectra: ions *m/z* 152 (fragmentor voltage 100 V) and 110 (200 V) for acetaminophen and 156 (100 V) and 114 (200 V) for acetaminophen-d_4. The metabolite concentration was calculated by comparison of the peak area of the metabolite to that of the deuterated internal standard.

2.9. CYP2D1 inhibition assay

Codeine *O*-demethylation was carried out as probe assay for CYP2D1 (Xu et al., 1995, 1997) and quinine was used as a specific inhibitor (Boobis et al., 1990; Kobayashi et al., 1989). Concentration range of codeine was 5–500 μM for K_m determination and that of quinine was 0.01–200 μM for K_i determination. The incubation was performed as described above for a period of 5 min except for the final volume of 500 μl. The reaction was terminated by adding 500 μl of carbonate buffer (1 M, pH 8.7), 5 ml of an ethyl acetate-diethyl ether mixture (1:1 v/v) and the internal standard morphine-d_3. After phase separation by centrifugation, the organic layer was evaporated to dryness and the residue was pentafluoropropionylated according to Maurer (2000). After evaporation to dryness, the residue was dissolved in 50 μl of ethyl acetate and an aliquot of 2 μl of this solution was analyzed by GC/MS. A Hewlett Packard (AT) 5890 Series II gas chromatograph combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software was used. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m × 0.2 mm I.D.), cross-linked methylsilicone, 330-nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow rate 1 ml/min; column temperature, programmed from 100–310 °C at 30 °C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: EI mode, ionization energy, 70 eV; ion source temperature, 220 °C; capillary direct interface heated at 260 °C. Selected ions used for quantification were *m/z* 414 for morphine and 417 for the internal standard morphine-d_3.

2.10. CYP3A2 inhibition assay

The midazolam α-hydroxylation assay (Ghosal et al., 1996; Gorski et al., 1994; Kronbach et al., 1989) was used for K_i determination of ketoconazol (Baldwin et al., 1995; Kotegawa et al., 2002). Concentration range of

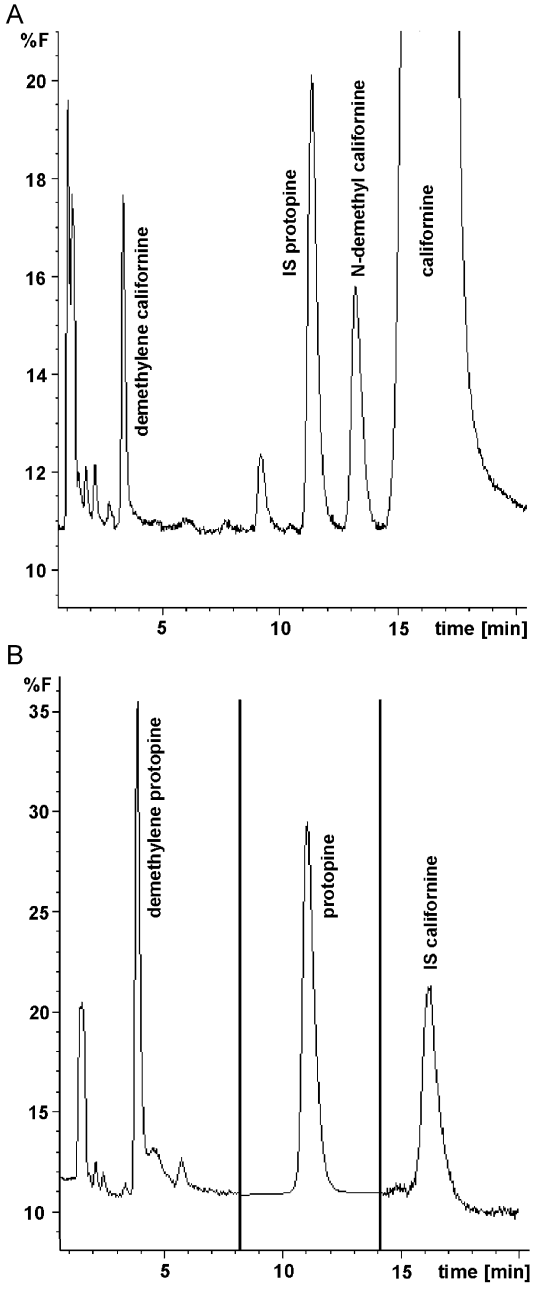

Fig. 1. Typical HPLC–FLD chromatograms of microsomal incubations of 50 μM each of californine (A) or protopine (B). Lines in B indicate the time of switch between the monitored wavelengths and gains.

midazolam for K_m determination was 1–200 μM and that of ketoconazole 0.1–100 μM for K_i determination. The incubations were performed as mentioned above and terminated after 5 min with 50 μl of acetonitrile. After addition of a methanolic solution of the internal standard diazepam and centrifugation, 2 μl was analyzed by LC/MS. Standard conditions were used as described above with a slightly modified gradient: 0–4 min 40% B (flow: 0.4 ml/min), 4–6 min 90% B (flow: 0.6 ml/min), 6–7 min 90% B (flow: 0.8 ml/min), 7–10 min 40% B (flow:

