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Investigation of the stereoselective in vitro metabolism of the chiral antiasthmatic/antiallergic drug flezelastine by high-performance liquid chromatography and capillary zone electrophoresis

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Abstract

An achiral HPLC method using a silica gel column as well as two independent chiral analytical methods by HPLC and capillary zone electrophoresis (CZE) were developed in order to investigate the in vitro metabolism of the racemic antiasthmatic/antiallergic drug flezelastine. The chiral HPLC analysis was performed on a Chiralpak AD column, which also allowed the simultaneous separation of the *N*-dephenethyl metabolite. The chiral separation by CZE was achieved by the addition of β-cyclodextrin to the run buffer. The stereoselectivity of the in vitro biotransformation of flezelastine was investigated using liver homogenates of different species. Depending on the species, diverse stereoselective aspects were demonstrated. The determination of the enantiomeric ratios of flezelastine and of *N*-dephenethylflezelastine after incubations of racemic flezelastine with liver microsomes revealed that porcine liver microsomes showed the greatest enantioselectivity of the biotransformation. (−)-Flezelastine was preferentially metabolized. After incubations with bovine liver microsomes the enantiomer of *N*-dephenethylflezelastine formed from (+)-flezelastine dominated. Incubations of the pure enantiomers of flezelastine with induced rat liver microsomes resulted in the stereoselective formation of a hitherto unknown metabolite, which was only detected in samples of (+)-flezelastine. Initial structure elucidation of the compound indicated that the new metabolite was most likely an aromatically hydroxylated derivative of the *N*-dephenethylflezelastine.

Keywords: Enantiomer separation; Flezelastine

1. Introduction

Flezelastine, 4-[(4-fluorophenyl)methyl]-2-[hexahydro-1-(2-phenylethyl)- 1H-azepin-4-yl]-1(2H)-phthalazinone, structure 1 in Fig. 1, is a new antiasthmatic/antiallergic drug, which was developed as the back-up candidate of the already marketed azelastine, structure 5 in Fig. 1 [1,2]. A further important property of the drug is the reversal of the multi-drug-resistance in the therapy with cytostatic drugs [3].

Due to its hexahydroazepinyl moiety, flezelastine (1) has a chiral centre. Both enantiomers have shown similar preclinical activity [1]. Therefore, the compound has been developed as the racemate.

Flezelastine (1) is metabolized in rat liver preparations to yield the *N*-dephenethyl derivative (2 in Fig. 1), the carboxylic acid metabolite (3 in Fig. 1), formed by oxidation and azepinyl ring opening, and the N-oxide (4 in Fig. 1).

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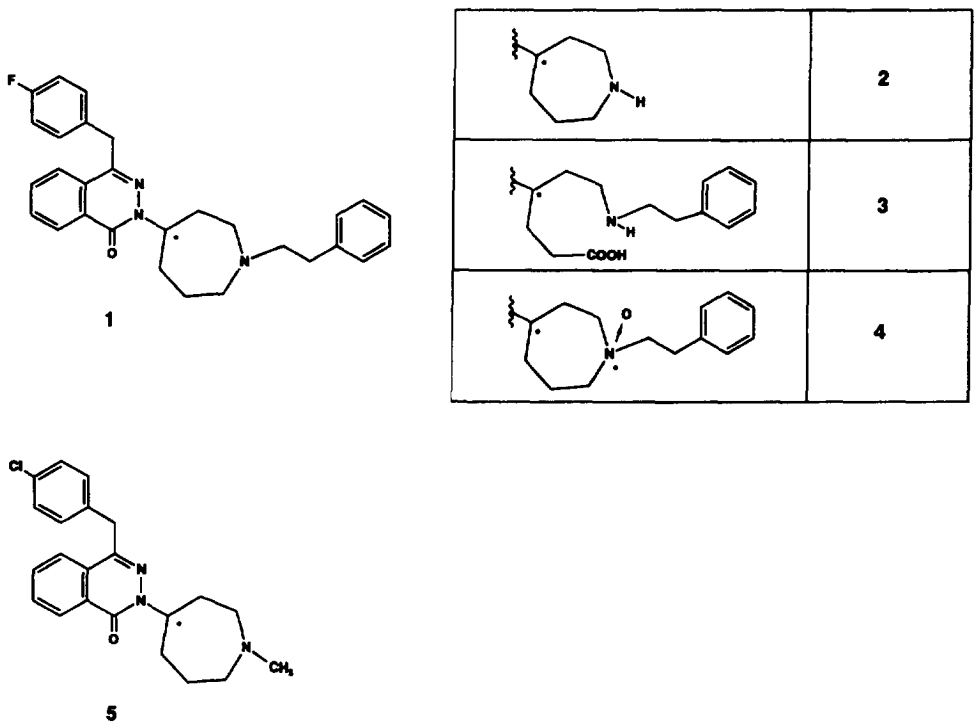

