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Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 555–567

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Capillary electrophoresis with (R)-(−)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine as chiral selector for separation of albendazole sulfoxide enantiomers and their analysis in human plasma

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

The electrokinetic separation and analysis of the enantiomers of albendazole sulfoxide (ABZSO), a sulfoxide with a sulfur stereogenic center hepatically formed during therapy with the anthelmintic drug albendazole (ABZ), is reported. Using aqueous or nonaqueous alkaline background electrolytes, ABZSO enantiomers cannot be separated via single use of common neutral cyclodextrins and negatively charged carboxymethyl-β-cyclodextrin. With the Pirkle-type (R)-(−)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine ((R)-DNBPG) chiral selector, however, ABZSO enantiomers do separate within a borate background electrolyte of pH 9.0–9.5 and can be detected by UV absorbance at 295 nm. Having untreated fused-silica capillaries and 50 mM (R)-DNBPG, enantiomeric resolution is dependent on capillary i.d., capillary length and operational temperature. Optimized separation is obtained for pH 9.25 and the lowest temperature setting. Preliminary data indicate that the same approach could be employed for analysis of the enantiomers of oxfenbendazole, a chiral anthelmintic sulfoxide employed in veterinary pharmacotherapy. Analysis of plasma extracts of patients under ABZ pharmacotherapy confirmed the known enantioselectivity in the sulfoxidation of ABZ with the (+)-ABZSO being the predominant enantiomer in blood. Commencing with 2 ml of plasma, enantiomers present at >1 μg/ml could be detected only, a limitation which is based upon the strong absorbance of the chiral selector. (R)-DNBPG and ABZSO are negatively charged at pH 9.0–9.5, which prevents the application of a partial filling technique. The mobility of (R)-DNBPG is significantly larger compared to that of ABZSO. A migrating plug–plug approach based upon a plug of (R)-DNBPG migrating across the sample plug in an electroosmosis free environment obtained via a dynamic coating produced by spermine is shown to provide chiral resolution but not increased sensitivity.

Keywords: Albendazole; Albendazole sulfoxide; Oxfenbendazole; Enantiomers; Chiral separation; Stereoselective metabolism

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0731-7085/02/$ - see front matter
PII: S0731-7085(01)00650-1

1. Introduction

Albendazole (ABZ, for structure see Fig. 1) is a benzimidazole anthelmintic drug that is used for eradication or reduction of the numbers of helmintic parasites in the intestinal tract or tissues of the body [1]. ABZ is a prochiral drug, which undergoes rapid metabolism by primary oxidation of the sulfide moiety to its major metabolite albendazole sulfoxide (ABZSO, Fig. 1), a compound that exhibits a sulfur stereogenic center and an enantioselective metabolism [2]. Of the two enantiomeric antipodes of ABZSO, the (+)-ABZSO was found to be the predominant enantiomer in blood of man [3–6], dog [6], rat [6], goat [7], sheep [7,8] and cattle [7,9]. Furthermore, using preparations of rat liver microsomes, Moroni et al. [10] demonstrated in vitro that the hepatic metabolism favors the formation of (+)-ABZSO. ABZSO has anthelmintic activity and is further oxidized to albendazole sulfone ($ABZSO_2$) [2,3,11], which is an achiral substance (Fig. 1). After oral administration, ABZ is usually undetectable in plasma, whereas ABZSO reaches its maximum concentration about 2–3 h after dosing [2,3,12–14]. For optimized therapy against tissue parasites, ABZSO plasma levels >1 μM (0.281 μg/ml) are recommended [14]. Similarly, fenbendazole (FeBZ, another benzimidazole anthelmintic drug that is used in veterinary pharmacotherapy) is metabolized to oxfenbendazole (OxBZ), which is a chiral sulfoxide metabo-

