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Use of chiral liquid chromatography for the evaluation of stereospecificity in the carbonyl reduction of potential benzo[*c*]fluorene antineoplastics benfluron and dimefluron in various species

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Abstract

Benfluron (B) [5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluorene-7-one hydrochloride] is a potential antineoplastic agent. In the organism, B undergoes a rapid phase I biotransformation through oxidative and reductive metabolic pathways. The carbonyl reduction of B leads to reduced benfluron, red-B, this is one of the principal pathways for the deactivation of this compound.

The structure of B was modified to suppress its rapid deactivation *via* the carbonyl reduction on C_7. Dimefluron, D (3,9-dimethoxy-benfluron) is one of the derivatives of B, in which an alternative metabolic pathway (*O*-desmethylation) prevails over the carbonyl reduction.

The goal of this study was to develop HPLC methods enabling chiral separations of the red-B and -D enantiomers. The separation of red-B enantiomers was successful done on a Chiralcel OD-R column (250 mm × 4.6 mm ID, 5 μm) using a mobile phase acetonitrile–1 M $NaClO_4$ (40:60, v/v). Another mobile phase, methanol–1 M $NaClO_4$ (75:25, v/v), had to be employed for the sufficient resolution of red-D enantiomers. Flow rate was 0.5 ml min^{-1} in both cases. Red-B was detected at 340 nm, red-D at 370 nm.

The above chiral HPLC methods were used for the study of the biotransformation of B and D in the microsomal fractions of liver homogenates prepared from various species (rat, rabbit, pig, guinea pig, goat and human). The enantiospecificity of the respective carbonyl reductases was evaluated and discussed for both prochiral compounds, B and D.

Keywords: Chiral HPLC; Potential antineoplastics; Benfluron and dimefluron; Carbonyl reduction metabolites

1. Introduction

Drug metabolism studies belong to the fundamental items to be resolved in the research and development of new drugs. The metabolism of drugs is usually extensively studied with regards to their oxidation. Less attention has been paid to their reductive metabolism, although it is well known that a variety of pharmacologically important substances undergo reductive reactions in their biotransformation [1]. Carbonyl reaction is a significant step in the phase I biotransformation of a great variety of aromatics, alicyclic and aliphatic carbonyl compounds, including pharmacologically active substances [2,3]. Carbonyl reduction has always been shown to

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be of significance in various inactivation processes of drug containing a carbonyl group, such as warfarin, haloperidol, daunorubicin and doxorubicin [4,5].

Benfluron (B) [5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluorene-7-one hydrochloride], first prepared in the Research Institute for Pharmacy and Biochemistry in Prague, is a typical representant of the first generation of potential antineoplastic agents exhibiting the activity against a broad spectrum of experimental tumors in vitro and in vivo [6]. The metabolism of B has been studied extensively in vivo as well as in vitro [7–21]. B undergoes a rapid phase I biotransformation through oxidative and reductive metabolic pathways [8,12,16]. The carbonyl reduction of B leads to C_7-reduced benfluron, 5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol (red-B), this is one of the principal pathways for the deactivation of this compound [8,12,16,20,21].

In recent years, the chemical structure of B has been modified with the aim to suppress its disadvantageous pharmacokinetic properties (low accessibility from the gastrointestinal tract and strong binding to tissue proteins) and to prevent from deactivating through carbonyl reduction.

Dimefluron (D) [3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluorene-7-one hydrochloride], is one of the developed derivates of B, in which an alternative metabolic pathway (*O*-desmethylation) prevails over the carbonyl reduction, although the C_7-reduced metabolite, 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol (red-D), was also detected [20–24]. Higher antineoplastic efficiency of D (in comparison with B) could be expected in vivo [25–27] and the respective studies have been done or are currently in progress.

As mentioned above, both B as well as D undergoes in organism the metabolic changes leading to reduced derivatives. The substituents on the carbon atom bearing the carbonyl group are different, thus this carbon is a prochiral centre. The reduction of such a carbonyl group produces then two enantiomers of red-B (red-D, respectively). The carbonyl reductases tend to be stereospecific [4].

The goal of this study was to develop an HPLC method enabling chiral separations of the red-B and -D enantiomers. The enantiospecificity of the respective carbonyl reductases was evaluated and discussed for both prochiral compounds, B and D in various species.

2. Experimental

2.1. Chemicals, solutions and material

Benfluron [5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride], $C_{21}H_{20}ClNO_2$, MW =353.84 g mol^{-1}, racemic 5-(2-dimethylaminoethoxy)-7*H*-benzo-[*c*]fluoren-7-ol hydrochloride $C_{21}H_{22}ClNO_2$, MW = 355.86 g mol^{-1}, dimefluron [3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-one hydrochloride]; $C_{23}H_{24}ClNO_4$, MW = 413.89 g mol^{-1}, racemic 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol hydrochloride, $C_{23}H_{26}ClNO_4$, MW = 415.91 g mol^{-1} (see Fig. 1) were synthesized in the laboratories of Institute of Experimental Biopharmaceutics and characterized using NMR experiments. Acetonitrile (ACN), chloroform, methanol (MeOH), ethanol (EtOH), hexane (Hex), 2-propanol, triethylamine (HPLC grade, Merck, Darmstadt, Germany), diethyl ether (analytical grade, Merck, Germany), hydrochloric acid 35%, sodium perchlorate (analytical grade, Lachema, Czech Republic), sodium borohydride (powder, 98+%, Janssen-Chimica, Belgium), ultra-high-quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga Ltd., Bucks, England) were used for the liquid–liquid extraction of biomatrices and chiral chromatography. Kieselgel 60H (Merck, Darmstadt, Germany) was used for preparative TLC.