Fig. 1. Structures of flezelastine (1); *N*-dephenethylflezelastine (2); carboxy metabolite (3); N-oxide (4); internal standard azelastine (5).

Many chiral drugs exhibit an enantioselective metabolism. To date, there are no data available about the stereoselectivity of the phase I metabolism of the enantiomers of flezelastine.

The present paper describes an achiral HPLC method for the analysis of flezelastine (1) and its metabolites in biological samples. Furthermore, a chiral HPLC assay was used in order to determine the enantiomeric ratios of flezelastine (1) and *N*-dephenethylflezelastine (2) after incubations of racemic flezelastine with liver microsomes. Moreover, a chiral capillary zone electrophoretic method was developed.

2. Experimental

2.1. Chemicals

Racemic flezelastine (1) and its enantiomers as well as *N*-dephenethylflezelastine (2) and the carboxylic metabolite (3) were a gift from ASTA Medica (Frankfurt/Main, Germany). Methanol, *n*-hexane and isopropanol were of HPLC-quality (Baker, Gross-Gerau, Germany). All other chemicals were of analytical grade. The components of the NADPH-regenerating system were purchased from Fluka (Neu-Ulm, Germany). Buffer solutions were prepared in double-distilled, deionized water and filtered (0.22 μm).

2.2. Apparatus

The HPLC system consisted of a 110B Beckman-Solvent Delivery Module pump (Munich, Germany), a Rheodyne sample injector (Model 7125, Rheodyne), equipped with a 20-μl loop, a fluorescence detector Merck-Hitachi F-1050 (Darmstadt, Germany), a Merck-Hitachi variable-wavelength UV monitor 655A or a Merck-Hitachi L 3000 diode-array detector and a Merck-Hitachi D-2000 chromato-integrator.

For the CZE analysis a Beckman P/ACE system or a Grom 100 system were used with an uncoated

fused-silica capillary (50 μm I.D., 40 cm effective length). Samples were introduced into the capillary by pressure (P/ACE system) or hydrodynamic injection (Grom 100 system). In each case the injection volume was 10 nl. Prior to use and between runs the capillary was rinsed for 2 min with 0.1 *M* phosphoric acid and for 1 min with the cyclodextrin-containing buffer. The measurements were performed with an applied voltage of 17.5 kV (field strength 372 V/cm) at a temperature of 20°C. The compounds were detected by on-column measurement of the UV absorption at 210 nm.

The mass spectrometer was a Finnigan MAT TSQ 7000 (Bremen, Germany).

2.3. Achiral HPLC

The separation of flezelastine (1) and its metabolites was achieved on a LiChrospher Si-60 column (5 μm particle size, 250×4 mm I.D., Merck, Darmstadt, Germany), equipped with a LiChrospher Si-60 guard column (5 μm particle size, 4×4 mm I.D.). The mobile phase consisted of 0.033% (v/v) perchloric acid in methanol. The flow-rate was 0.5 ml/min for the first 17 min of each analysis. A flow-rate of 0.9 ml/min was applied throughout the remainder of the analysis.

For the assays a fluorescence detector (λ_{ex}=210 nm, λ_{em}=360 nm) was used.

The limit of detection was 125 μg/ml for flezelastine (1).

2.4. Chiral HPLC

Direct chromatographic resolution of flezelastine (1) and its metabolite *N*-dephenethylflezelastine (2) was achieved on a Chiralpak AD column (10 μm particle size, 250×4.6 mm I.D., Baker, Gross-Gerau, Germany), equipped with a guard column (10 μm particle size, 50×4.6 mm I.D.) containing the same material. The mobile phase consisted of *n*-hexane–2-propanol–diethyl amine (88:12:0.5, v/v), operated at a flow-rate of 1.0 ml/min.

The compounds were detected with an UV detector set at 292 nm.

The limit of detection was 140 μg/ml for flezelastine (1).