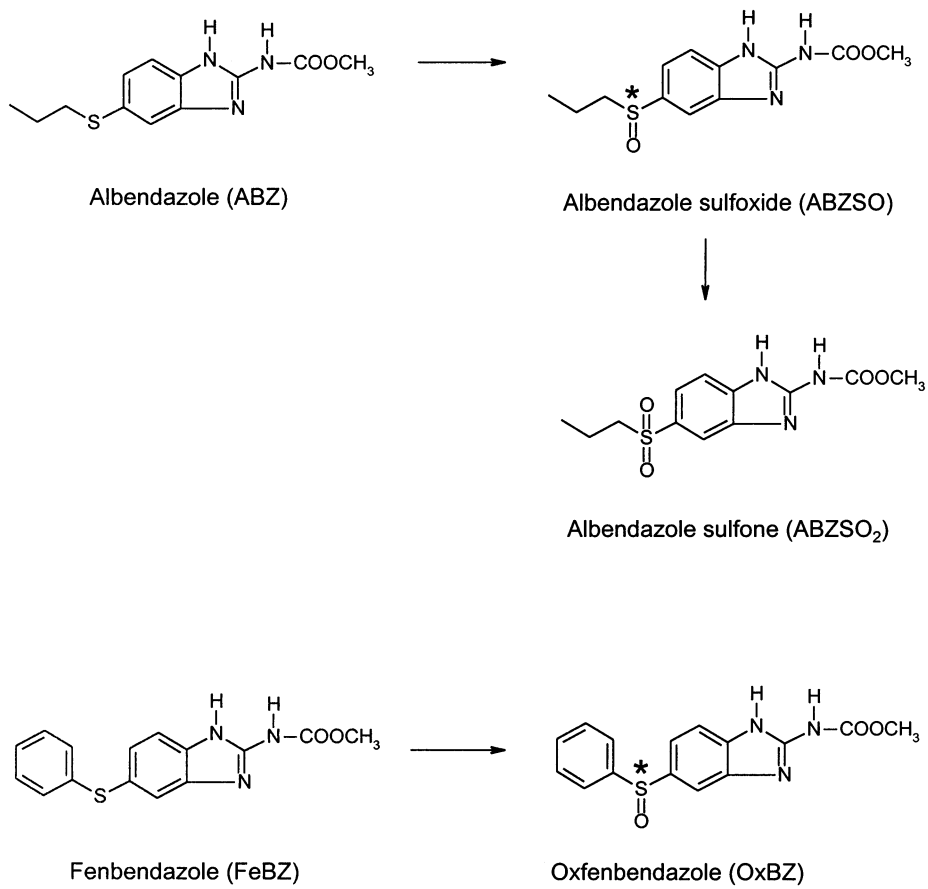


Fig. 1. Chemical structures of ABZ and its two metabolites ABZSO and $ABZSO_2$, together with those of FeBZ and OxBZ. The asterisks mark the sulfur stereogenic centers.

lite as well (Fig. 1). The enantioselectivity in the sulfoxidation of FeBZ has briefly been mentioned in the Refs. [2,15] but, to our knowledge, not further exploited.

The enantioselective chromatographic separation of chiral sulfoxides has been the focus of innumerable investigations for both analytical and preparative purposes (for further information refer to Refs. [16–22]). Analysis of ABZSO enantiomers by high-performance liquid chromatography (HPLC) using chiral stationary phases derived from (S)-*N*-(3,5-dinitrobenzoyl)-tyrosine [2,6,7,15], cellulose tribenzoate referred to as Chiracel OB-H [4], amylose *tris*(3,5-dimethylphenyl carbamate) referred to as Chiralpak AD [3,5] and α_1-acid glycoprotein also referred to as AGP [8,9] has been reported and applied to the elucidation of the stereoselectivity of the ABZ sulfoxidation in man and various animals. During the past few years, chiral separations by capillary electromigration methods have been studied extensively (for reviews refer to Refs. [23–29]) and were successfully applied to bioanalytical drug monitoring [28,29]. For enantiomeric separation under electrokinetic conditions, a chiral selector (such as a cyclodextrin (CD), a crown ether, a protein or a bile acid, to name but a few) and proper buffer conditions (pH, ionic strength, solvent, additives etc.) are required.