2.2. Preparation of synthetic rac. 5-(2-dimethylaminoethoxy)-7H-benzo[c]fluoren-7-ol and rac 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7H-benzo[c]fluoren-7-ol

Base of benfluron (0.84 g, 2.65 mmol) or dimefluron (1 g, 2.65 mmol) was dissolved in methanol (40 ml). Sodium borohydride (0.3 g, 7.94 mmol) was added and the reaction mixture was stirred under ambient temperature for 1 h and then refluxed for half of an hour. Methanol was removed under reduced pressure, the residue was diluted by 5% aqueous NaOH (20 ml), and C_7-reduced dimefluron was extracted into ethyl acetate. The organic layer containing the product was dried over anhydrous Na_2SO_4. After ethyl acetate removal, the crude base of rac. 3,9-dimethoxy-5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol (base of rac. 5-(2-dimethylaminoethoxy)-7*H*-benzo[*c*]fluoren-7-ol, respectively) was obtained.

Preparative thin-layer chromatography was used for isolation of red-B and -D from reaction mixtures. Chromatographic layers of Kieselgel 60H (0.3 mm thick on 20×20 glass plates) were prepared from a suspension in triethylamine-methanol 1:1 (v/v) using a Camag Automatic TLC Plate Coater (Muttenz, Switzerland) and left to dry for about 20 min. The reaction mixture was dissolved in a minimum volume of the mobile phase and the solution was transferred onto the start of a chromatographic plate and developed in a chloroform–methanol–triethylamine mixture (80:10:5, v/v). After chromatography, the individual fluorescence bands appertaining to red-B or -D were scraped off from the silica gel. Each individual purified product was eluated from silica gel with methanol using a sintered-glass filter (No. 4, porosity 40 μm) and filtrate was evaporated to dryness. The identity of the compounds was confirmed by NMR analysis.

A Varian Mercury-Vx BB 300 NMR spectrometer was used for the NMR analyses of the synthetic standards of red-B and -D metabolites. The NMR spectra were recorded at 300 MHz for ^{1}H, and 75 MHz for ^{13}C. Chemical shifts are given as δ values in ppm, the coupling constants are given in

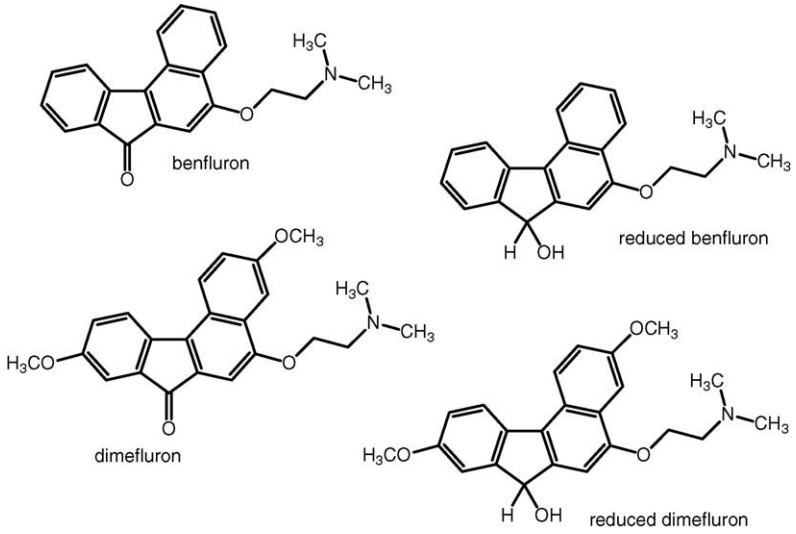

Fig. 1. Structures of the compounds under study.

Hz. Analytical sample (15–20 mg) was dissolved in deuterated dimethylsulfoxide (0.7 ml) and the solution was transferred *via* pipette into an NMR tube (203 mm length, 5 mm diameter).

2.2.1. NMR analysis of red-B

^{1}H NMR (300 MHz, DMSO) δ 8.65 (1H, d, J = 8.51 Hz, H1), 8.28 (1H, dd, J = 8.51 Hz, J = 1.10 Hz, H4), 8.20 (1H, d, J = 7.69 Hz, H11), 7.73–7.65 (1H, m, H2), 7.62 (1H, d, J = 7.69 Hz, H8), 7.60–7.53 (1H, m, H3), 7.46–7.38 (1H, m, H10), 7.31–7.22 (2H, m, H6, H9), 5.90 (1H, d, J = 7.42 Hz, OH), 5.47 (1H, d, J = 7.41 Hz, H7), 4.39–4.25 (2H, m, OCH_2), 2.83 (2H, t, J = 5.49 Hz, NCH_2), 2.30 (3H, s, CH_3).

