Capillary electrophoresis to assess drug metabolism induced in vitro using single CYP450 enzymes (Supersomes™): Application to the chiral metabolism of mephenytoin and methadone

Capillary electrophoresis (CE) with multiwavelength absorbance detection is demonstrated to be an effective tool for the assessment of in vitro drug metabolism studies using microsomes containing single human cytochrome P450 enzymes (CYPs) expressed in baculovirus-infected insect cells (Supersomes™). Mephenytoin (MEPH), dextromethorphan, diclofenac, caffeine, and methadone (MET) were successfully applied as test substrates for CYP2C19, CYP2D6*1, CYP2C9*1, CYP1A2, and CYP3A4, respectively. For each system, the CE-based assay could be shown to permit the simultaneous analysis of the parent drug and its targeted metabolite. Using a chiral micellar electrokinetic capillary chromatography assay, the aromatic hydroxylation of MEPH catalyzed by CYP2C19 could thereby be confirmed to be highly stereoselective, an aspect that is in agreement with data obtained via urinary analysis after intake of racemic MEPH by extensive metabolizer phenotypes. The MET to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) conversion was investigated with a chiral zone electrophoresis assay. Incubation of racemic and nonracemic MET with CYP3A4 revealed no stereoselectivity for the transformation to EDDP, whereas no EDDP formation was observed with CYP1A2. CYP2C9 and CYP2C19 provided enhanced formation of R-EDDP and CYP2D6 incubation resulted in the preferential conversion to S-EDDP. Investigations using racemic MET and human liver microsomes revealed a modest stereoselectivity with an R/S EDDP ratio < 1 which is similar to the in vivo findings in urine.

**Keywords:** Caffeine / Chiral capillary electrophoresis / CYP450 / Dextromethorphan / Diclofenac / in vitro drug metabolism / Mephenytoin / Methadone DOI 10.1002/elps.200305493

1 Introduction

Drug metabolism studies have made and are continuing to make fundamental contributions to drug development, to the understanding of in vivo molecular reactions and mechanisms of drug action, to the elucidation of drug toxicity and are part of the material required for drug registration and thus application of a drug in pharmacotherapy.

Xenobiotics are mostly metabolized through the liver, the major detoxification organ. Exogenous compounds are thereby converted into hydrophilic, active or inactive compounds that can be readily eliminated by the kidneys or other organs. The conversions are mostly oxidations and are catalyzed by liver enzymes, the so-called cytochrome P450 enzymes (CYPs) and the flavin monoxygenases (FMOs). The CYPs metabolize endogenous and xenobiotic compounds and are classified into families (first number), subfamilies (letter) and isoforms (final number). The major enzymes involved in the metabolism of drugs are CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 [1–3]. In humans, CYP1A1 is predominantly extrahepatic. CYP1A2 is universally expressed and accounts for about 13% of the total immunoquantified human hepatic CYPs. The subfamily CYP3A represents about 30% and the universally expressed CYP3A4 is the major adult isoform. The subfamily CYP2C accounts for about 20% of total liver metabolism.
CYPs. The CYP2C9 and CYP2C19 isoforms are polymorphically expressed and the CYP2C9 quantitatively dominates over the CYP2C19. CYP2D6 represents less than 2% of the total liver CYPs and is polymorphically expressed [2].