To the best of our knowledge, the electrokinetic separation of the enantiomers of sulfoxides with a sulfur stereogenic center has not yet been described. This and our departmental activities associated with the pharmacotherapy of humans with ABZ [13,14] prompted us to investigate the analysis of the enantiomers of ABZSO by chiral capillary zone electrophoresis (CZE). This paper reports (i) the first CZE data obtained with various CDs and *N*-(3,5-dinitrobenzoyl) derivatives as chiral selectors for analysis of ABZSO enantiomers, (ii) optimization of ABZSO enantiomeric resolution in presence of (R)-(−)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine ((R)-DNBPG) in forced air thermostated and liquid cooled fused-silica capillaries, (iii) analysis of ABZSO enantiomers using a plug of (R)-DNBPG migrating through the sample plug in an electroosmosis free environment, and (iv) analysis of ABZSO enantiomers in extracts of human plasma. Furthermore, the separation of OxBZ enantiomers in presence of chiral *N*-(3,5-dinitrobenzoyl) derivatives is also briefly discussed.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and the organic solvents acetonitrile (Biosolve, Walkenswaard, The Netherlands) and dichloromethane (Chemicals Limited, Walkerburn, Scotland) were of HPLC grade. ABZSO and OxBZ were a gift from SmithKline Beecham Pharmaceuticals (Brantfort, UK). (R)-DNBPG, (S)-(+)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine ((S)-DNBPG), (S)-(+)-*N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine, (R)-(−)-*N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine, and *N*-(3,5-dinitrobenzoyl)-L-leucine were purchased from Aldrich (Buchs, Switzerland). α-CD, β-CD, (2-hydroxypropyl)-β-CD, γ-CD, (2-hydroxypropyl)-γ-CD and carboxymethyl-β-CD were obtained from Fluka (Buchs, Switzerland), heptakis-(2,6-di-*O*-methyl)-β-CD from Cyclolab (Budapest, Hungary) and 2,3,6-trimethyl-β-CD from FDS Publications (Trowbridge Wilts, UK). Disodium tetraborate decahydrate, boric acid, sodium hydrogen carbonate, disodium carbonate decahydrate were from Merck (Darmstadt, Germany) and spermine tetrahydrochloride was from Fluka (Buchs, Switzerland).

2.2. Plasma samples and extraction procedure

Patient plasma samples were obtained from the departmental routine drug assay laboratory where they were received for therapeutic drug monitoring of ABZSO using HPLC [14]. Our own plasma was used as blank. All plasma samples were stored at −20 °C in polypropylene vials. For liquid–liquid extraction of ABZSO, 2.0 ml of patient plasma, 1 ml of blank plasma or 1 ml of fortified blank plasma were combined with 1.0 ml of 0.25 M carbonate buffer (pH 10.3) and 5 ml of dichloromethane in a 10 ml glass tube. After

gentle shaking of the closed tube for 10 min using a horizontal shaker and centrifugation at about $1500 \times g$ (3000 rpm) for 10 min, the aqueous (upper) phase was removed and the organic phase was evaporated to dryness at 40 °C employing a gentle stream of air. The residue was redissolved in 100 μl of acetonitrile.

2.3. Instrumentation and running conditions with forced air capillary cooling

CZE measurements were performed on a 270A-HT CE system (Applied Biosystems, San Jose, CA) that was equipped with a fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of 75, 50 and 25 μm i.d. and 75.5–119.3 cm (54.5–98.3 cm to the detector) total length. If not stated otherwise, the temperature control was set to 31 °C. Before use, the capillaries were rinsed for 20 min with 1 M NaOH, 20 min with 0.1 M NaOH and 20 min with water by applying a vacuum of 67.7 kPa at the outlet end. Between runs capillaries were typically rinsed for 3 min with 0.1 M NaOH, 2 min with water and 2 min with background electrolyte (BGE) (25 μm i.d. capillaries were washed only with BGE for ~ 30 min). Sample was introduced by applying a vacuum of 16.9 kPa for 1–5 s. The voltage applied was 7–30 kV (anode at injection end) and the current was between 8 and 61 μA. The wavelength used for detection was 280 nm. A PC equipped with a PC Integration Pack (Kontron Instruments, Basel, Switzerland) was employed for data registration, evaluation and storage.