^{13}C NMR (75 MHz, DMSO) δ 154.6, 147.6, 147.3, 140.6, 129.5, 128.7, 127.8, 126.1, 125.6, 125.6, 125.2, 124.7, 124.0123.1, 121.7, 102.8, 74.3, 66.9, 57.9, 45.9.

2.2.2. NMR analysis of red-D

^{1}H NMR (300 MHz, DMSO) δ 8.51 (d, 1H, J = 9.34 Hz, H1), 8.04 (d, 1H, J = 8.51 Hz, H11), 7.59 (d, 1H, J = 2.75 Hz, H4), 7.30 (dd, 1H, J = 9.34 Hz, J = 2.75 Hz, H2), 7.22 (s, 3H, H6), 7.20 (d, 1H, J = 2.61 Hz, H8), 6.94 (dd, 1H, J = 8.51 Hz, J = 2.61 Hz, H10), 5.87 (bs, 1H, OH), 5.39 (s, 1H, CH), 4.34–4.23 (m, 1H, OCH_2), 3.88 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 2.82 (t, 2H, J = 5.27 Hz, NCH_2), 2.30 (s, 6H, NCH_3).

^{13}C NMR (75 MHz, DMSO) δ 158.1, 156.7, 152.8, 149.9, 143.6, 133.3, 126.9, 126.6, 125.7, 124.2, 122.1, 119.1, 113.5, 111.2, 103.6, 102.0, 74.1, 67.1, 57.9, 55.5, 55.2, 45.9.

2.3. Biological material

Young adult intact males, sexually matured, 3–5 animals from each species, were used for experiments. All animals used were healthy and have not been subjected to any pharmacological treatments. Wistar rats (*Rattus novergicus* var. *alba*, 10–14 weeks old) and guinea pigs (*Cavia aperea* var. *porcellus*, weight 450–500 g) were fed a standard diet and sacrificed by decapitation under ether anaesthesia. Rabbits (*Oryctolagus cuniculus* f. *domesticus*, Chinchilla race) were fed a standard diet and sacrificed by cervical spine dislocation at the age of 4 month. Farm animals, boars (*Sus scrofa* f. *domestica*, Landrace breed) and bucks (*Capra aegagrus* f. *hircus*, Czech white goat breed), were bred under the usual farm conditions in Czech Republic. They were slaughtered at the age of 7–9 months. All species were subjected to an overnight starvation with free access to tap water. The liver (without any macroscopic alterations) was removed immediately after death of animal. The whole liver or *lobus sinister* or *dexter lateralis* were cut into small pieces, and stored frozen in liquid nitrogen. The human liver samples from three male donors (49, 56 and 64 years old) were obtained from the Cadaver Donor Programme of Transplant Centre of Faculty of Medicine, Charles University, Hradec Králové. Cut part of liver (*lobus hepatis sinister*) in ice-cooled Eurocollins solution was transported from the hospital to the laboratory and after that it was stored in the freezer (−80 °C) till preparation of subcellular fractions.

2.4. Preparation of subcellular fractions

Frozen liver samples were thawed at room temperature (up to 15 min) and homogenised at the w/v ratio of 1:6 in 0.1 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogeniser and sonication with Sonopuls (Bandeline, Germany). The microsomal fractions were isolated by fractional ultracentrifugation of the liver homogenate with the same buffer. A re-washing step (followed by a second ultracentrifugation) was included at the end of the microsome preparation procedure. Microsomes were finally resuspended in the same buffer containing 20% glycerol (v/v) and were stored at −80 °C. Protein concentrations were assayed using the

bicinchoninic acid method [28]. Concentrations of protein ranged between 6 and 8 mg ml^{-1}.

2.5. Incubation of hepatic microsomal fraction with benfluron and dimefluron

Suitable conditions for biotransformation assays (substrate and coenzyme concentrations, time of incubation) were chosen according to previous experiments [23]. The microsomal or cytosolic suspensions (0.1 ml in 0.1 M Na-phosphate buffer, pH 7.4) were incubated with 0.6 μmol of coenzymes (mixture NADPH and NADH) and with 0.5 μmol of substrates (benfluron or dimefluron, respectively). The total volume of reaction mixtures was 0.3 ml. First type of blank samples did not contain substrates, while in second types of blanks the buffer was added instead of microsomes. The incubations were carried out at 37 °C for 30 min under aeration and were terminated by addition of 0.1 ml of 26% aqueous ammonia solution.

2.6. Sample preparation of the incubates

When the in vitro enzymatic reaction (see Section 2.5) was accomplished by addition of aqueous ammonia solution, ethyl acetate (0.7 ml) was added into the incubation mixture and the content of the tube was vortexed-mixed for 1 min. After centrifugation (2000 × *g*, 12 min), the tubes were stored in a deep freezer (−75 °C for 30 min) until the lower aqueous layer froze to ice. The upper ethyl acetate layer containing the analytes was decanted into another clean 3-ml tube. The extraction of aqueous layer was repeated once more with the same volume of ethyl acetate as described above. Both ethyl acetate extracts were poured together and the solvent was evaporated (water bath 45 °C, stream of nitrogen). The dry extract in the glass tube was reconstituted in 600 μl of the mobile phase and transferred into the vial of autosampler. An amount of 100 μl of the sample was injected into the chromatographic column.