2.5. Chiral CZE

The enantioseparation of flezelastine (1) and *N*-dephenethylflezelastine (2) was achieved by the addition of 16.3 m*M* β-cyclodextrin to the run buffer consisting of 50 m*M* phosphate buffer, pH 3.75. The electrode buffer consisted of 100 m*M* phosphate buffer, pH 3.75. The samples were dissolved in 5 m*M* phosphate buffer, pH 3.75, containing 20% methanol.

The decrease in the concentration of the buffers led to field strength differences between the zones enhancing the enantioseparation. This method is called stacking.

The limit of detection was about 500 μg/ml.

2.6. In vitro biotransformation studies

For in vitro studies of the biotransformation of flezelastine (1) human, rat, bovine and porcine liver microsomes were used. The human liver microsomes were obtained from ASTA Medica. All other microsomes were prepared in our laboratories.

The fresh livers were homogenized at 4°C and fractionated by a classical set of centrifugation steps according to the method of Dayer [4]. Protein concentrations were determined by the method of Bradford [5] with bovine serum albumin as the protein standard.

Male Sprague–Dawley rats were induced with phenobarbitone (50 mg/kg for 6 days) prior to the preparation of the liver homogenates.

The incubations were carried out according to the following scheme: 50 μg (≈0.11 μmol) of racemic flezelastine (1) or of the enantiomers, 250 μl of the liver microsome preparations and 500 μl of the NADPH-regenerating system consisting of NADP, isocitrate, isocitrate dehydrogenase and magnesium chloride were transferred into glass tubes. The samples were vigorously stirred during the incubation period of 24 h at 37°C. Following the incubation the samples were adjusted to pH 10 by the addition of 0.1 *M* sodium hydroxide. 3 ml of cyclohexane–ethyl acetate (40:10, v/v) were added and mixed on a mechanical shaker for 10 min. The suspensions were centrifuged at 2500 *g* for 10 min and the organic layer was transferred into fresh glass tubes.

The process was repeated in order to complete the extraction. The combined organic layers were evaporated to dryness at room temperature under a stream of nitrogen.

For the achiral analysis samples of the internal standard azelastine (5) as a solution in methanol were added and also dried under nitrogen. The residue was dissolved in 200 μl methanol. 20 μl were injected into the HPLC system.

The chiral HPLC method was used without the addition of an internal standard. The residue was dissolved in 200 μl of the mobile phase and 20-μl aliquots were injected.

Due to the limited sensitivity in CZE, the residue was dissolved in 10 μl methanol and 40 μl 5 m*M* phosphate buffer, pH 3.75. 5–10 nl were introduced into the capillary.

2.7. Cytochrome P450 model system for the biomimetic monooxygenation

A 50-μg amount (≈0.14 μmol) of *N*-dephenethylflezelastine (2) was incubated in 3 ml of 0.1 *M* phosphate buffer, pH 6.0, containing Fe^{2+}, ascorbic acid and H_2O_2 for 3 h at 37°C. Following the incubation 2 ml of 1 *M* aqueous Na_2CO_3 were added and the samples were extracted with 4 ml of cyclohexane–ethyl acetate (40:10, v/v) for 10 min on a mechanical shaker. After centrifugation at 2500 *g* for 5 min the organic layer was transferred into glass tubes. The extraction step was repeated and the combined organic layers were dried at room temperature under a stream of nitrogen. The residue was dissolved in 100 μl methanol and 20-μl aliquots were injected into the achiral HPLC system.

2.8. Mass spectrometric experiments

The extracts of the microsomal incubations were injected either directly into the mass spectrometer for tandem MS analysis or separated by achiral HPLC coupled to the MS (LC–MS). Furthermore, fractions of the metabolites were collected after achiral chromatography and the mobile phase was evaporated to dryness under a stream of nitrogen. The fractions were analyzed by MS and LC–MS. The ionization was performed by electrospray (ESI, 20 eV).

3. Results and discussion

3.1. Achiral determination of flezelastine and its metabolites in in vitro incubations with liver microsomes (achiral assay)

The in vitro biotransformation of flezelastine was investigated using human, rat, bovine and porcine liver microsomes. The achiral analysis revealed that the microsomes of three species metabolized flezelastine (1) to the *N*-dephenethyl derivative (2), that the microsomes of all species metabolized 1 to the carboxylic acid metabolite (3) and to the N-oxide (4). Only after incubations with porcine liver microsomes was no formation of 2 observed.