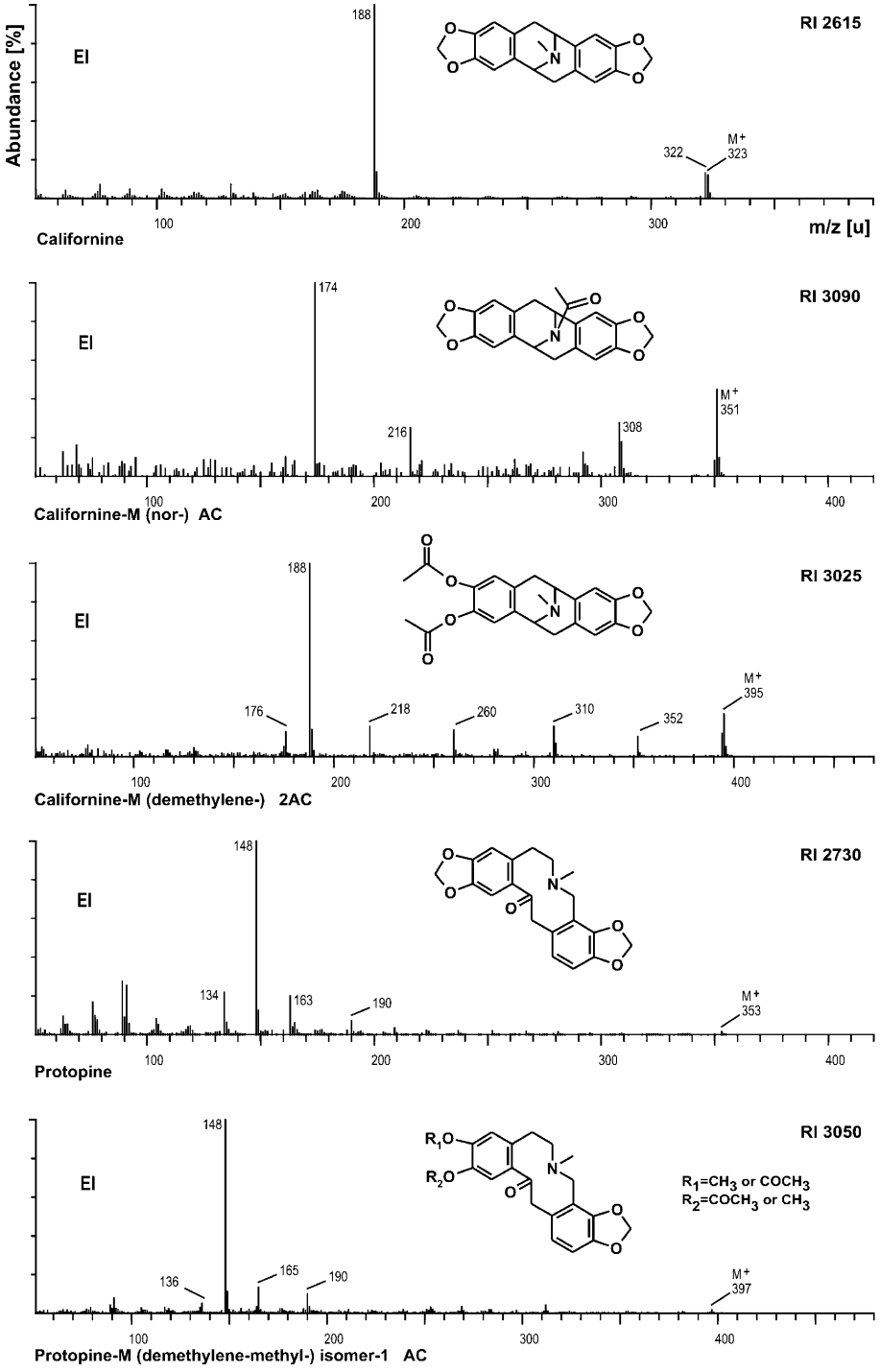

Fig. 2. EI mass spectra, gas chromatographic retention indices (RI) and structures of californine and protopine and their metabolites after acetylation. The axes are only labelled for spectrum 1.

0.4 ml/min). Selected ions for quantification were *m*/*z* 285 for diazepam and *m*/*z* 342 for α-hydroxy midazolam (fragmentor voltage 100 V).

2.11. Inhibition of californine and protopine metabolism

Inhibitor effects on the rat liver microsomal californine demethylenation and *N*-demethylation as well as protopine demethylenation activity were determined at substrate concentrations near K_m (5 μM for californine and 6 μM for protopine) and at inhibitor concentrations 10 times the isoenzyme-selective K_i value (see Table 2): 6 μM for α-naphthoflavone, 50 μM for quinine and 2 μM for ketoconazole. Control incubations were performed without inhibitor but corrected for the methanol content. Immunoinhibition experiments with CYP2C11 selective antiserum were performed as described above and were compared with control incubations using goat nonimmune serum. Inhibition of reactions was expressed as the percentage of decrease in metabolite production based on the peak areas of the metabolites obtained from incubations in presence or absence of inhibitors. V_{max} values and activities were expressed as peak