2.7. Chiral chromatography of benzo[c]fluorene derivatives in the incubation mixtures

Routine chromatographic analyses were performed using a Thermo Electron (formerly Thermo Finnigan) chromatograph (San Jose, CA, USA). The chromatographic system was composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100 μl sample loop, UV6000 LP photodiode array detector with Light Pipe Technology, SN4000 system controller and a data station with the ChromQuest 4 analytical software (Thermo Electron, Inc., San Jose, CA, USA) working under the Windows 2000 operating system (Microsoft Corporation). During the development of an HPLC method, a polarimetric detector Chiralyser (IBZ Meβtechnik, Hannover, Germany) was used for identification of (+)- and (−)-enantiomer of reduced metabolites of benfluron and dimefluron.

A polysaccharide, cellulose-based reversed phase chiral column 250 mm × 4.6 mm packed with Chiralcel® OD-R, 10 μm (Daicel Chemical Industries, Ltd., Tokyo, Japan), precolumn LiChroCART® 4-4 (Merck, Darmstadt, Germany) with a C-18 achiral reversed phase.

A mixture of 1 M aqueous $NaClO_4$ and acetonitrile (60:40, v/v) was applied to the separation and determination of benfluron and its phase I metabolites, including C_7-reduced benfluron (red-B) enantiomers. Benfluron and its metabolites were separated over 50 min.

Likewise, a mixture of 1 M aqueous $NaClO_4$ and methanol (25:75, v/v) was applied to the separation and determination of dimefluron and its phase I metabolites, inclusive of C_7-reduced dimefluron (red-D) enantiomers. Dimefluron and its metabolites were separated over 90 min.

Flow rate was 0.5 ml min^{-1} in both cases. Red-B was detected at 340 nm, red-D at 370 nm.

2.8. Calibration

Standard 10^{-3} M stock solutions [31.9 mg of red-B (base) and 37.9 mg of red-D (base)] and each in 100 ml of the respective UHQ-water solution containing equimolar amount of hydrochloric acid were prepared. Lower concentrations of each compound were obtained by dilution with UHQ water. A calibration series of C_7-reduced benfluron (C_7-reduced dimefluron, respectively) with the concentrations 2.5, 10.0, 17.5, 25.0, and 35.0 nmol ml^{-1} of each analyte was made. All points of calibration curve were made in six replicates. This calibration series was measured using the UV detector under the condition mentioned in Section 2.7 and evaluated using an external standard method.

2.9. Testing and statistical evaluation of the analytical procedure

On-line statistical processing of the calibration analyses by the least-squares method was performed automatically using the ChromQuest 4, software. The linearity of the calibration curve from the aqueous solutions of rac. C_7-reduced benfluron (rac. C_7-reduced dimefluron, respectively) was tested and evaluated for each of the enantiomers [$y = kx + q$, where x is the half concentration of rac. C_7-reduced benfluron (rac. C_7-reduced dimefluron) and y the corresponding peak-area of each enantiomer of C_7-reduced benfluron (C_7-reduced dimefluron). The correlation coefficient (r) was also expressed. The accuracy was determined as a relative error (%) found on the standard curve. The precision of the method, expressed as the relative standard deviation (percentage of coefficients of variation; R.S.D. = 100 S.D./mean), was also assessed. Both statistical parameters were calculated for each concentration level. The range of the applicability of the HPLC method was enclosed within the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). The lower limit of quantification (LLOQ) was determined as the lowest concentration on the standard calibration curve, which was measured

with a precision of 20% and accuracy of 80 or 120%. Upper limit of quantification (ULOQ) was equal to the highest concentration in the 5-level calibration. The recoveries of individual enantiomers of red-B and -D were also calculated [29,30].

3. Results and discussion

3.1. Chiral chromatography of benzo[c]fluorene derivatives

The carbonyl reduction of a prochiral 7*H*-benzo[*c*]fluorene-7-one derivative (B or D) leads to the formation of two enantiomers. Their enantiomeric ratio is dependent on the conditions of the respective reaction. Reduction of B or D using sodium borohydride in methanol (see Section 2.2) gave a racemic mixture of both enantiomers (enantiomeric ratio 1:1), because the access of hydride anion to the electrophilic carbon of carbonyl group is possible from both sides of 7*H*-benzo[*c*]fluorene-7-one molecule with the same probability.

On the other hand, in an enzymatically catalyzed reduction, when a planar 7*H*-benzo[*c*]fluorene-7-one molecule is fixed on the surface (or in some cavity) of apoenzyme and the prochiral carbonyl is accessible preferentially from one side of the planar molecule, the enantiomeric ratio could be shifted in favour of one of the enantiomers. These differences could be observed using chiral separation methods.

For these purposes, suitable chiral chromatographic columns (chiral selectors) had to be searched for and new bioanalytical chiral HPLC methods were developed, validated and used for the evaluation of interspecies differences in the stereospecificity of reductive metabolism of B and D.