2.4. Instrumentation and running conditions with liquid capillary cooling

A BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA) was employed. It was equipped with untreated 50 μm i.d. fused-silica capillaries (Polymicro Technologies) of 50 cm (45.4 cm to the detector) or 104.6 cm (100 cm) total lengths that were mounted in a user assembled cartridge (Bio-Rad). Injection of sample was effected by applying positive pressure (5 or 10 psi s). The temperatures of cartridge and carousel were maintained at 20 °C and detection was effected at 295 nm. In the morning, the capillary was rinsed with 0.1 M NaOH and water (10 min each). Before each experiment the capillary was rinsed with 0.1 M NaOH for 3 min, water for 2 min and BGE for 2 min. For identification purposes, the fast scanning detection mode (range: 250–360 nm at 5 nm intervals) was employed. BioFocus integration software (version 3.01, Bio-Rad) was employed for data conversion and evaluation. If not stated otherwise, a BGE composed of 30 mM $Na_2B_4O_7$ and 50 mM (R)-DNBPG (pH 9.0) was used and a constant voltage of 20 kV (currents of 50 and 104.6 cm capillaries were 48 and 19 μA, respectively) was applied. The chiral selector was added freshly every day. Achiral data were gathered employing a BGE comprising 30 mM $Na_2B_4O_7$ (pH 9.2) and application of a constant voltage of 20 kV (current in 50 cm capillary: 32 μA).

3. Results and discussion

3.1. CZE of albendazole sulfoxide and use of cyclodextrins as chiral selectors

ABZSO has pKa values of 0.3 and 9.7 and is thus neutral between about pH 2 and 8 [30]. Using a BGE composed of 30 mM $Na_2B_4O_7$ at pH 9.4, ABZSO is partially negatively charged and was determined to produce a sharp, narrow peak migrating in opposite direction compared to the electroosmotic flow (Fig. 2). The same was found to be true for $ABZSO_2$ (pKa unknown), here applied as impurity of ABZSO. The electroosmotic mobility was 5.80×10^{-8} $m^2/V\,s$ and the effective mobilities of ABZSO and $ABZSO_2$ were calculated to be -0.42×10^{-8} and -0.70×10^{-8} $m^2/V\,s$, respectively. In a strongly acidic environment ($pH < 2$), ABZSO becomes positively charged.

To separate the enantiomers of ABZSO, the interaction of ABZSO with various CDs was investigated in a 75 μm i.d. fused-silica capillary of 58.7 cm total (37.7 cm effective) length that was mounted into the ABI 270A-HT. Using neutral CDs (α-CD, β-CD, (2-hydroxypropyl)-β-CD, γ-CD, (2-hydroxypropyl)-γ-CD, heptakis-(2,6-di-*O*-

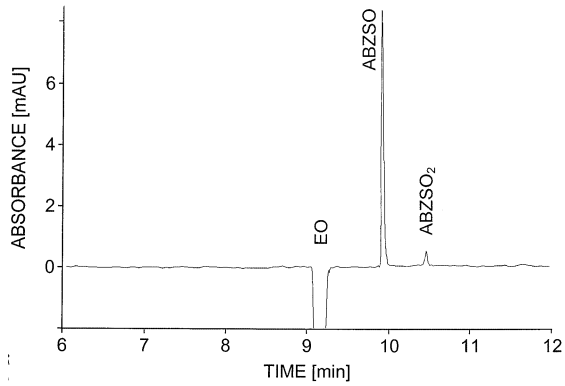
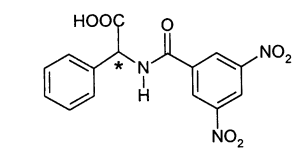


Fig. 2. Achiral CZE electropherogram recorded on the ABI 270A-HT for ABZSO in a 50 μm i.d. fused-silica capillary of 80 (100) cm effective (total) length using a BGE comprising 30 mM $Na_2B_4O_7$ (pH 9.4). A constant voltage of 25 kV (current: 25 μA; power: 0.625 W/m) was applied. The sample comprised 183 μg/ml ABZSO ($ABZSO_2$ as impurity of ABZSO standard) dissolved in acetonitrile and was injected during 2 s. EO refers to the electroosmotic flow.