Drug metabolism studies can be performed in vivo after drug intake and following its fate via analysis of the temporal drug and metabolite concentrations in body fluids and tissues. Alternatively, in vitro systems which mimic hepatic functions are important tools in the field of pharmacological and toxicological research. Freshly isolated hepatocytes which express most of the functional activities of the intact liver, differentiated liver-derived cell lines and microsomes containing single or multiple CYPs can be used to study the metabolism of xenobiotics in vitro [2, 4]. About one fourth of the therapeutically administered drugs are metabolized to various isomeric substances whose biological activity may well reside predominantly in one form. The majority of these are racemic mixtures of synthetic chiral drugs. It is well known that liver enzymes are able to discriminate between stereoisomers [1, 5, 6]. Thus, stereospecific drug monitoring is often required and widely accomplished via the use of chromatographic methods with chiral stationary phases [7] or capillary electromigration methods exhibiting chiral selectors, such as various cyclodextrins (CDs), in the buffer [8, 9]. Capillary electrophoresis (CE) in the formats of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) had been successfully applied for the assessment of chiral and achiral drug biotransformation. Most of this work is based upon analysis of the parent drug and/or its metabolites in body fluids and tissues and only few articles report the use of CE for the study of the metabolism of xenobiotics in vitro [8, 9]. The examples of in vitro work listed here are those in which the stereoselective drug metabolism was investigated. Using preparations with rat liver microsomes, CZE with sulfobutyl-β-CD as chiral selector was employed to study the metabolism of the anthelminthic drug praziquantel [10] and the metabolism of the sedative drug thalidomide was investigated with carboxymethyl-β-CD [11] and the combined use of sulfobutyl-β-CD and β-CD [12]. Incubations of flecainide with human, rat, bovine, and porcine liver microsomes were analyzed by CZE in the presence of β-CD [13] and the stereoselectivity of the mephenytoin (MEPH) metabolism in an incubation with human liver microsomes (HLM) was monitored by MEKC using taurodeoxycholic acid and β-CD as chiral buffer additives [14]. The number of achiral applications reported in the literature is not much larger [15] and all these incubations were performed with liver microsomes from animals or humans, i.e., preparations which contain multiple CYPs.

As CE was successfully applied to the analysis of drugs and metabolites after incubation of the parent drug with liver microsomes, the aim of our work was to investigate drug metabolism using microsomes with single human CYPs that were expressed in baculovirus-infected insect cells (Autographa californica). The microsomes are referred to as Supersomes™ and are claimed to provide very high levels of catalytic activity. In this paper, CE data obtained for the biotransformation of five test substrates (Fig. 1) after incubation with their specific enzyme are presented together with a careful investigation of the stereoselectivity of the methadone (MET) to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) conversion obtained after incubation of MET with CYP2D6, CYP3A4, CYP2C9, CYP2C19, CYP1A2 Supersomes. The latter data are compared to those obtained after incubation of MET with liver microsomes and to those monitored in vivo via analysis of urines of patients that received either racemic MET or R-MET. To the best of our knowledge, these are the first CE results document-
ing the in vitro formation of drug metabolites using Super-
"somes"*, i.e., microsomal preparations that contain single human CYPs.

2 Material and methods

2.1 Chemicals, Supersomes*, liver microsomes, NADPH regenerating system, origin of urine samples, and stock solutions of drug substrates

All chemicals used were of analytical or research grade. Racemic MEPH was from Siegfried (Zofingen, Switzerland) and 4-hydroxymephentoin (4-OH-MEPH) was synthesized in-house according to Karlaganis et al. [16]. Dextromethorphan (as hydrobromide), dextrorphan (as tartrate) were a kind gift of F. Hoffmann-La Roche (Basel, Switzerland). Racemic MEPH was from Siegfried (Zofingen, Switzerland). Diclofenac was purchased from Spirig (Egerkingen, Switzerland). Heptakis(2,6-di-methyl)-β-CD (DIMEB, product CY-2004) was from Cyclolab (Budapest, Hungary). Baculovirus-insect-cell-expressed human CYP3A4 + P450 Reductase + Cytochrome b5 Supersomes*, human CYP2C19 + P450 Reductase + Cytochrome b5 Supersomes*, human CYP2D6*1 + P450 Reductase Supersomes*, human CYP1A2 + P450 Reductase Supersomes*, and human CYP2C9*1 (Arg 144) + P450 Reductase + Cytochrome b5 Supersomes* were purchased from Gentest (Woburn, MA, USA) and paraxanthine, β-CD, hydroxypropyl-β-CD (OHP-β-CD) were from Fluka (Buchs, Switzerland). Heptakis(2,6-di-O-methyl)-β-CD was from Merck (Darmstadt, Germany), and Na2B4O7 was from Haenseler (Herisau, Switzerland). Baculovirus-insect-cell-expressed human CYP3A4 + P450 Reductase + Cytochrome b5 Supersomes*, human CYP2C19 + P450 Reductase + Cytochrome b5 Supersomes*, human CYP2D6*1 + P450 Reductase Supersomes*, human CYP1A2 + P450 Reductase Supersomes*, and human CYP2C9*1 (Arg 144) + P450 Reductase + Cytochrome b5 Supersomes* were purchased from Gentest (Woburn, MA, USA, distributed through Anawa Trading, Wangen, Switzerland). The mixed gender pool of HLM, containing CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A, with a protein concentration of 20 mg/mL in 250 mM sucrose and the NADPH regenerating system were also from Gentest (BD Biosciences). The regenerating system comprises two solutions, solution A composed of 26.0 mM NADP+, 66 mM glucose-6-phosphate and 66 mM MgCl₂ and solution B containing 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. The microsomes were stored in small aliquots of 25 or 50 pmol at −80°C and the NADPH regenerating system was kept at −18°C until use. The MET urines were from patients receiving racemic MET here in Switzerland or R-MET in Germany. The urines containing MEPH stemmed from patients whose hydroxylator phenotype status was assessed in our departmental drug assay laboratory. All urines were stored at −18°C until analysis. Aqueous stock solutions of dextromethorphan (2 mg/mL), diclofenac (2 mg/mL), MET (1 mg/mL), and caffeine (16 mg/mL) were prepared. The stock solution of MEPH (2 mg/mL) was in acetonitrile.