The structures of the *N*-dephenethyl (2) and the carboxylic metabolite (3) were confirmed by tandem MS analysis of incubated samples in comparison to the references. The identity of the N-oxide (4) is discussed in Section 3.3.

The microsomes of the different species revealed quantitative differences in the formation of the metabolites. After incubations of racemic flezelastine with bovine and porcine liver microsomes the metabolites 3 and 4 dominated, whereas in incubations

Table 1
Distribution of the metabolites of flezelastine (1) after incubation of the pure enantiomers with liver microsomes of different species

Metabolites	Liver microsomes			
	Man	Rat	Bovine	Porcine
(+)-Flezelastine (1)				
2	+++	+++++	++	–
3	++++	++	+	++
4	+	++++	+++	+++++
A	–	+++	–	–
(−)-Flezelastine (1)				
2	++++	++++	+	–
3	+++	+++	++++	++++
4	++	++	++++	++++
A	–	–	–	–

with human and rat liver microsomes the preferential formation of 2 was observed.

To investigate the stereoselectivity of the metabolism of flezelastine the enantiomers were separately incubated. Table 1 presents the relative distribution of the metabolites after incubation of the enantiomers of flezelastine (1) with liver microsomes of different species.

Mainly quantitative differences in the formation of the metabolites were observed. For instance, the incubation of (+)-flezelastine (1) with human liver microsomes resulted in a higher formation of 3 and a lower formation of 2 and 4 compared to the incubation of (−)-flezelastine (1) (Fig. 2). The studies with phenobarbitone-induced rat liver microsomes showed also qualitative differences in the biotransformation of the enantiomers. A hitherto unknown metabolite

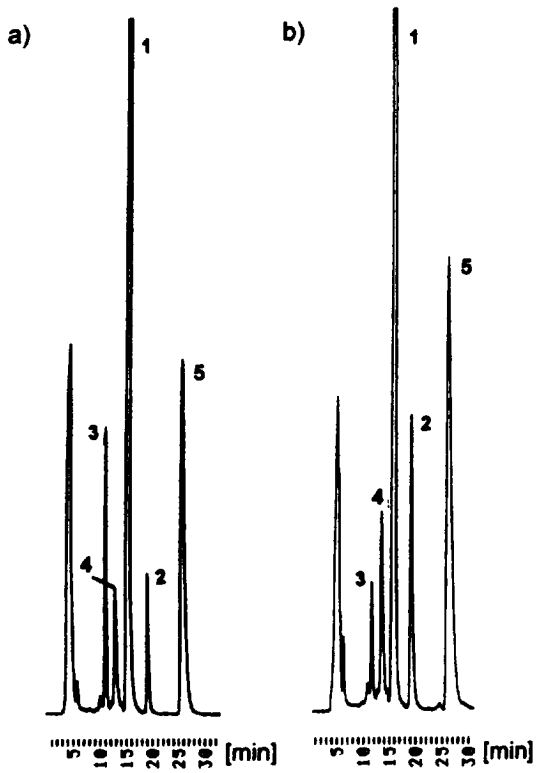

Fig. 2. Representative chromatograms of the achiral analysis after incubation of (a) (+)- and (b) (−)-flezelastine (1) with human liver microsomes. For chromatographic conditions, see Section 2.

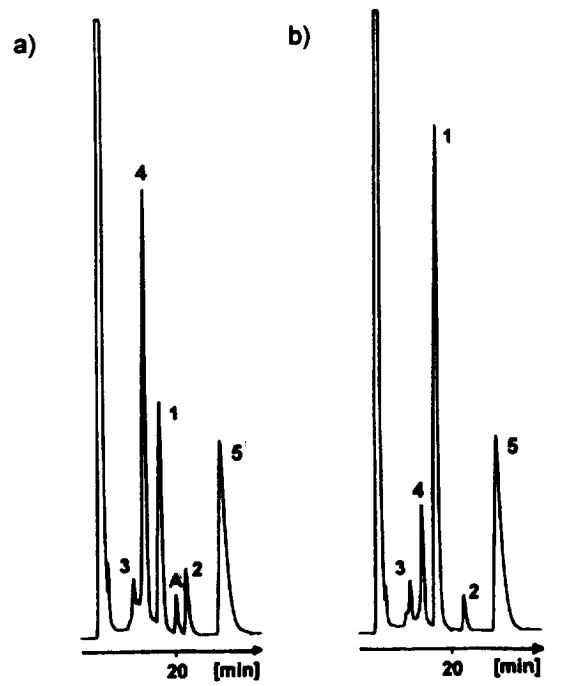

Fig. 3. Representative chromatograms of the achiral analysis after incubation of (a) (+)- and (b) (−)-flezelastine (1) with liver microsomes of phenobarbitone-induced rats. For chromatographic conditions, see Section 2.