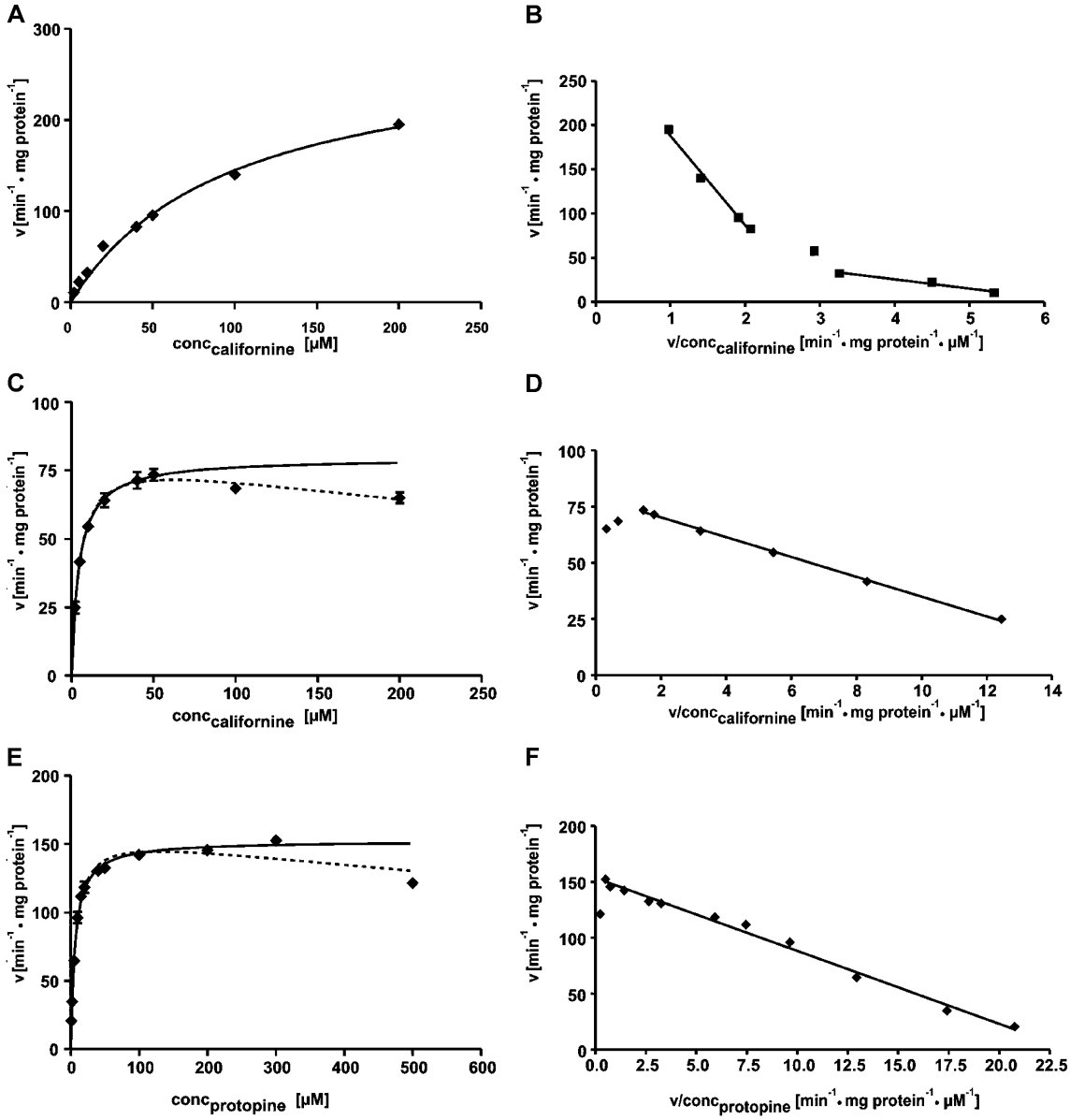

Fig. 3. Michaelis–Menten (A, C, E) and Eadie–Hofstee plots (B, D, F) for californine *N*-demethylation (A, B), for californine demethylenation (C, D) and for protopine demethylenation (E, F). Squares are means of triplicate measurements with error bars indicating S.E. (V_{max} values given as dimensionless peak area ratio per min and mg microsomal protein). Solid lines in A, C and E are results of fitting the data with nonlinear regression to Eq. (1), dotted lines in C and E to Eq. (3).

Table 1
Kinetic data of the studied metabolic reactions of californine and protopine (V_{max} values given as dimensionless peak area ratio per min and mg microsomal protein)

Metabolic reaction	K_m [μM][a]	K_m [μM][a]	K_i [μM][a]	V_{max} [min/mg protein]	V_{max} [min/mg protein]
Californine *N*-demethylation (high affinity)	4.5 ± 4.7[b]	–	–	22.9 ± 13.7[b]	–
Californine *N*-demethylation (low affinity)	161.3 ± 16.7[b]	–	–	311.8 ± 39.4[b]	–
Californine demethylenation	4.6 ± 0.4[c]	5.0 ± 0.5[d]	750.3 ± 173.5[d]	79.6 ± 1.7[c]	83.3 ± 2.6[d]
Protopine demethylenation	6.2 ± 0.3[c]	7.1 ± 0.6[d]	2297.0 ± 532.6[d]	152.4 ± 1.5[c]	160.7 ± 4.0[d]

[a] Best fit value ± S.E.
[b] Kinetic data estimated by nonlinear regression according to Eq. (2).
[c] Kinetic data estimated with truncated data sets by nonlinear regression according to Eq. (1).
[d] Kinetic data estimated by nonlinear regression according to Eq. (3), presenting the results of estimation of K_m and K_i for substrate inhibition.

area ratios of the metabolite vs. internal standard per min and mg microsomal protein.