2.2 Microsomal incubations

Incubations were performed in duplicate and essentially according to the recommendations of the manufacturer of the microsomal preparations [17]. If not stated otherwise, 25 µL NADPH regenerating solution A, 5 µL regenerating solution B, xx µL stock solution of the drug of interest and 445-xx µL 100 mM KH2PO4 (pH 7.4) solution were quickly vortexed in an Eppendorf vial and incubated for 10 min at 37°C in an Eppendorf Thermomixer. Then, if not stated otherwise, 25 µL of the CYP or HLM aliquot was quickly thawed and added to the mixture, vortexed, and incubated for the required amount of time (between 1 and 72 h) without shaking. The incubation was stopped by cooling the mixture on ice. The 500 µL incubation mixture comprised 25 pmol CYP, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride. For the incubations with CYP2C19 and CYP2C9, 50 mM phosphate buffer (pH 7.4) and 100 mM Tris buffer (pH 7.5), respectively, were used instead of the 100 mM KH2PO4 (pH 7.4) solution.

2.3 Sample pretreatment

The total amount (500 µL) of the incubated solution was used for sample pretreatment. Substrates and their metabolites were extracted using methods taken from the literature [18–22]. MET and its metabolite were extracted using solid-phase extraction (SPE) whereas liquid/liquid extraction (LLE) was employed for all other cases. Extraction principles and reconstitution media for all samples are listed in Table 1. Patient urines were pretreated with the same methods. For MET, extractions were performed with 1 mL urine and the dried residues were reconstituted in 200 µL water. For MEPH, 5 mL of urine was extracted and reconstituted in 100 µL of running buffer without β-CD and isopropanol alcohol.

2.4 Electrophoretic instrumentation and running conditions

The measurements were performed on a BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA), equipped with a 50 µm ID untreated capillary.
Table 1. Overview of experimental conditions used for CE analysis of in vitro incubations

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite(s)</th>
<th>CE mode</th>
<th>Separation buffer</th>
<th>Sample pre-treatment&lt;br&gt;Composition</th>
<th>pH</th>
<th>Ref.</th>
<th>Extraction principle</th>
<th>Reconstitution volume&lt;br&gt;(mL)/medium</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-, S-MET</td>
<td>R-, S-EDDP</td>
<td>Chiral CZE</td>
<td>100 mM NaH₂PO₄, H₂PO₄, 2 mM DIMEB and 10% methanol</td>
<td>2.3</td>
<td>[23]</td>
<td>SPE (Bond Elut Certify)</td>
<td>50 µL water</td>
<td>[18]</td>
<td></td>
</tr>
<tr>
<td>S-Mephenytoin (applied as racemate)</td>
<td>S-4-OH- Mephenytoin</td>
<td>Chiral MEKC</td>
<td>5.6 mM Na₂B₄O₇, 8.4 mM Na₂HPO₄, 95 mM SDS, 40 mM β-CD, and 8% isopropyl alcohol</td>
<td>9.1</td>
<td>[19]</td>
<td>LLE (dichloroethane)</td>
<td>50 µL in separation buffer without β-CD and isopropyl alcohol</td>
<td>[19]</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Dextrorphan</td>
<td>CZE</td>
<td>140 mM Na₂B₄O₇</td>
<td>9.4</td>
<td>[20]</td>
<td>LLE (hexane, 10% butanol)</td>
<td>50 µL 5-fold diluted separation buffer</td>
<td>[20]</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>4’OH-Diclofenac</td>
<td>CZE</td>
<td>20 mM Na₂B₄O₇</td>
<td>9.2</td>
<td>[24]</td>
<td>LLE (dichloromethane, 5% isopropyl alcohol)</td>
<td>50 µL 5-fold diluted separation buffer</td>
<td>[21]</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>Paraxanthine, theobromine</td>
<td>MEKC</td>
<td>75 mM SDS, 6 mM Na₂B₄O₇ and 10 mM Na₂HPO₄</td>
<td>9.0</td>
<td>[22]</td>
<td>LLE (chloroform/isopropanol)</td>
<td>50 µL separation buffer</td>
<td>[22]</td>
<td></td>
</tr>
</tbody>
</table>