A was only detected in incubations of racemic and (+)-flezelastine (1) (Fig. 3). It was formed in incubations of *N*-dephenethylflezelastine (2) as well.

3.2. Investigation of the structure of the new metabolite A

3.2.1. Diode-array detection

UV spectra recorded by the DAD detector revealed a bathochromic shift of the maximum in accordance with a phenolic hydroxylation (Fig. 4).

3.2.2. Cytochrome P450 model system for biomimetic monooxygenations

In order to get further evidence of the existence of the aromatically hydroxylated metabolite A, *N*-dephenethylflezelastine (2) was incubated with a cytochrome P450 model system, which executes preferentially phenolic hydroxylations. The reaction mix-

Fig. 4. DAD-UV spectra of *N*-dephenethylflezelastine (2) and metabolite A.

ture constituted a combination of the Udenfriend and the Fenton reaction systems [6]. The achiral HPLC analysis revealed that a product showing the same chromatographic behaviour as metabolite A was formed.

3.2.3. Mass spectrometric experiments

Mass spectrometric experiments were carried out with the fractions of the isolated metabolite A, with extracts of the incubations of *N*-dephenethylflezelastine (2) as well as with the fractionated product of the incubations of 2 with the cytochrome P450 model system. Similar mass spectra were obtained. They showed a molecular peak at *m/z* 368 (Fig. 5). This peak was 16 mass units higher than the molecular mass of 2. Therefore, a hydroxylated derivative of 2 was assumed. The fragment peak at *m/z* 271 (Fig. 5) indicated an aromatic hydroxylation in the phthalazinone moiety of the molecule. The non-hydroxylated fragment at *m/z* 254 was not found. Thus, hydroxylation in the hexahydroazepin ring or in the phenethyl side chain was excluded. The exact position of the hydroxy group in the phthalazinone ring could not be determined from the mass spectra.

3.3. Investigation of the structure of the flezelastine-N-oxide (4)

In order to confirm the structure of the flezelastine-N-oxide (4), a reference was synthesized [7]. The mass spectra of the reference and of the isolated metabolite from incubated samples of racemic flezelastine (1) were similar. A molecular peak at

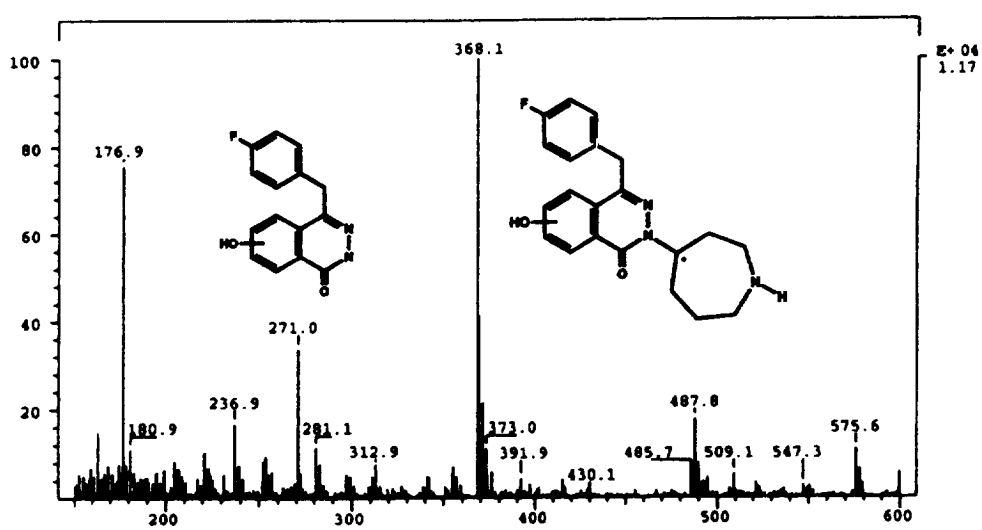

Fig. 5. Mass spectrum of the fraction of metabolite A after electrospray ionisation (ESI).