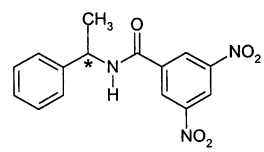
methyl)-β-CD and 2,3,6-trimethyl-β-CD) at concentrations of about 10–50 mM (for β-CD 2.5–10 mM only) in 25 mM aqueous borate buffers at pH 9.3, no chiral resolution was achieved. With increasing concentration of the chiral selector, only peak broadening was observed and the resolution between ABZSO and $ABZSO_2$ was noted to become decreased. Furthermore, chiral separation could not be improved via addition of urea (6 mM) to the BGE. Using a 15 mM phosphate buffer at pH 7.1 containing 4–20 mM of the negatively charged carboxymethyl-β-CD as chiral selector (at this pH ABZSO is uncharged and migrates only as complex with the charged CD), no chiral separation occurred and a broad, small and asymmetric peak was observed. In non-aqueous capillary electrophoresis (NACE) with 25 mM tetraborate in methanol:NMF 1:3 (v/v) at pH* 9.3, no chiral separation was observed with α-CD, β-CD, (2-hydroxypropyl)-β-CD, γ-CD and 2,3,6-trimethyl-β-CD (at about 50–100 mM each). Compared to aqueous systems, peak broadening was noted to be smaller. This is in agreement with the theory, which predicts that the interaction of the analyte with the cavity of CD is weaker in a more hydrophobic medium and association constants are lower compared to aqueous systems [31,32]. Based upon the poor interaction between ABZSO and the investigated CDs, no further work was undertaken with these types of chiral selectors.

3.2. Use of *N*-(3,5-dinitrobenzoyl) derivatives as chiral selectors in capillary electrophoresis

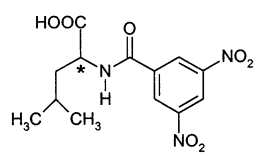
In HPLC, stationary phases featuring chiral *N*-(3,5-dinitrobenzoyl) derivatives have been used for separation of the enantiomers of sulfoxides [16,17], including ABZSO [2]. In analogy of the work of Lienne et al. [2], (S)-(+)-*N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine, (R)-(−)-*N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine, (S)-DNBPG, (R)-DNBPG and *N*-(3,5-dinitrobenzoyl)-L-leucine (for structures see Fig. 3) were considered as buffer additives for chiral separation of ABZSO and OxBZ. The two methylbenzylamine selectors were found to be insufficiently soluble in water and were thus not further investigated. The L-leucine derivative, (R)-DNBPG and



N-(3,5-dinitrobenzoyl)-α-phenylglycine



N-(3,5-dinitrobenzoyl)-α-methylbenzylamine



N-(3,5-dinitrobenzoyl)-leucine

Fig. 3. Chemical structures of the *N*-(3,5-dinitrobenzoyl) derivatives considered as chiral selectors in this work. The asterisks mark the chiral centers.

(S)-DNBPG are sufficiently soluble in an alkaline BGE (pH > 8.2) and are negatively charged. Using a borate buffer at pH 9 comprising 20–50 mM *N*-(3,5-dinitrobenzoyl)-L-leucine was found to be capable of partially separating the enantiomers of OxBZ, but not those of ABZSO (data not shown). Using (R)-DNBPG, chiral resolution of ABZSO and OxBZ enantiomers became possible (Figs. 4 and 5). The same was noted to be true for (S)-DNBPG. However, as (R)-DNBPG is better soluble and significantly cheaper than (S)-DNBPG, (R)-DNBPG was chosen as chiral selector for all further experiments.