a) The sample volume was 500 µL.

3 Results and discussion
3.1 CZE analysis of in vitro incubations of test substrates for CYP2D6, CYP2C9 and CYP1A2

For the assessment of the use of CE assays for the analysis of the activity of Supersomes™, configurations with known test substrates were elucidated (Fig. 1). CYP2D6 is polymorphically expressed in the human liver and is responsible for the metabolism of a number of drugs, including bufuralol, propranolol, codeine, and dextromethorphan. The CYP2D6*1 investigated here is the most common allele in Caucasians and that found in extensive metabolizers of the sparteine/debrisoquine type [25]. In order to test the activity of the human CYP2D6*1 dextromethorphan was chosen as substrate. Dextromethorphan (d-(-)-3-methoxy-N-methylmorphinan) becomes O-demethylated and is thereby converted to dextrorphan (d-(-)-3-hydroxy-N-methylmorphinan, for structures refer to Fig. 1). This metabolism is inhibited in poor metabolizers and dextromethorphan can therefore be employed as a test compound for CYP2D6 phenotyping [20]. In our setup, dextromethorphan was determined to become rapidly demethylated (Fig. 2). After a 2 h incubation of 0.1 mM dextromethorphan, both the parent drug and its metabolite dextrorphan could be monitored in the extract (Fig. 2A), whereas after 24 h of incubation, an increased dextrorphan peak and no dextromethorphan were detected (Fig. 2B). Peak identification was accomplished by spiking and via spectral analysis (Figs. 3A and B). Thus, the CZE assay (for conditions see Table 1 and legend of Fig. 2) appears to be an efficient approach to test the activity of the enzyme product.

CYP2C9 is a polymorphic enzyme that catalyzes the metabolism of many drugs, including S-warfarin, phenytoin, diclofenac, losartan, and most of the nonsteroidal anti-inflammatory drugs. Diclofenac is converted to 4’OH-
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CE for assessment of drug metabolism in vitro

Figure 2. CZE electropherograms obtained from liquid-liquid extracts of 0.1 mM dextromethorphan incubated with CYP2D6 for (A) 2 h and (B) 24 h. The applied voltage was a constant 14 kV (current about 94 μA) and the capillary was thermostated at 25°C. Samples were injected by applying a positive pressure of 4 psi × s. The depicted electropherograms are those for 200 nm. The arrow (B) marks the position of dextromethorphan. Peak identification: dextromethorphan (DX) and its metabolite dextrorphan (DR).

Figure 3. Normalized UV-absorbance spectra of (A) dextromethorphan and (B) dextrorphan. Overlays of the monitored spectra with those obtained with the respective standard compound are presented.

diclofenac (Fig. 1), its major metabolite, and other compounds [26]. It was chosen as a substrate for human CYP2C9*1 (Arg144) and found to be converted rapidly when applied with an initial concentration of 0.1 mM (Fig. 4). After 1 h of incubation, the analyzed extract revealed two peaks, one for diclofenac at about 4.5 min and one at about 6.2 min (Fig. 4A). The diclofenac peak became significantly smaller and the metabolite peak much larger after 2 h incubation (Fig. 4B), whereas after 3 h, a complete metabolism of diclofenac was observed (Fig. 4C). Unfortunately, no standard compound for 4’OH-diclofenac was available. However, based upon the knowledge that 4’OH-diclofenac is the major metabolite produced by CYP2C9 [26], the peaks detected at about 6.2 min in the electropherograms of Fig. 4 can be assumed to be 4’OH-diclofenac. The normalized spectra of diclofenac and the metabolite are presented in Figs. 5A and B, respectively.