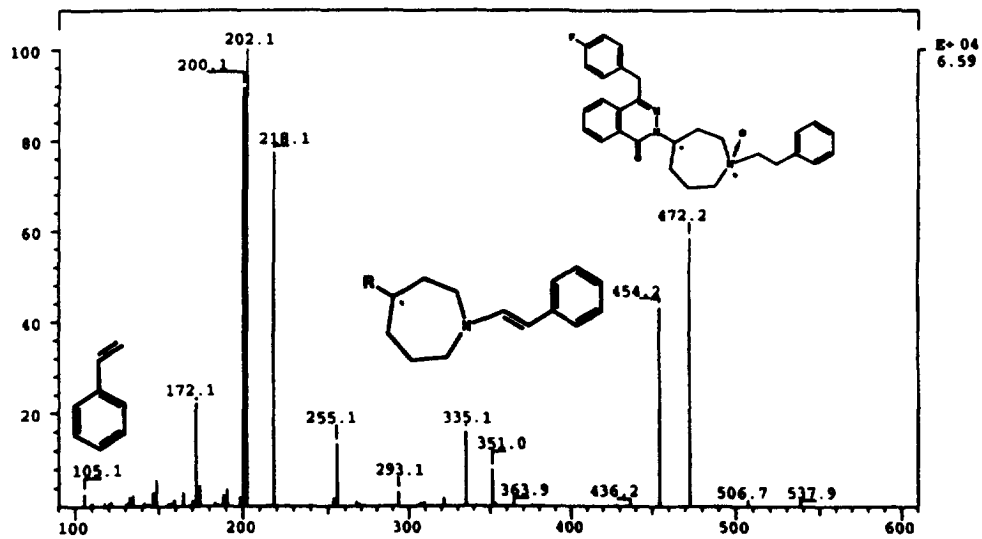

Fig. 6. Mass spectrum of the fraction of flezelastine-N-oxide (4) after electrospray ionisation (ESI).

m/z 472 was obtained (Fig. 6). The fragment peak at m/z 454 corresponded to dehydroflezelastine (Fig. 6), which resulted from the N-oxide (4) by Cope-elimination. The also possible phenolic hydroxylated derivative was excluded, as the recorded DAD-spectra did not reveal a bathochromic shift of the maximum.

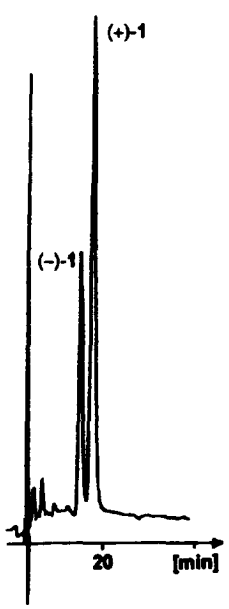

Fig. 7. Representative chromatogram of the chiral HPLC analysis after incubation of racemic flezelastine (1) with porcine liver microsomes. For chromatographic conditions, see Section 2.

3.4. Determination of the enantiomeric ratios of flezelastine (1) and N-dephenethylflezelastine (2) after incubations of racemic 1 with liver microsomes of different species (chiral assay)

3.4.1. HPLC

The determination of the enantiomeric ratios of flezelastine (1) and *N*-dephenethylflezelastine (2) after incubations of racemic 1 with liver microsomes of different species was performed on a Chiralpak AD column. Simultaneous enantioseparation of neither the carboxylic acid metabolite (3) nor of the N-oxide (4) was possible. Therefore, only the enantiomeric ratios of 1 and 2 were determined.

Rat and human liver microsomes did not metabolize flezelastine (1) stereoselectively. No preferential formation of 2 from either enantiomer of 1 was observed.

After incubations of racemic 1 with bovine liver microsomes stereoselective formation of 2 was observed. The enantiomer of 2 which resulted from (+)-1 dominated. Thus, the ratio of 2 from (−)-1 to 2 from (+)-1 was about 0.22 (±0.04).