In presence of (R)-DNBPG, the effective electrophoretic mobility of ABZSO was found to become higher. The data presented in Fig. 4A were obtained with 50 mM (R)-DNBPG and otherwise similar conditions as for Fig. 2. The electroosmotic mobility was 5.56×10^{-8} m^2/V s and the effective mobilities of the firstly and secondly detected ABZSO enantiomers were calculated to be -1.134×10^{-8} and -1.151×10^{-8} m^2/V s, respectively. Having an effective capillary length of 80 cm and the forced air temperature control set at 31 °C, the mobility difference of 0.017×10^{-8} m^2/V s led to an enantiomeric resolution (Rs) of 0.48. Rs was calculated using $Rs = 2(t_2 - t_1)/(W_1 + W_2)$ where t_i and W_i represent the detection time and peak width of enantiomer i, respectively. Firstly and secondly detected enantiomers are denoted with subscripts 1 and 2, respectively. An Rs value of ≥ 1.4 represents baseline resolution. A detailed discussion of the optimization of ABZSO enantiomer separation with (R)-DNBPG as chiral selector is presented in Section 3.3.

3.3. Optimization of ABZSO enantiomer separation with (R)-(−)-N-(3,5-dinitrobenzoyl)-α-phenylglycine

To achieve baseline resolution of both ABZSO enantiomers, the impact of the BGE composition (pH, concentrations of chiral selector and coion, addition of CDs and small amounts of organic solvents), the capillary dimensions (i.d. and length) and the running temperature were investigated. Using 50 μm i.d. capillaries of 54 (75) cm effective (total) length, the forced air temperature control set at 31 °C and 50 mM (R)-DNBPG, the pH of the borate BGE was varied between 8.4–9.9 (pH set by addition of 0.5 M NaOH). Chiral

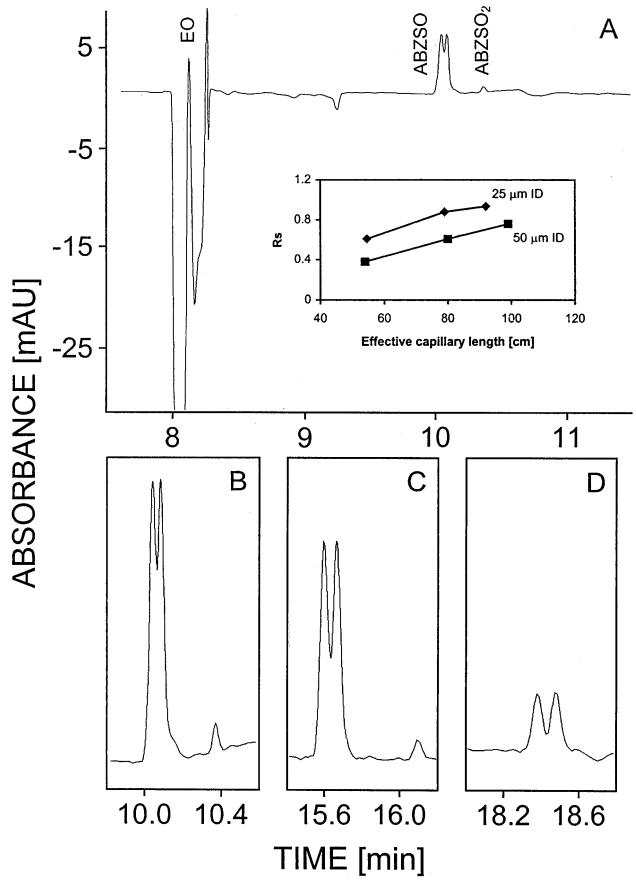


Fig. 4. Chiral electropherograms obtained at pH 9 on the ABI 270A-HT for ABZSO with 50 mM (R)-DNBPG as chiral selector having (A) and (B) a 50 μm i.d. capillary of 80 cm effective (100 cm total) length and a 30 mM borate BGE, (C) a 50 μm i.d. capillary of 99 cm effective (120 cm total) length and a 30 mM borate BGE, and (D) a 25 μm i.d. capillary of 92 cm effective (113 cm total) length and a 100 mM borate BGE. A constant voltage of 30 kV was applied in all cases. Currents and power levels were (A) and (B) 32 μA and 0.96 W/m, (C) 27 μA and 0.675 W/m, and (D) 13 μA and 0.345 W/m. The sample comprised 183 μg/ml ABZSO ($ABZSO_2$ as impurity of ABZSO standard) dissolved in acetonitrile and was injected during 3, 3, 2 and 8 s, respectively. EO refers to the electroosmotic flow. The insert in panel A depicts the Rs values obtained for the separation of ABZSO enantiomers in capillaries of various lengths.