2.12. Statistical analysis

All statistics were calculated using GraphPad Prism 3.02 software (San Diego, CA) designed for nonlinear regression analysis. The Michaelis–Menten parameters K_m and V_{max} were calculated by fitting kinetic data to a one- or two-binding site model or the substrate inhibition model. K_i values were calculated according to Cheng and Prusoff (1973) and statistical significance of inhibition was determined using the nonparametric one-tailed Mann–Whitney test.

3. Results

3.1. Characterization of rat liver microsomes

The determined microsomal protein concentration according to Bradford was 35.3 mg/ml and the total CYP content was 1.4 nmol/mg protein.

3.2. Determination of californine, protopine and their metabolites

For sensitive determination of the alkaloids and their metabolites, an HPLC–FLD procedure was developed, which required no special sample workup. Typical chromatograms of microsomal incubations of 50 μM each of californine (A) or protopine (B) are shown in Fig. 1. The retention times were as follows: 3.4 min for demethylene californine, 13.7 min for *N*-demethyl californine, 17.8 min for californine, 3.9 min for demethylene protopine, and 12.0 min for protopine. Peak identity was confirmed by GC/MS. The electron ionization (EI)–MS spectra of the parent compounds and metabolites are presented in Fig. 2. The molecular weights were confirmed by PICI–MS (Paul and Maurer, 2003).

3.3. Enzyme kinetics

Fig. 3 shows the Michaelis–Menten (A, C, E) and Eadie–Hofstee plots (B, D, F) of californine *N*-demethylation (A, B), of californine demethylenation (C, D) and of protopine demethylenation (E, F). Californine demethylenation and protopine demethylenation showed apparently linear Eadie–Hofstee plots. Californine *N*-demethylation exhibited clear biphasic kinetics with at least one low and one high affinity component each, which could be readily seen in the respective Eadie–Hofstee plot. In Table 1, the K_m and V_{max} values of the studied metabolic reactions are listed. V_{max} values are expressed as arbitrary values, because quantification could not be carried out without reference standards. They are given as dimensionless peak area ratio per min and mg microsomal protein. The determined K_m and K_i values of the specific CYP isoenzyme index reactions and the used chemical inhibitors are given in Table 2.

3.4. Inhibition studies

Fig. 4 shows the metabolite formation rates in the presence of the given inhibitors (percent of control mean without inhibitors) for californine *N*-demethylation (A), for californine demethylenation (B), and for protopine demethylenation (C). Californine *N*-demethylation was inhibited by ketoconazole (74%), quinine (28%), and α-naphthoflavone (21%), whereas anti-rat CYP2C11 serum caused no inhibition. Californine demethylenation was inhibited by quinine (81%), anti-rat CYP2C11 serum (76%), ketoconazole (43%), and α-naphthoflavone (5%). Protopine demethylenation was inhibited by anti-rat CYP2C11 serum (91%), quinine (75%), ketoconazole (40%), and α-naphthoflavone (25%). All inhibitions were significant using an one-tailed Mann–Whitney test ($P < 0.05$).

Table 2
CYP isoenzymes assayed, index reactions, chemical inhibitors, and the determined K_m and K_i values

CYP	Index reaction	K_m [μM][a]	Inhibitor	K_i [μM]
1A2	phenacetin deethylation	3.8 ± 3.5[b]	naphthoflavone	0.6
2D1	codeine *O*-demethylation	30.2 ± 3.2	quinine	5.0
3A2	midazolam α-hydroxylation	6.3 ± 0.9	ketoconazole	0.2

[a] Best fit value ± S.E.
[b] $K_{m,1}$ according to Eq. (2).