Caffeine is metabolized through CYP1A2 to paraxanthine (for structure see Fig. 1), as well as other compounds, and CYP1A2 in vivo activity is thus typically determined by using caffeine as a substrate [27, 28]. Caffeine metabolism has previously been investigated with microsomal preparations containing lymphoblastoid cell lines transfected with human CYPs [29]. In that work, samples were analyzed by HPLC. Data obtained with the human CYP1A2 enzyme being expressed from baculovirus-infected insect cells are depicted in Fig. 6. As suggested by the manufacturer, double concentrations of solutions A and B were employed. The extract of a 72 h incubation of 4 mM caffeine and having 50 pmol of the enzyme was analyzed by MEKC in a capillary of 104.6 cm total length and according to the assay described in [22]. The peak detected at about 18 min is the 1-N-demethylated metabolite theobromine. At 20 min the parent compound caffeine is monitored, whereas the 3-N-demethylated metabolite, paraxanthine, was detected after about 24 min. Peaks were confirmed by reanalysis of the extract fortified by theobromine and paraxanthine and by spectral analysis (data not shown). Other metabolites, including 1-methylxanthine and 1,7-dimethyluric acid, were not detected.
Figure 4. CZE electropherograms of liquid-liquid extracts obtained after incubation of 0.1 mM diclofenac with CYP2C9 for (A) 1 h, (B) 2 h and (C) 3 h. The depicted wavelength is 200 nm, the constant voltage was 20 kV (current about 25 μA) and the capillary was held at 25°C. Injection of the samples occurred with a positive pressure of 5 psi × s. Peak identification: diclofenac (DICLO) and its metabolite 4’OH-diclofenac (4’OH-DICLO).

Having 0.4 mM caffeine and incubation time intervals up to 72 h, no metabolites could be detected (data not shown). The slow metabolite formation observed in these in vitro experiments is in contrast to the rapid in vivo formation of paraxanthine after caffeine intake [22]. The example is interesting because it exhibits the formation of more than one metabolite produced by a single enzyme and also shows the difficulty of their in vitro formation. This abnormally low rate of microsomal caffeine biotransformation was previously reported by Grant et al. [30], an aspect that is important to bear in mind when working with microsomal preparations. Similarly, incubations of 0.1 mM debrisoquine with CYP2D6 Supersomes™ and using the CZE assay of Lanz et al. [31] for sample analysis did provide a small metabolite peak only. In vivo biotransformation in extensive metabolizers, however, provided large amounts of urinary S-4-hydroxydebrisoquine [31]. Thus, data obtained via in vitro experiments should be used with caution when correlated with in vivo observations [32].

Figure 5. Normalized UV-absorbance spectra of (A) diclofenac and (B) the metabolite peak that is assumed to be 4’OH-diclofenac. (A) represents an overlay of the monitored spectrum with that of the standard compound.

Figure 6. MEKC electropherogram of a liquid-liquid extract obtained after incubation of 4 mM caffeine with 50 pmol CYP1A2 for 72 h. For analysis, a 50 μm ID fused-silica capillary of 100 cm (104.6 cm) effective (total) length was employed. The applied constant voltage was 20 kV (current about 18 μA), the temperature was 25°C, and sample injection occurred with positive pressure at 5 psi × s. The data at 200 nm are displayed. Peak identification: caffeine (CA) and its metabolites theobromine (TB) and paraxanthine (PX).
3.2 Chiral MEKC for the in vitro and in vivo confirmation of the CYP2C19 hydroxylation of MEPH