Eleven various chiral columns were tested in the development of the optimal conditions for enantiomeric separations of red-B and -D. Solutions of racemic red-B ($c = 0.2\ mg\ ml^{-1}$) and racemic red-D ($c = 0.4\ mg\ ml^{-1}$) in 2-propanol were injected into the chiral column and the resolution factor was

Table 1
Results of the enantioselective separation of red-B

Column	Parameters	Particle size (μm)	Mobile phase (v/v)	Flow rate (ml min^{-1}), temperature (°C)	Commentary
Chiralcel OD-R	250 mm × 4.6 mm	10	ACN–1 M $NaClO_4$ (60:40)	0.5, 40	Partly separated
			ACN–1 M $NaClO_4$ (40:60)	0.5, 40	Baseline separated, analysis time about 20 min
			ACN–1 M $NaClO_4$ (40:60)	0.5, 25	Baseline separated; optimal condition
			ACN–0.5 M $NaClO_4$ (40:60)	0.5, 40	Decrease of resolution
			ACN–0.25 M $NaClO_4$ (40:60)	0.5, 40	Decrease of resolution
			ACN–0.5 M $NaClO_4$ (39:61)	0.5, 40	Baseline separated, analysis time about 20 min
			ACN–0.5 M $NaClO_4$ (30:70)	0.5, 40	Baseline separated, analysis time about 30 min
Chiralcel OD-H	250 mm × 4.6 mm	10	Hex–2-propanol (90:10)	1.0, 40	No separation, analysis time about 23 min
			Hex–2-propanol (75:25)	1.0, 40	No separation, analysis time about 8.5min
			Hex–EtOH (80:20)	1.0, 30	No separation, analysis time about 13 min
Chiralcel OB-H	250 mm × 4.6 mm	10	Hex–2-propanol (65:35)	0.7, 40	No separation, analysis time about 22 min
Caltrex Chiral R IV	250 mm × 4.0 mm	5	Hex–EtOH (40:60)	0.9, 40	No separation
Caltrex Chiral R VIII	250 mm × 4.0 mm	5	Hex–EtOH (30:70)	0.7, 40	No separation
Caltrex Chiral R X	250 mm × 4.0 mm	5	Hex–2-propanol (75:25)	1.0, 30	No separation
Chiral AGP	100 mm × 4.0 mm	5	12 mM Na_2HPO_4–2-propanol (95:5) pH = 7.03	0.8, 25	No separation
			12mM Na_2HPO_4–MeOH (88:12) pH = 7.03	0.6, 25	Partly separated
Chiral HSA	150 mm × 4.0 mm	5	12mM Na_2HPO_4–MeOH (88:12) pH = 7.03	0.6, 25	No separation
(R.R) Whelk-0-1	250 mm × 4.0 mm	5	Hex–2-propanol (60:40)	1.0, 40	No separation
Kromasil KR 100-5CHI – DMB	250 mm × 4.6 mm	5	Hex–2-propanol (60:40)	1.0, 40	No separation, analysis time about 3.8 min
	250 mm × 4.6 mm	5	Hex–EtOH (95:5)	1.0, 40	No separation, analysis time about 3.8 min
Chiraspher	250 mm × 4.0 mm	5	Hex–EtOH (50:50)	0.6, 30	No separation
			Hex–EtOH (75:25)	0.6, 30	No separation

Table 2
Results of the enantioselective separation of red-D

Column	Parameters	Particle size (μm)	Mobile phase (v/v)	Flow rate (ml min^{-1}), temperature (°C)	Commentary
Chiralcel OD-R	250 mm × 4.6 mm	10	ACN–1 M $NaClO_4$ (40:60)	0.5, 40	No separation
			Gradient elution*	0.5, 40	Without effect
			EtOH–1 M $NaClO_4$ (29:71)	0.5, 25	Partly separation, low resolution
			(MeOH–EtOH (7:3))–1 M $NaClO_4$ (75:25)	0.5, 40	Partly separation, low resolution
			(MeOH–EtOH (8.5:1.5))–1 M $NaClO_4$ (75:25)	0.5, 40	Partly separation, low resolution
			(MeOH–EtOH (9:1))–1 M $NaClO_4$ (75:25)	0.5, 40	Partly separation, low resolution
			MeOH–1 M $NaClO_4$ (75:25)	0.5, 40	Sufficient separation
			MeOH–1 M $NaClO_4$ (75:25)	0.5, 25	Sufficient separation, optimal condition
Chiralcel OD-H	250 mm × 4.6 mm	10	Hex–2-propanol (70:30)	1.0, 40	No separation
			Hex–2-propanol (75:25)	1.0, 40	No separation
			Hex–EtOH (80:20)	0.8, 30	No separation
Chiralcel OB-H	250 mm × 4.6 mm	10	Hex–2-propanol (60:40)	0.5, 40	No separation
			Hex–2-propanol (75:25)	0.5, 40	No separation
Caltrex Chiral R IV	250 mm × 4.0 mm	5	Hex–EtOH (40:60)	0.9, 40	No separation
Caltrex Chiral R VIII	250 mm × 4.0 mm	5	Hex–EtOH (30:70)	0.7, 40	No separation
Caltrex Chiral R X	250 mm × 4.0 mm	5	Hex–2-propanol (75:25)	1.0, 30	No separation
			Hex–2-propanol (10:90)	1.0, 30	No separation
Chiral AGP	100 mm × 4.0 mm	5	10 mM CH_3COOH, pH = 4.51	0.8, 25	No separation
			10 mM CH_3COOH–2-propanol (95:5) pH = 4.51	0.8, 25	No separation
			12mM Na_2HPO_4–2-propanol (95:5) pH = 6.58	0.8, 25	No separation
			12mM Na_2HPO_4–ACN (82:18) pH = 7.03	0.6, 25	No separation
			12 mM Na_2HPO_4–ACN (95:6) pH = 6.58	0.6, 25	No separation
			12 mM Na_2HPO_4–MeOH (82:18) pH = 6.58	0.6, 25	No separation
Chiral HAS	150 mm × 4.0 mm	5	12mM Na_2HPO_4–MeOH (82:18) pH = 7.03	0.6, 25	No separation
(R.R) Whelk-0-1	250 mm × 4.0 mm	5	Hex–2-propanol (95:5)	1.0, 40	No separation
			Hex–2-propanol (60:40)	1.0, 40	No separation
			Gradient elution**	1.0, 40	Without effect
Kromasil KR 100-5CHI – DMB	250 mm × 4.6 mm	5	Hex–2-propanol (40:60)	1.0, 40	No separation
			Hex–2-propanol (60:40)	1.0, 40	No separation
			Hex–2-propanol (90:10)	1.0, 40	No separation
			Hex–EtOH (95:5)	0.7, 40	No separation
Chiraspher	250 mm × 4.0 mm	5	Hex–EtOH (50:50)	0.6, 30	No separation
			Hex–EtOH (75:25)	0.6, 30	No separation