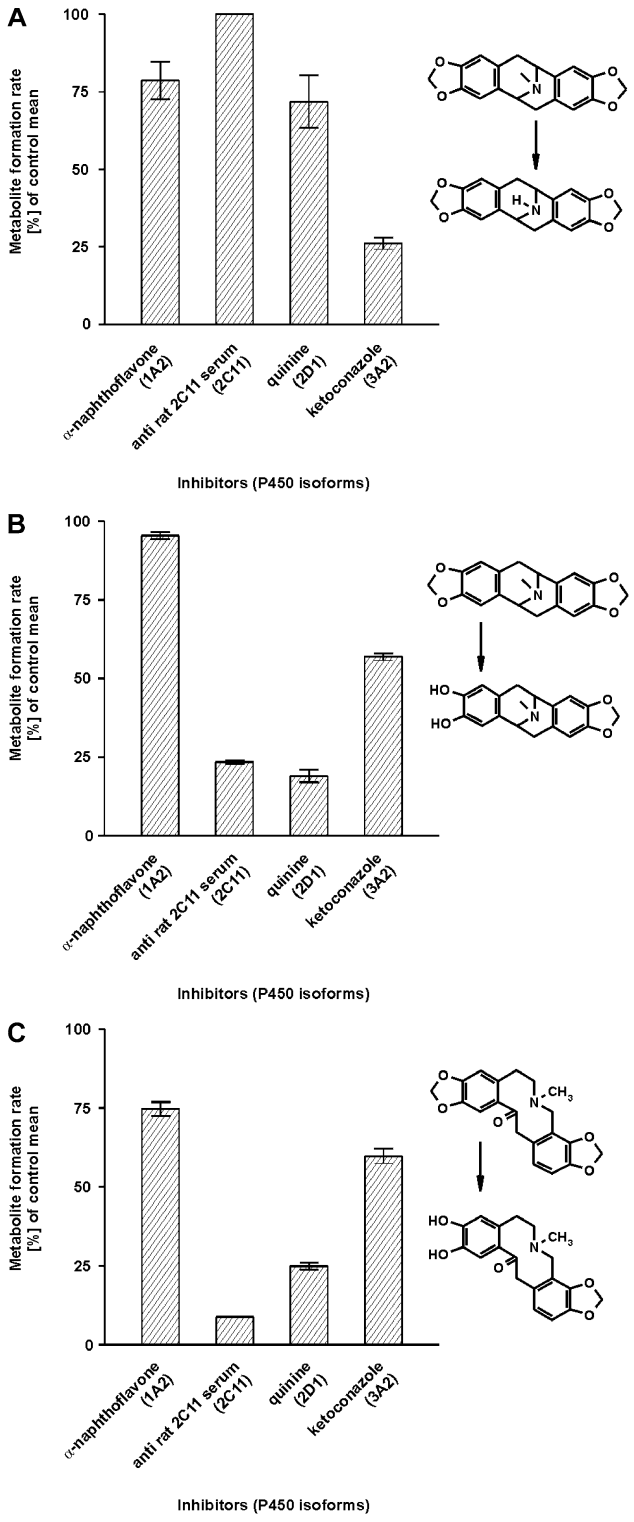

Fig. 4. Metabolite formation rates in presence of the given inhibitors (percent of control mean without inhibitors) for californine *N*-demethylation (A), for californine demethylenation (B), and for protopine demethylenation (C). Each bar represents the mean of four incubations ± S.E.

4. Discussion

The metabolic pathways of californine and protopine in rats have recently been described (Paul and Maurer, 2003). Incubations with rat liver microsomes and NADPH indicated that the initial metabolic steps (californine *N*-demethylation and demethylenation and protopine demethylenation) were catalyzed by *P*450. Therefore, in the present study, the influence of the main CYP isoenzymes on these steps was investigated using rat liver microsomes and specific inhibitors. In order to prevent possible oxidation of the formed catechols, superoxide dismutase was added to the incubation mixture, because it prevents oxidation by scavenging reactive oxygen species (Hiramatsu et al., 1990).

An HPLC–FLD procedure was developed for determination of californine, protopine and their metabolites in the supernatant of microsomal incubation mixtures. The advantages of this procedure were that no extensive sample preparation was necessary and that the sensitivity and selectivity for the investigated analytes were higher compared to HPLC-UV. The confirmation of the peak identity by GC/MS was required, because reference substances of the monitored metabolites were not available. The parent compounds showed a linear response of the FLD signals. Carrying the same chromophore, linearity has to be claimed for the metabolites as well. For this reason, use of peak area ratios instead of absolute metabolite concentrations was possible. This neither affected the conclusions from the kinetic calculations nor those from the inhibition studies.

Kinetic assays were performed under initial rate conditions, a prerequisite for Michaelis–Menten kinetics (Clarke, 1998). The concentration ranges of the substrates for determination of K_m and V_{max} values were limited by the detectability of the formed metabolites in the lower range and by the solubility of the substrates in the upper range.

For inhibition experiments, K_i values for three isoenzyme-specific chemical inhibitors (α-naphthoflavone for CYP1A2, quinine for CYP2D1, and ketoconazole for CYP3A2) were estimated by measurement of the decrease in metabolite formation rate of specific probe substrates. Since no specific chemical inhibitor was available, CYP2C11 was inhibited by specific antibodies. The determined K_m and K_i values (Table 2) were in the range of calculations by other authors, except for quinine, which had a higher K_i value for the investigated rat microsome batch. Boobis et al. (1990) determined a K_i of quinine of 1.7 μM for the debrisoquine 4-hydroxylation. This was three times below the K_i of 5 μM for codeine *O*-demethylation, determined in this study. Phenacetin *O*-deethylation showed biphasic kinetics as described by Boobis et al. (1981) and Kobayashi et al. (2002). The high affinity reaction ($K_{m,\ 1}$ given in Table 2) was specified as catalyzed by CYP1A2 (Sesardic et al., 1990). The kinetic parameters were determined according to Eq. (2) and the corresponding $K_{m,\ 1}$ was selected as substrate concentration for the K_i determination assay of α-naphthoflavone. It should also be considered that

CYP2D1 is only one of six CYP2D isoenzymes in the rat. However, these were very similar and difficult to purify from rat tissues, because of their highly homologous amino acid sequences. As a consequence, very little information on the similarities and/or differences in catalytic properties among these six isoenzymes is available at present (Hiroi et al., 2002). Inhibition studies were performed at inhibitor concentrations 10 times the K_i values. These inhibitor concentrations did not affect the oxidative metabolism of other isoenzyme-selective substrates (Bourrie et al., 1996).