resolution of ABZSO enantiomers was found to be possible at a pH > 8.5 and best separation was observed between at pH 9.0–9.5 (Rs = 0.38 for the two edge values). Enantiomeric separation was noted for configurations with ≥ 40 mM (R)-DNBPG with highest resolution being obtained at 50–60 mM of the chiral selector. Furthermore, at the expense of about 10% elongated run times, a BGE concentration increase to 100 mM $Na_2B_4O_7$ led to a small increase of the resolution (Rs increase from 0.57 to 0.61, assessed in 25 μm i.d. capillary). Furthermore, incremental addition of ACN (4.8–11%, v/v) to the BGE comprising 30 mM $Na_2B_4O_7$ and 50 mM (R)-DNBPG (pH 9) resulted in a gradual loss of resolution. The effective mobility of the analyte was determined to decrease, which indicates a weakening of the interaction between ABZSO and the chiral selector. Similarly, addition of (2-hydroxypropyl)-β-CD (2.2–50 mM) to the same BGE resulted in a decrease of the effective mobility of the analyte and a decrease in the chiral resolution. Thus, BGEs composed of 50 mM (R)-DNBPG and 30 or 100 mM borate were employed for all the following studies.

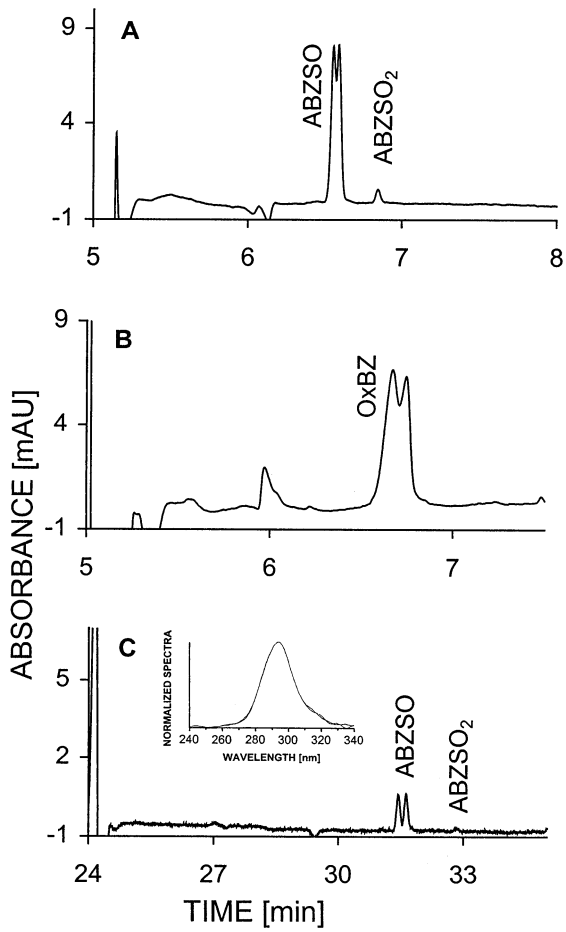


Fig. 5. Chiral electropherograms obtained on the BioFocus with a pH 9 BGE composed of 30 mM $Na_2B_4O_7$ and 50 mM (R)-DNBPG and (A) and (C) for analysis of ABZSO (234 μg/ml in acetonitrile) in 50 μm i.d. capillaries of (A) 45.4 cm effective (50 cm total) length and (C) 100 cm effective (104.6 cm total) length and (B) for analysis of OxBZ (100 μg/ml in methanol) using the capillary of 50 cm total length. The applied voltage was 20 kV in all cases. The currents and power levels were (A) 48 μA and 0.48 W/m, (B) 45 μA and 0.45 W/m, and (C) 19 μA and 0.37 W/m. Sample injection occurred at 5 psi s. The insert in panel C depicts the normalized UV spectra of the ABZSO enantiomers extracted from the data of panel A.