CYP2C19 is a polymorphically expressed CYP and is well known for the stereoselective 4-hydroxylation of MEPH (Fig. 1, refer to [19, 33, 34]). In Caucasian and Asian populations, less than 5% and 12–20%, respectively, are poor metabolizer phenotypes [2]. Using chiral MEKC, the stereoselective metabolism of MEPH could easily be confirmed via analysis of MEPH and 4-OH-MEPH in human urine [19] and was also briefly discussed in vitro by incubation with HLM [14]. Thus, it was of interest to investigate the performance of the CYP2C19 when incubated together with MEPH. Figure 7A depicts data obtained after a 2 h incubation of 0.1 mM racemic MEPH with CYP2C19. In this electropherogram, both MEPH enantiomers were detected shortly after 13 min. For 4-OH-MEPH, however, the S-enantiomer was monitored only (peak at about 9 min). Not surprisingly, the S-enantiomer of MEPH shows a decreased peak compared to that of R-MEPH. Peak assignment was accomplished via spiking with racemic MEPH and racemic 4-OH-MEPH (Fig. 7B) and via comparison of absorption spectra (data not shown, [19]). Both enantiomers of 4-OH-MEPH were detected in the fortified sample. These results confirm that CYP2C19 is indeed responsible for the stereoselective hydroxylation of MEPH. Furthermore, it appears that R-MEPH is not affected by CYP2C19. This does not come as a surprise as R-MEPH is known to be preferentially demethylated to nirvanol [35]. Thus, using CYP2C19 (S), S-MEPH is converted to S-4-OH-MEPH, whereas R-MEPH is not metabolized.

The chiral MEKC assay was previously employed to distinguish between extensive and poor metabolizers for MEPH via the monitoring of S-4-OH-MEPH in extracts prepared from unhydrolyzed urine that was collected during the 0–8 h interval after intake of 100 mg racemic MEPH [19]. Extensive metabolizers could thereby be recognized by the presence of S-4-OH-MEPH, whereas no S-4-OH-MEPH could be detected in the urinary extracts for poor metabolizers. Figure 7C depicts data obtained with a 2-fold diluted urine extract of an extensive metabolizer. As was the case in the in vitro incubation experiment (Fig. 7A), S-4-OH-MEPH could be detected only (Fig. 7C). Peak identification was again accomplished via spiking (Fig. 7D) and spectral analysis (data not shown, for examples refer to [19]). Furthermore, for the example presented in Fig. 7D, R-MEPH and S-MEPH were determined to coelute with endogenous interferences. Such interferences were not always present [19] and have no impact on the classification of the hydroxylator phenotype status.

![Chiral MEKC electropherograms obtained after liquid-liquid extraction of](image)

Figure 7. Chiral MEKC electropherograms obtained after liquid-liquid extraction of (A) a 2 h incubation of 0.1 mM racemic MEPH with CYP2C19 that was stopped on ice with 250 μL acetonitrile, (B) the 2-fold diluted sample spiked with 12.5 μg/mL racemic MEPH and 12.5 μg/mL racemic 4-OH-MEPH, (C) a 2-fold diluted sample of 5 mL extracted patient urine, and (D) sample of (C) spiked with the same amount of racemic MEPH and racemic 4-OH-MEPH as for (B). The applied constant voltage was 20 kV (current about 35 μA) and the capillary was held at 20°C. Samples were injected by applying a positive pressure of 5 psi × s. The depicted wavelength is 195 nm. Peak identification: R- and S-enantiomers of MEPH and its metabolite 4-OH-MEPH.

3.3 Chiral CZE for the assessment of the MET metabolism

MET has become the most widely used drug for opiate dependency treatment and is also administered for the management of chronic pain. The chemical structures of MET and its cyclic metabolite EDDP are shown in Fig. 1. The MET to EDDP conversion (N-demethylation of MET followed by a spontaneous cyclization) is known to be
mainly catalyzed through CYP3A4 [32, 36, 37]. Other potential enzymes involved are CYP2C9, CYP2C19, CYP1A2, and CYP2D6 [37–39]. Both MET and EDDP are chiral compounds (Fig. 1). The two MET enantiomers show different pharmacodynamic and pharmacokinetic parameters, including protein binding, and R-MET is the enantiomer with the higher pharmacological activity [40]. While in some countries, including Germany, R-MET is predominantly employed, it is more often the racemate, such as here in Switzerland, which is administered. For the latter case, the concentration of R-MET in urine was determined to be higher than that of S-MET [18, 23, 41, 42]. Furthermore, the R/S ratios of MET in serum were found to be below or above 1 [40, 43–45], whereas those in saliva were noted to be ≥ 1 [43].