As shown in Fig. 3, californine *N*-demethylation was not saturable over the tested substrate range (A) and showed a clear concave biphasic course in the corresponding Eadie–Hofstee plot (B). It could be concluded that a minimum of two different CYP isoenzymes with different K_m values were involved in this metabolic step (Clarke, 1998). Consequently, K_m and V_{max} values for a high and a low affinity component were estimated according to Eq. (2) (Table 1).

The results from the inhibition experiments given in Fig. 4A indicated that californine *N*-demethylation was mainly catalyzed by CYP3A2 with minor involvement of CYP2D1 and CYP1A2. Obviously, CYP2C11 did not participate in the californine *N*-demethylation. The biphasic course of the plots suggested high and low affinity components among the involved isoenzymes with clear differences in the K_m values.

Californine demethylenation (Fig. 3C, D) and protopine demethylenation (E, F) showed a classical hyperbolic kinetic profile. However, a decrease in metabolite formation rate at high substrate concentrations in the Michaelis–Menten plot of californine demethylenation indicated substrate inhibition, one of the most common deviations from classical hyperbolic kinetics (Hutzler and Tracy, 2002; Lin et al., 2001). This kinetic behaviour could be explained by the model, that a second molecule of substrate can bind to the enzyme–substrate complex to form an inactive ternary complex (Copeland, 2000). For the determination of K_m and V_{max}, data were fitted to the appropriative kinetic model (Eq. (3)), using nonlinear regression analysis. Substrate inhibition is usually realized only at high substrate concentrations. Therefore, in a second approach, only the low concentration range of 2–50 μM was considered, using the classic Michaelis–Menten Eq. (1). Classic Michaelis–Menten kinetics were applied because of the linear Eadie–Hofstee plot (Table 1). Nevertheless, different isoenzymes with similar K_m values could be involved (Clarke, 1998). The results were virtually the same for both approaches and the estimated K_m values were taken as substrate concentrations for the inhibition studies. Substrate inhibition could also be discussed for protopine demethylenation, but only at substrate concentrations higher than 300 μM.

The CYP isoenzyme patterns involved in the demethylenations of californine and protopine were very similar (Fig. 4B and C, respectively). Both metabolic reactions (californine demethylenation and protopine demethylenation) showed marked involvement of CYP2D1 and especially CYP2C11. The latter isoenzyme is known to be the most abundant one in male rat liver microsomes (about 50% of total CYP (Soucek and Gut, 1992)). Californine demethylenation and protopine demethylenation were also catalyzed by rat CYP3A2. CYP1A2 may also contribute to the demethylenations, since there was a minor but significant inhibition by α-naphthoflavone. Although various enzymes took part in demethylenations of californine and protopine (Fig. 4), the Michaelis–Menten and Eadie–Hofstee plots (Fig. 3) did not indicate biphasic kinetics. There are two explanations for this findings. Firstly, the differences in the apparent K_m values of californine demethylenation and protopine demethylenation catalyzed by CYP2D1 and CYP2C11 would be too little and therefore impossible to identify in the plots (Clarke, 1998). Secondly, CYP3A2 and CYP1A2 exhibited such marginal activity compared to CYP2C11 and CYP2D1, that this components would not notably influence the plots.

The obtained results on metabolism in rats may be useful for predictions of the principal CYP isoenzymes in the metabolic oxidations of californine and protopine also in humans. Future studies using human liver microsomes will show whether the CYP-dependent metabolism of the alkaloids in rats and humans is comparable. This is a prerequisite for further toxicological risk assessment using this rat model. For instance, formation of catechol metabolites is discussed to contribute to toxicity of designer drugs of the methylenedioxyphenylalkylamine type, like methylenedioxymethamphetamine (Kraemer and Maurer, 2002). The catechol metabolites may also play a role in a possible toxicity of the *Eschscholtzia* alkaloids.

Acknowledgements

The authors would like to thank Dr. David Stevens (Physiology, Homburg), Carsten Kratzsch, Frank T. Peters, Gabi Ulrich, Armin A. Weber (Toxicology, Homburg), and Dr. Anke Esperester (Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim) for their assistance and support.

References

Baldwin, S.J., Bloomer, J.C., Smith, G.J., Ayrton, A.D., Clarke, S.E., Chenery, R.J., 1995. Ketoconazole and sulphaphenazole as the respective selective inhibitors of *P*4503A and 2C9. Xenobiotica 25, 261–270.

Boobis, A.R., Kahn, G.C., Whyte, C., Brodie, M.J., Davies, D.S., 1981. Biphasic *O*-deethylation of phenacetin and 7-ethoxycoumarin by human and rat liver microsomal fractions. Biochem. Pharmacol. 30, 2451–2456.

Boobis, A.R., Sesardic, D., Murray, B.P., Edwards, R.J., Singleton, A.M., Rich, K.J., Murray, S., de-la-Torre, R., Segura, J., Pelkonen, O., Pasanen, S., Kobayashi, S., Zhi-Guang, T., Davies, D.S., 1990. Species variation in the response of the cytochrome *P*-450-dependent monooxygenase system to inducers and inhibitors. Xenobiotica 20, 1139–1161.

