Interaction of chlormezanone enantiomers with rat liver microsomes

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SUMMARY

Both chlormezanone enantiomers, for the first time obtained by enantiospecific HPLC with a 100% yield, bind to oxidized cytochrome P-450 in rat liver microsomes with a binding curve according to type I, similar to hexobarbital but less pronounced. There are no differences between the binding curves of the two enantiomers. Ethylmorphine N-demethylation, ethoxycoumarin and ethoxyresorufin O-deethylation are inhibited by both chlormezanone enantiomers at 0.1–1 mM concentrations: no differences could be found. Luminol and lucigenin amplified chemiluminescence indicating the formation of reactive oxygen species was not influenced by either enantiomer in concentration ranges between millimolar and micromolar, whereas hydrogen peroxide formation was inhibited. NADPH/Fe stimulated lipid peroxidation was not influenced. Scavenger activity could not be demonstrated: the zymosan stimulated whole blood chemiluminescence was not influenced significantly.

INTRODUCTION

Chlormezanone (CM) is a well recommended, racemic, centrally-acting muscle relaxant, developed as early as 1958 (1). Using enantiospecific HPLC base-line separation of the enantiomers, recoveries of nearly 100% with a 1.0 g load per run and a purity of >99% chromatographically were achieved (2). All experiments are not influenced by the splitting of the S–C bond (2). However, the fate of this drug in the organism was successfully investigated only recently (3–5). According to these results, the S–C bond is split autoprotolytically, the participation of cytochrome P-450 could be excluded unequivocally. The primary products of cleavage, which could be isolated, are 4-chlorobenzaldehyde and 2-carboxyethane-sulfinic-acid-N-methylamide. The following reaction steps are controlled enzymatically, they are not cytochrome P-450 dependent. Up to 70% of the dose administered orally
appeared as 4-chlorohippuric acid, 5–10% as the glucuronide of 4-chlorobenzoic acid and a small amount as free 4-chlorobenzoic acid. The sulfur containing moiety appeared as unstable 2-carboxy-ethane-sulfinic-acid-N-methylamide, which was split at its amide bond. The enantiomers show an enantiospecific plasma protein binding, which may partly explain the different plasma half life times (Pt1/2) in human volunteers (47 h and 24 h). On the other hand, binding of racemic CM to cytochrome P-450 has been proven (4) and, as there are many examples of a different binding of enantiomers to different cytochrome P-450s (6,7), the binding of the two enantiomers to microsomal cytochrome P-450 and their interference with different mono-oxygenase and oxidase functions seemed to be worth investigating.

First we determined, with rat liver microsomes, the binding type and range of affinity of the CM enantiomers to oxidized cytochrome P-450 and its interaction with the mono-oxygenase reactions ethylmorphine N-demethylation and ethoxycoumarin and ethoxyresorufin O-deethylation and the oxidase function (formation of reactive oxygen species and of stimulated lipid peroxidation), to get information on enantiomer specific interactions with the binding and biotransformation of drugs and endogenous substances as a possible mechanism of unwanted side effects. Only weak interactions of racemic CM with the cytochrome P-450 system have been demonstrated in a previous paper (4). Inhibition of mono-oxygenase functions could be observed in high concentrations only. Uncoupling effects and scavenger activity could not be demonstrated for racemic CM (4); nevertheless, these aspects have also been investigated with both enantiomers, as great differences between enantiomers with respect to cytochrome P-450 interactions might be expected – one enantiomer even inhibiting the interaction of the other one at the cytochrome P-450 level, so that interactions of one enantiomer cannot be detected if racemates are investigated.

**MATERIALS AND METHODS**

**Chemicals**

CM (m.p. 113.3°C) was a kind gift from Sanofi Winthrop GmbH, Munich, Germany. The enantiomers were obtained by preparative HPLC using an OD-column 50mm x 500 mm (Daicell, Tokyo, Japan), UV detector at 229 nm, eluent n-hexane/EtOH (1/1, v/v), flow 70 ml/min. Yield was 100%, purity >99% (chromatographically): (+)-CM, m.p. = 154.0°C, [α]D20 = +50.4° (acetone), +31.3° (THF); (-)-CM, m.p. = 154.2°C, [α]D20 = -50.2° (acetone), -30.85° (THF).

Blauschke et al. (8) separated CM firstly by low pressure chromatography using microcrystalline cellulose triacetate as column material [eluent CH3OH/H2O (95/5, v/v)], yield: (+)-CM 65% and (-)-CM 54%, m.p. 153°C, [α]D22 = +49.3° and -49.6° (acetone), respectively.

**Animals and experiments**

Liver microsomes from 60-day-old male outbred Han:Wist rats were used. The animals were housed in plastic cages under standardized conditions (light-dark cycle 12/12 h, temperature 22 ± 2°C, humidity 55 ± 10%, pellet diet Altromin 1316 and water ad libitum) (9). The rats were decapitated under ether anesthesia, the livers were removed and homogenized in 0.1 M sodium phosphate buffer pH 7.4 (1:3, w/v). The homogenate was centrifuged at 9000 g for 20 min at 0°C. The supernatant was mixed with 25 mM magnesium chloride solution (1:3.5, v/v) and centrifuged at 9000 g for 20 min at 0°C. The sediment was washed and rehomogenized in sodium phosphate buffer at 4°C (10). Protein was determined with a modified Biuret reaction (11).

To determine the CM interaction with cytochrome P-450, spectral changes of cytochrome P-450 in microsomal suspension by CM (1 mM) were analysed using a Unicam UV/visible spectrometer UV4 (ATA Unicam, Cambridge, UK). Binding spectra of the two model substrates hexobarbital and aniline served as positive controls (12).

The influence of CM on different mono-oxygenase reactions of P-450 was investigated using different model reactions for different cytochrome P-450s: ethylmorphine N-demethylation (EMND) (13), ethoxycoumarin O-deethylation (ECOD) (14) and ethoxyresorufin O-deethylation (EROD) (15). 9000 g liver supernatant was used.

The influence of CM on the oxidative function of cytochrome P-450 was studied by measuring the NADPH/Fe**^3+**-dependent formation of reactive oxygen species (ROS) in liver microsomes. ROS were assayed with the luminol (LM) and lucigenin (LC) amplified chemiluminescence (CL) using a Berthold-AutoLumat LB 953 (Berthold GmbH & Co, D-7547 Wildbad, Germany). The reaction mixture contained iron, the amplifiers LM or LC, microsomes (0.5 mg protein/ml for LM-CL, 0.05 mg/ml for LC-CL) and sodium phosphate buffer pH 7.4. Addition of NADPH started a reduction of molecular oxygen to superoxide anion radical, which can dismutate spontaneously or be catalyzed by superoxide dismutase to form hydrogen peroxide (H2O2). Photon emission was measured over 3.5 min and given as relative light units (RLU) in counts per minute (CPM) per mg protein (16,17). The iron stimulated NADPH-dependent