The turnover of (−)-1 was faster after incubations with porcine liver microsomes. During the incuba-

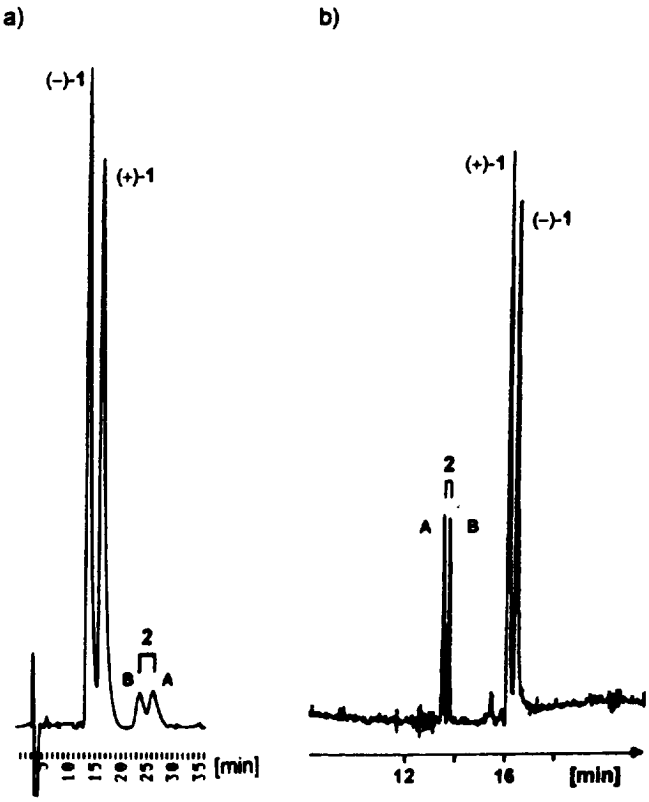

Fig. 8. Chiral separations. (a) HPLC, (b) CZE after incubation of racemic flezelastine (1) with human liver microsomes. For chromatographic (a) and electropherographic (b) conditions, see Section 2. 2A: from (+)-1, 2B: from (−)-1.

tion period between 6 h and 24 h the ratio of (−)-1 to (+)-1 was about 0.6 (±0.012). Thus, the porcine species showed the greatest stereoselectivity in the biotransformation of flezelastine (1). Fig. 7 presents a chromatogram after incubation (6 h) of racemic 1 with porcine liver microsomes.

3.4.2. CZE

To confirm the results of the chiral HPLC assay a CZE method with β-cyclodextrin as the chiral selector in the run buffer was employed.

Incubations of racemic flezelastine (1) with human and rat liver microsomes for a period of 24 h were analysed by CZE. The results confirmed the non-stereoselective metabolism of flezelastine in these two species obtained by HPLC analysis. Furthermore, the preferential turnover of (−)-1 in porcine liver microsomes and the stereoselective formation of 2 in bovine liver microsomes was also confirmed by CZE.

3.4.3. Comparison of the two analytical methods

Fig. 8 shows the chromatogram as well as the electropherogram of an incubation (24 h) of racemic flezelastine (1) with human liver microsomes.

The chiral HPLC analysis was influenced by matrix effects and possible coelution of peaks of different metabolites. This led to peak broadening and incomplete separations. However, the chiral CZE method yielded sharp peaks and more complete enantioseparations.

4. Conclusions

The in vitro biotransformation of flezelastine (1) showed species dependence. Incubations of racemic

1 with human, rat, bovine and porcine liver microsomes revealed that three species metabolized 1 to the *N*-dephenethyl metabolite (2), that all species metabolized 1 to the carboxylic acid metabolite (3) and to the flezelastine-N-oxide (4), but in each case to a different extent. Only after incubations with porcine liver microsomes was no formation of 2 observed.

Incubations of the pure enantiomers of flezelastine (1) with phenobarbitone-induced rat liver microsomes revealed a stereoselective formation of the new metabolite A, which was only detected in incubated samples of (+)-1. Elucidation of the structure using a cytochrom P450 model system, MS- as well as DAD-analyses confirmed the assumption of an aromatic hydroxylated derivative of *N*-dephenethylflezelastine (2).

Chiral analyses of incubations of racemic flezelastine (1) with liver preparations of different species were performed by HPLC as well as by CZE. Human and rat liver microsomes did not metabolize 1 stereoselectively. After incubations with porcine liver microsomes the turnover of (−)-1 was faster than the turnover of (+)-1. After incubations with bovine liver microsomes stereoselective formation of 2 was observed.

Because of its higher efficiency the CZE assay turned out to be more suitable for the analysis of the enantiomeric ratios in biological samples.

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