Bourrie, M., Meunier, V., Berger, Y., Fabre, G., 1996. Cytochrome *P*450 isoform inhibitors as a tool for the investigation of metabolic reactions

catalyzed by human liver microsomes. J. Pharmacol. Exp. Ther. 277, 321–332.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Butler, M.A., Iwasaki, M., Guengerich, F.P., Kadlubar, F.F., 1989. Human cytochrome *P*-450PA (*P*-450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. Proc. Natl. Acad. Sci. U. S. A. 86, 7696–7700.
Cheney, R.H., 1964. Therapeutic potential of eschscholtziae californicae herba. Q. J. Crude Drug 3, 413–416.
Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.
Clarke, S.E., 1998. In vitro assessment of human cytochrome *P*450. Xenobiotica 28, 1167–1202.
Copeland, R.A., 2000. Kinetics of single-substrate enzyme reactions. In: Copeland, R.A. (Ed.), Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis. Wiley, NY, pp. 109–144.
Duke, J.A., 1985. CRC Handbook of Medicinal Herbs. CRC Press, Boca Raton, FL.
Gertig, H., 1965. Alkaloids of *Eschscholtzia californica* Cham: 3. Preparative separation of alkaloid fractions obtained from benzene root extract. Acta Pol. Pharm. 22, 271–279.
Ghosal, A., Satoh, H., Thomas, P.E., Bush, E., Moore, D., 1996. Inhibition and kinetics of cytochrome *P*4503A activity in microsomes from rat, human, and cDNA-expressed human cytochrome *P*450. Drug Metab. Dispos. 24, 940–947.
Gorski, J.C., Hall, S.D., Jones, D.R., Vandenbranden, M., Wrighton, S.A., 1994. Regioselective biotransformation of midazolam by members of the human cytochrome *P*450 3A (CYP3A) subfamily. Biochem. Pharmacol. 47, 1643–1653.
Gottlieb, A.A., 2002. Concise Encyclopedia of Legal Herbs and Chemicals with Psychoactive Properties. http://www.erowid.org/library/books_online/legal_highs/legal_highs.shtml.
Guedon, D., Cappelaere, N., Simanek, V., 1990. HPLC analysis of the main alkaloids from *Eschscholtzia californica* cham. Phytochem. Anal. 1, 77–82.
Haensel, R., Keller, K., Rimpler, H., Schneider, G., 1993. Hagers Handbuch der pharmazeutischen Praxis, 5th ed. Springer, Berlin.
Hiramatsu, M., Kumagai, Y., Unger, S.E., Cho, A.K., 1990. Metabolism of methylenedioxymethamphetamine: formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. J. Pharmacol. Exp. Ther. 254, 521–527.
Hiroi, T., Chow, T., Imaoka, S., Funae, Y., 2002. Catalytic specificity of CYP2D isoforms in rat and human. Drug Metab. Dispos. 30, 970–976.
Hutzler, J.M., Tracy, T.S., 2002. Atypical kinetic profiles in drug metabolism reactions. Drug Metab. Dispos. 30, 355–362.
Kardos, J., Blasko, G., Simonyi, M., 1986. Enhancement of gamma-aminobutyric acid receptor binding by protopine-type alkaloids. Arzneim.-Forsch. 36, 939–940.
Ko, F.N., Wu, T.S., Lu, S.T., Wu, Y.C., Huang, T.F., Teng, C.M., 1989. Antiplatelet effects of protopine isolated from Corydalis tubers. Thromb. Res. 56, 289–298.
Kobayashi, S., Murray, S., Watson, D., Sesardic, D., Davies, D.S., Boobis, A.R., 1989. The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. Biochem. Pharmacol. 38, 2795–2799.
Kobayashi, K., Urashima, K., Shimada, N., Chiba, K., 2002. Substrate specificity for rat cytochrome *P*450 (CYP) isoforms: screening with cDNA-expressed systems of the rat. Biochem. Pharmacol. 63, 889–896.
Kotegawa, T., Laurijssens, B.E., von Moltke, L.L., Cotreau, M.M., Perloff, M.D., Venkatakrishnan, K., Warrington, J.S., Granda, B.W., Harmatz, D.J., Greenblatt, D.J., 2002. In vitro, pharmacokinetic, and pharmacodynamic interactions of ketoconazole and midazolam in the rat. J. Pharmacol. Exp. Ther. 302, 1228–1237.
Kraemer, T., Maurer, H.H., 2002. Toxicokinetics of amphetamines: metabolism and toxicokinetic data of designer drugs, of amphetamine, methamphetamine and their *N*-alkyl derivatives [review]. Ther. Drug Monit. 24, 277–289.
Kraemer, T., Bickeboeller-Friedrich, J., Maurer, H.H., 2000. On the metabolism of the amphetamine-derived antispasmodic drug mebeverine: gas chromatography–mass spectrometry studies on rat liver microsomes and on human urine. Drug Metab. Dispos. 28, 339–347.
Kronbach, T., Mathys, D., Umeno, M., Gonzalez, F.J., Meyer, U.A., 1989. Oxidation of midazolam and triazolam by human liver cytochrome *P*450IIIA4. Mol. Pharmacol. 36, 89–96.
Lin, Y., Lu, P., Tang, C., Mei, Q., Sandig, G., Rodrigues, A.D., Rushmore, T.H., Shou, M., 2001. Substrate inhibition kinetics for cytochrome *P*450-catalyzed reactions. Drug Metab. Dispos. 29, 368–374.
Manske, R.H.F., Shin, K.H., 1965. The alkaloids of papaveraceous plants, LIII. *Eschscholtzia californica* cham. Can. J. Chem. 43, 2180–2182.
Maurer, H.H., 2000. Methods for GC–MS. In: Pfleger, K., Maurer, H.H., Weber, A. (Eds.), Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites, Part 4. Wiley, Weinheim, pp. 3–241.
Maurer, H.H., Bickeboeller-Friedrich, J., Kraemer, T., Peters, F.T., 2000. Toxicokinetics and analytical toxicology of amphetamine-derived designer drugs (“Ecstasy”). Toxicol. Lett. 112, 133–142.
Nakajima, M., Kobayashi, K., Oshima, K., Shimada, N., Tokudome, S., Chiba, K., Yokoi, T., 1999. Activation of phenacetin *O*-deethylase activity by alpha-naphthoflavone in human liver microsomes. Xenobiotica 29, 885–898.
Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver micosomes: I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370–2378.
Ono, S., Hatanaka, T., Hotta, H., Satoh, T., Gonzalez, F.J., Tsutsui, M., 1996. Specificity of substrate and inhibitor probes for cytochrome *P*450s: evaluation of in vitro metabolism using cDNA-expressed human *P*450s and human liver microsomes. Xenobiotica 26, 681–693.
Paul, L.D., Maurer, H.H., 2003. Studies on the metabolism and toxicological detection of the *Eschscholtzia californica* alkaloids californine and protopine in urine using gas chromatography–mass spectrometry. J. Chromatogr., B, Biomed. Sci. Appl. 789, 43–57.
Raetsch, C., 1998. Enzyklopädie der Psychoaktiven Pflanzen. Wissenschaftliche Verlagsgesellschaft, Stuttgart.
Rolland, A., Fleurentin, J., Lanhers, M.C., Younos, C., Misslin, R., Mortier, F., Pelt, J.M., 1991. Behavioural effects of the American traditional plant *Eschscholzia californica*: sedative and anxiolytic properties. Planta Med. 57, 212–216.
Rolland, A., Fleurentin, J., Lanhers, M.C., Misslin, R., Mortier, F., 2001. Neurophysiological effects of an extract of *Eschscholzia californica* Cham. (Papaveraceae). Phytother. Res. 15, 377–381.
Sesardic, D., Boobis, A.R., Edwards, R.J., Davies, D.S., 1988. A form of cytochrome *P*450 in man, orthologous to form d in the rat, catalyses the *O*-deethylation of phenacetin and is inducible by cigarette smoking. Br. J. Clin. Pharmacol. 26, 363–372.
Sesardic, D., Edwards, R.J., Davies, D.S., Thomas, P.E., Levin, W., Boobis, A.R., 1990. High affinity phenacetin *O*-deethylase is catalysed specifically by cytochrome *P*450d (*P*450IA2) in the liver of the rat. Biochem. Pharmacol. 39, 489–498.
Shimada, T., Mimura, M., Inoue, K., Nakamura, S., Oda, H., Ohmori, S., Yamazaki, H., 1997. Cytochrome *P*450-dependent drug oxidation activities in liver microsomes of various animal species including rats, guinea pigs, dogs, monkeys, and humans. Arch. Toxicol. 71, 401–408.
Siegel, R.K., 1976. Herbal intoxication. Psychoactive effects from herbal cigarettes, tea, and capsules. JAMA 236, 473–476.
Song, L.S., Ren, G.J., Chen, Z.L., Chen, Z.H., Zhou, Z.N., Cheng, H., 2000. Electrophysiological effects of protopine in cardiac myocytes: inhibition of multiple cation channel currents. Br. J. Pharmacol. 129, 893–900.