In order to determine the impact of the individual CYPs on MET, racemic MET was incubated with the CYP3A4, CYP2C9, CYP2C19, CYP2D6, CYP1A2 Supersomes™ (Fig. 8). After 24 h incubation, MET and EDDP were extracted according to the SPE method described by Lanz and Thormann [18] and EDDP formation was analyzed by chiral CZE using the method of Frost et al. [23]. In that configuration, the enantiomers of EDDP were detected after about 14 min, whereas those of MET after about 16 min (Fig. 8A). Peaks were identified via reanalysis of the samples spiked with racemic mixtures of EDDP and MET and by spectral analysis (data not shown, for examples refer to [46]). Having CYP3A4 (Fig. 8A), the mean (n = 2) enantiomeric R/S ratio (calculated on the basis of relative peak heights) for EDDP and MET were determined to be 0.91 and 1.06, respectively. Enantiomeric R/S ratios obtained after SPE of racemic EDDP and MET spiked into water and saline (0.9% NaCl) solution revealed similar values (Table 2). Furthermore, incubation of nonracemic MET mixtures that were obtained by recycling isotachophoresis [47] with the same enzyme revealed unequal production of EDDP enantiomers (Fig. 9). The sample with the higher amount of S-MET (Fig. 9A) was determined to produce a higher amount of the firstly detected EDDP enantiomer. The opposite was found to be true for the second sample (Fig. 9B). Thus, with the assumption that there is no conversion of the chiral center during biotransformation, these data suggest that S-EDDP is migrating ahead of R-EDDP, an assignment that is opposite to that reported by Frost et al. [23]. In contrast to MET [18], no pure EDDP enantiomers were available to directly prove this assignment. The analysis of a urine from a patient that received R-MET, however, confirmed that R-EDDP is indeed the secondly detected EDDP enantiomer (see below). The inserts in Fig. 9 depict the enantiomeric ratio of the nonracemic MET mixtures that were incubated with CYP3A4 Supersomes™. The R/S enantiomeric ratios of MET remained the same as applied (Table 2). Thus, the CYP3A4 metabolism appears not to be stereoselective which is in agreement with the literature. The lack of stereoselectivity for the formation of EDDP by incubation of R-MET and S-MET with microsomes from human lymphoblastoid cells containing expressed CYP3A4 was previously described by Foster et al. [37] using an achiral HPLC assay for analysis of their samples.
Table 2. Enantiomeric ratios of MET and EDDP

<table>
<thead>
<tr>
<th>Sample compounds</th>
<th>Sample matrix or incubation medium</th>
<th>MET R/S enantiomeric ratio&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>EDDP R/S enantiomeric ratio&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>rac-MET and EDDP (5 μg/mL each)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1.05</td>
<td>0.88</td>
</tr>
<tr>
<td>rac-MET and EDDP (5 μg/mL each)</td>
<td>NaCl (0.9%)</td>
<td>0.97</td>
<td>0.91</td>
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<td>rac-MET (200 μM)</td>
<td>HLM</td>
<td>1.11</td>
<td>0.73</td>
</tr>
<tr>
<td>Nonrac. MET (R/S = 0.56)&lt;sup&gt;c)&lt;/sup&gt;</td>
<td>CYP3A4</td>
<td>0.55</td>
<td>0.39</td>
</tr>
<tr>
<td>Nonrac. MET (R/S = 2.07)&lt;sup&gt;d)&lt;/sup&gt;</td>
<td>CYP3A4</td>
<td>2.10</td>
<td>2.22</td>
</tr>
<tr>
<td>Patient urine no. 8 of [18]</td>
<td>Urine</td>
<td>1.52</td>
<td>0.59</td>
</tr>
<tr>
<td>Mean of 8 patient urines of [18]</td>
<td>Urine</td>
<td>1.53</td>
<td>0.63</td>
</tr>
</tbody>
</table>