* 0–20 min ACN–1 M $NaClO_4$ (30:70, v/v); 20–40 min ACN–1 M $NaClO_4$ (10:90, v/v); 40–50 min ACN–1 M $NaClO_4$ (10:90, v/v); 50–70 ACN:1 M $NaClO_4$ = 30:70; 70–90 min ACN–1 M $NaClO_4$ (50:50, v/v); 90–120 min ACN–1 M $NaClO_4$ (50:50, v/v).
** 0 min Hex–2-propanol (100:0, v/v); 30 min Hex–2-propanol (0:100, v/v).

evaluated as a principal criterion for the selection of suitable conditions of enantioselective separation of red-B and -D. The results are shown in Table 1 for red-B and in Table 2 for red-D, respectively.

The best separation of enantiomers in racemic red-B were achieved on a Chiralcel OD-R column, with cellulose tris (3,5-dimethylphenylcarbamate) as chiral selector using a mobile phase acetonitrile–1 M aqueous $NaClO_4$ (40:60, v/v). Flow rate was 0.5 ml ml^{-1} and the separation was performed at ambient temperature (see Fig. 2). Increase of the temperature led to the acceleration of analysis but the resolution of enantiomers was worse. An analogous effect was observed after the decrease of natrium perchlorate concentration in the mobile phase. From the whole spectrum of chiral selectors (see Tables 1 and 2), also the AGP-column seemed to give promising results, but the peak symmetry and resolution was not sufficient on this column. The separation properties of the other columns tested were not appropriate for our purpose.

Reduced dimefluron differs from the structure of reduced benfluron by the presence of two methoxy groups on C_3 and

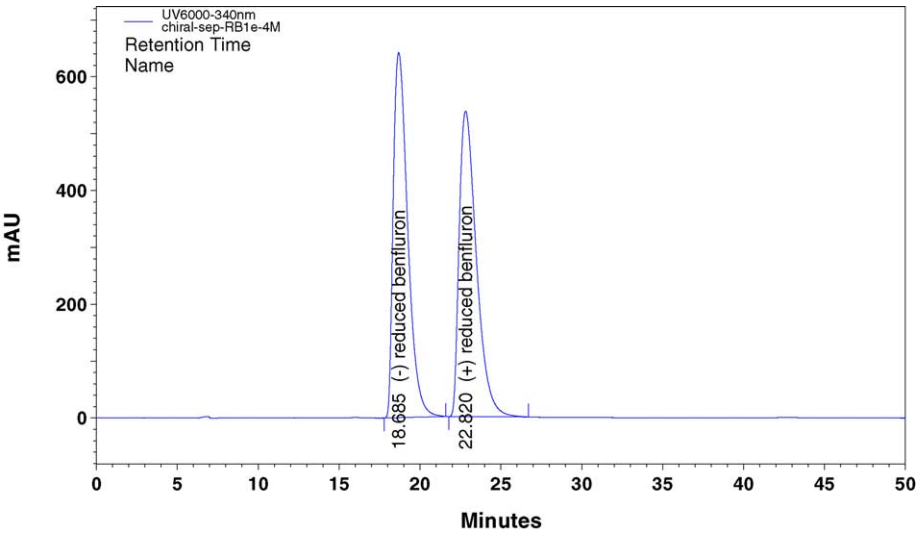

Fig. 2. Chiral separation of red-B under optimal conditions (see Table 1).