Soucek, P., Gut, I., 1992. Cytochromes *P*-450 in rats: structures, functions, properties and relevant human forms. Xenobiotica 22, 83–103.

Ustunes, L., Laekeman, G.M., Gozler, B., Vlietinck, A.J., Ozer, A., Herman, A.G., 1988. In vitro study of the anticholinergic and antihistaminic activities of protopine and some derivatives. J. Nat. Prod. 51, 1021–1022.

Vincieri, F.F., Celli, S., Mulinacci, N., Speroni, E., 1988. An approach to the study of the biological activity of *Eschscholtzia californica* Cham. Pharmacol. Res. Commun. 20 (Suppl. 5), 41–44.

Xu, B.Q., Aasmundstad, T.A., Bjorneboe, A., Christophersen, A.S., Morland, J., 1995. Ethylmorphine *O*-deethylation in isolated rat hepatocytes. Involvement of codeine *O*-demethylation enzyme systems. Biochem. Pharmacol. 49, 453–460.

Xu, B.Q., Aasmundstad, T.A., Christophersen, A.S., Morland, J., Bjorneboe, A., 1997. Evidence for CYP2D1-mediated primary and secondary O-dealkylation of ethylmorphine and codeine in rat liver microsomes [published erratum appears in Biochem. Pharmacol. 1997 Jun 15;53(12):1945]. Biochem. Pharmacol. 53, 603–609.