**a)** Mean values (n = 2) after chiral CE analysis of solid-phase extracts  
**b)** Ratio of relative peak heights  
**c)** Fraction 13 of MinipHor RITP run of [47] (total MET concentration of about 200 μM)  
**d)** Fraction 15 of MinipHor RITP run of [47] (total MET concentration of about 200 μM)

Data obtained with CYP2C9 and CYP2C19 incubations are presented in Figs. 8B and C, respectively. The electropherograms reveal a stereoselective formation of EDDP with these enzymes. The mean enantiomeric R/S ratio for EDDP was found to be 2.78 in the case of CYP2C9 and 3.03 for the incubation with CYP2C19. The opposite was found to be true with CYP2D6 for which an R/S EDDP ratio of 0.39 was determined (Fig. 8D). The enantiomeric R/S ratios for EDDP and MET were determined to be 0.73 and 1.11, respectively (Table 2). These data suggest that the formation of EDDP is stereoselective. However, no marked stereoselectivity is observed for the MET enantiomers. This is distinctly different to the case with CYP2C19 that metabolizes MEPH to S-4-OH-MEPH in a truly stereoselective manner (Fig. 7). Furthermore, the in vitro results obtained with HLM were compared to the in vivo metabolism data obtained via analysis of a urine of a patient under racemic MET therapy (Fig. 10B). The enantiomeric R/S ratios of EDDP and MET for that sample were calculated to be 0.59 and 1.52, respectively, data that compare well to those published previously using a different chiral CE assay [18] (Table 2). The data obtained with the urine of a patient who received R-MET are presented in Fig. 10C. Two peaks were monitored, one for R-MET and one for R-EDDP. Comparison with data obtained after spiking the urinary extract with racemic MET and racemic EDDP (Fig. 10D), resulted in unambiguous assignment of the enantiomers of EDDP.
Thus, the urinary data obtained by chiral CZE are in complete agreement with those found by chiral HPLC (urinary R/S EDDP ratio < 1 [40–42]). The enantiomeric R/S ratio of EDDP obtained after incubation with HLM is somewhat higher compared to that observed in vivo. The metabolism of MET to EDDP is known to be stereoselective [18, 23, 40–44], but all the steps involved are not yet clear. A recent study revealed the binding of MET to plasma proteins as being stereoselective [40]. R-MET was found to exhibit a greater unbound fraction and total renal clearance compared with S-MET. This suggests a differential renal clearance mechanism for the excretion of the unchanged MET enantiomers and thus the unequal amounts determined in urine. These considerations, however, are not applicable to EDDP as insufficient data are available.

4 Concluding remarks

MEKC and CZE with multiwavelength absorbance detection are demonstrated to be suitable methods for analysis of extracts of in vitro incubations of drugs with specific human CYPs that were expressed in baculovirus-infected insect cells. MEPH, dextromethorphan, diclofenac, caffeine, and MET were successfully applied as test substrates for CYP2C19, CYP2D6*1, CYP2C9*, CYP1A2, and CYP3A4, respectively. For each system, the CE-based assay could be shown to permit the simultaneous analysis of the parent drug and its metabolite (S-4-OH-MEPH, dextrorphan, 4’-OH-diclofenac, theobromine, and EDDP, respectively) in a rapid and simple way. Using a chiral MEKC assay, the aromatic hydroxylation of MEPH catalyzed by CYP2C19 could thereby be confirmed to be highly stereoselective, an aspect that is in agreement with data obtained via urinary analysis after intake of racemic MEPH by extensive metabolizer phenotypes. Chiral CZE data obtained after incubation of racemic and nonracemic MET with Supersomes revealed that CYP3A4 is the major enzyme for EDDP formation but that this N-demethylation followed by a spontaneous cyclization is not stereoselective. Incubations with CYP2C19, CYP2C9*1 and CYP2D6 provided small amounts of EDDP with R- or S-EDDP being preferentially formed. Using pooled HLM revealed a larger amount of S-EDDP compared to R-EDDP with S-MET, however, being slightly but not significantly reduced compared to R-MET. Thus, the stereoselectivity being observed via analysis of urines after intake of racemic MET is more likely to be caused by differential protein binding and renal clearance of the enantiomers rather than the activity of single enzymes. CE analysis of incubations with Supersomes provides an exciting tool to assess the enzyme(s) responsible for a metabolic step and the stereoselectivity of this process.

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