C_9 carbons. These methoxy groups seems to be promising for the pharmacokinetic and xenobiochemical properties [22,23] but the chiral separation of red-D is negatively influenced by this substitution. When the same conditions as for red-B had been applied on chiral separation of red-D, the separation of red-D enantiomers failed. The organic component of mobile phase was by sequel replaced with ethanol and then with methanol. In first case, the enantiomers were only in part resolved. Better results were achieved with methanol as an organic modifier. The mobile phase was then optimised to the final composition methanol–1 M aqueous $NaClO_4$ (75:25, v/v); flow rate was 0.5 ml min^{-1} at ambient temperature (see Fig. 3).

The peak identity and purity was tested using UV6000 diode-array detector, the same very characteristic UV spectra were obtained for both enantiomers of each compound (red-B or -D). Optical rotation was characterized using a Chiralyser polarimetric detector. (−)-Enantiomer of both compounds left the column as first (t_R = 18.685 min for red-B, t_R = 20.405 min for red-D) followed by (+)-enantiomer (t_R = 22.820 min for red-B, t_R = 22.702 min for red-D).

Although only the percentual enantiomeric ratio of red-B (red-D, respectively) after the incubation of the microsomal fractions of various species was evaluated, the validation of an external standard method for both enantiomers of red-B (red-D, respectively) was performed. The calibration curve was found to be linear [$y = 1.43 \times 10^5 x – 8.4 \times 10^4$ for (−)-enantiomer of red-B; $y = 1.43 \times 10^5 x – 8.9 \times 10^4$ for (+)-enantiomer of red-B; $y = 9.89 \times 10^5 x – 7.2 \times 10^4$ for (−)-enantiomer of red-D; $y = 1.09 \times 10^5 x – 5.7 \times 10^4$ for (+)-enantiomer of red-D] in the range of 5.0–17.5 nmol ml^{-1} with

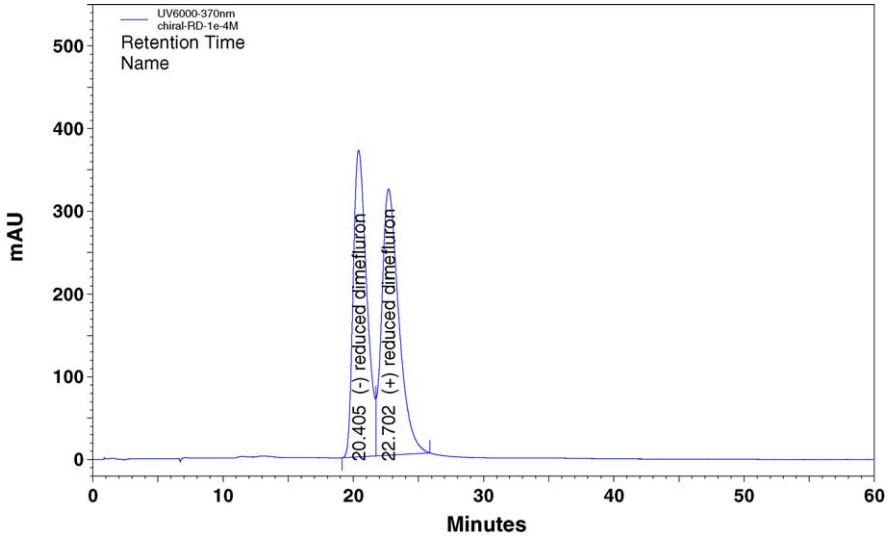

Fig. 3. Chiral separation of red-D under optimal conditions (see Table 2).

$r = 0.999$ for both red-B and -D. The accuracy and precision for red-B and -D ranged in the intervals 81.9–103.7% and 0.04–4.0%. LLOQ for red-B (red-D) was 2.5 nmol ml^{-1}. ULOQ was in both cases 17.5 nmol ml^{-1}. Recovery ranged in the interval (72.9–74.5%).

3.2. Enantiomeric ratios of red-B and -D in the incubation mixtures of various species

The extracts from the incubation mixtures were analysed under the optimal conditions for each enantiomer. Differences among species in the metabolism of both compounds (B and D) were observed. The carbonyl reduction of B led predominantly to (+)-red-B in all species except rat. The amount of (−)-red-B was inconsiderable in comparison with (+)-enantiomer, whereas in the metabolisms of rats prevailed (−)-red-B. In some cases, the enantiomeric excess was not specified exactly, because some small amounts of interfering peaks were co-eluted with red-B (see Table 3).

Table 3
Comparison of the enantiomeric excess of red-B

Species	(−)-Red-B	(+)-Red-B	e.e. (−)-red-B vs. (+)-red-B
Red-B			
Rat	Prevalent	Minor	90.2% vs. 9.8%
Guinea pig	Minor	Prevalent	–
Rabbit	Not detected	Prevalent	0% vs. 100%
Goat	Minor	Prevalent	–
Pig	Minor	Prevalent	–
Human	Not detected	Prevalent	0% vs. 100%

Different results have been found out for red-D. Rat metabolized B mainly to (−)-red-B as D was metabolized to (+)-red-D. More interesting was the comparison of metabolites by rabbits, B was converted completely to the (+)-red-B

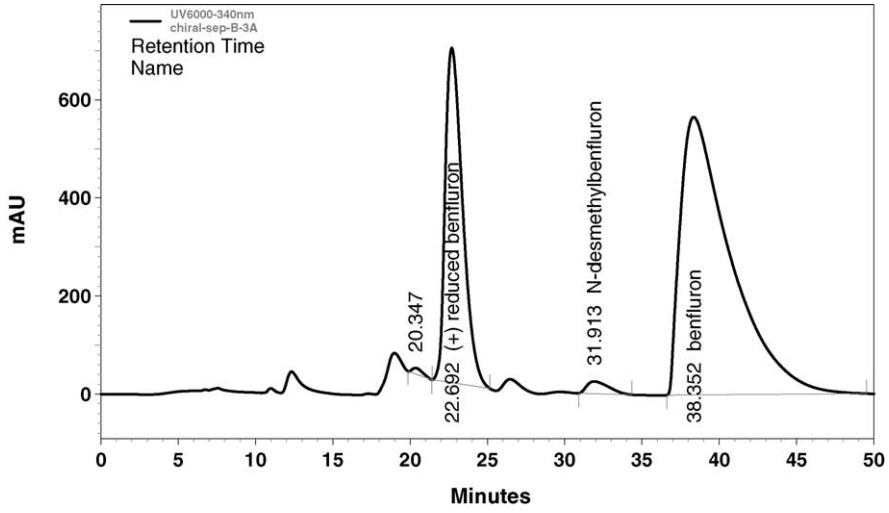

Fig. 4. Chromatogram of benfluron after incubation in rabbit's microsomes.

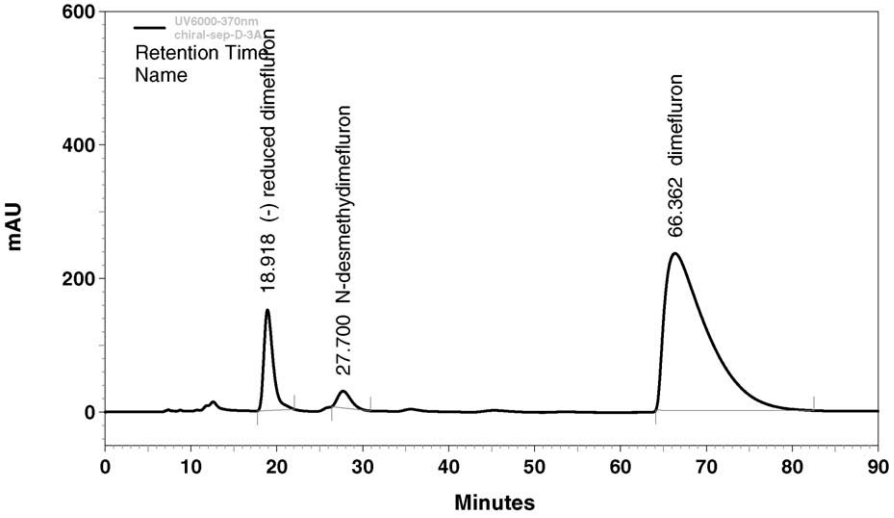

Fig. 5. Chromatogram of dimefluron after incubation in rabbit's microsomes.

Table 4
Comparison of the enantiomeric excess of red-D

Species	(−)-Red-D	(+)-Red-D	e.e. (−)-red-D vs. (+)-red-D
Red-D			
Rat	Minor	Prevalent	19.3% vs. 80.7%
Guinea pig	Minor	Prevalent	23.6% vs. 76.4%
Rabbit	Prevalent	Not detected	100% vs. 0%
Goat	Minor	Prevalent	28.2% vs. 71.8%
Pig	Comparable	Comparable	47.0% vs. 53%
Human	Prevalent	Minor	64.6% vs. 35.4%

(see Fig. 4), but D was converted completely to the (−)-red-D (see Fig. 5). Pig's reductase seems to be not stereospecific in conversion of D to the red-D metabolite, the enantiomeric excess was nearly 1:1, but B undergoes the conversion largely to the (+)-red-B. Results summary is shown in Tables 3 and 4.

4. Conclusions

Optimalized chromatographic conditions for chiral separation of red-B and -D were searched out. The best separation was achieved on column Chiralcel OD-R, with cellulose tris (3,5-dimethylphenylcarbamate as chiral selector). The red-B enantiomers were separated successfully using a mobile phase acetonitrile–1 M aqueous $NaClO_4$ (40:60, v/v). Another mobile phase, methanol–1 M aqueous $NaClO_4$ (75:25, v/v), had to be employed for the sufficient resolution of red-D enantiomers. Flow rate was 0.5 ml min^{-1} at ambient temperature in both cases. Red-B was detected at 340 nm, red-D at 370 nm.

The developed analytical procedures were successfully applied to in vitro study of the biotransformation of B and D in the microsomal fractions of liver homogenates prepared from six species (rat, rabbit, pig, guinea pig, goat and human).

Variances in the reductive metabolism were found out in the biotransformation of both parent compounds, i.e. B and D. Interesting results gave the comparison of all species under study. The carbonyl reduction was stereospecific in all cases and led predominately to one enantiomeric form. Only carbonyl-reductase of pig seems not to be stereospecific, if D was used as substrate. The amounts of both enantiomers are comparable in this case.

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