Chiral separation of ABZSO in the presence of 50 mM (R)-DNBPG was studied in capillaries of 50 and 25 μm i.d. using the instrument with forced air temperature control that was set at 31 °C. Almost complete resolution was obtained in 25 μm i.d. capillaries (insert of Fig. 4A). Up to an effective length of 92 cm (113 cm total length), an increase of enantiomeric separation was observed (insert Fig. 4A; Rs = 0.95 for the conditions of Fig. 4D). In longer capillaries, resolution became somewhat lower (data not shown). As the use of 25 μm i.d. capillaries provides smaller detector responses (compare Fig. 4D with Fig. 4B and C) and requires much longer time intervals for capillary rinsing (e.g. more than 0.5 h for a capillary of 113 cm total length mounted on the ABI 270A-HT), the work with these capillaries was discontinued. In 50 μm i.d. capillaries, the resolution of the ABZSO enantiomers was determined to increase with the capillary length (insert Fig. 4A). Having an effective capillary length of 99 cm (120 cm total length) an enantiomeric resolution Rs of 0.76 was obtained (Fig. 4C).

The data presented in Fig. 5 were obtained on the BioFocus 3000, which featured water cooled capillaries with the temperature of the recirculating water set to 20 °C. For the data of Fig. 5A, the electroosmotic mobility was calculated to be 3.86×10^{-8} m^2/V s and the effective mobilities of

the firstly and secondly detected ABZSO enantiomers were -0.977×10^{-8} and -0.990×10^{-8} m^2/V s, respectively. Corresponding values for the electropherogram of Fig. 5C were determined to be 3.61×10^{-8}, -0.835×10^{-8} and -0.856×10^{-8} m^2/V s, respectively. Having effective capillary lengths of 45.4 and 100 cm, respectively, the mobility differences of 0.013×10^{-8} and 0.021×10^{-8} m^2/V s led to enantiomeric resolutions Rs of 0.40 and 0.99, respectively, values that are higher compared to those obtained in the corresponding air-cooled capillary (Fig. 4A–C). The lower temperature appears to have a beneficial effect on enantiomeric resolution.

The influence of the temperature on chiral resolution of ABZSO enantiomers was assessed via comparison of data obtained with air- and liquid-cooled capillaries. The ABI instrument features thermostated forced air convection for the maintenance of a constant capillary temperature. The temperature of the forced air cannot be set lower than 5 °C above room temperature (i.e. 31 °C in our experiments). The actual temperature within the capillary is known to be linearly dependent upon applied power. For our instrumental configuration, the fluid temperature raise within the capillary is estimated at about 14 °C per applied power level of 1 W/m [33]. Thus, the data presented in Fig. 4 with power levels of 0.345–0.96 W/m can be expected to represent enantiomer separations performed at intracapillary temperatures between about 36 and 44 °C. Having liquid capillary cooling as in the BioFocus, the temperature raise as function of the electrical power dissipated within the capillary is much smaller (about half compared to air convection [33]). Thus, for the data given in Fig. 5A and C, which were generated with a cooling fluid temperature of 20 °C and an applied electric power of 1.92 and 0.37 W/m, respectively, the intracapillary temperatures are estimated to be in the order of 33 and 23 °C, respectively. Furthermore, experiments performed with the cooling fluid temperature set to 15 °C provided a small increase in resolution, whereas no resolution was observed with a setting of 40 °C. In the latter case, power levels of 1.41 and 2.48 W/m (estimated intracapillary temperatures of about 50 and 58 °C, respectively) were applied. In conclusion, the performed studies indicate that best enantiomeric separation was achieved at the lowest intracapillary temperature and that no chiral separation is obtained at temperatures above about 45 °C. Thus, experiments with plasma extracts were performed using the instrument with liquid capillary cooling (conditions of Fig. 5C with a intracapillary fluid temperature of about 23 °C) and having a detection wavelength of 295 nm (cf. inset of Fig. 5C).

3.4. Analysis of albendazole sulfoxide enantiomers in human plasma using (R)-(−)-N-(3,5-dinitrobenzoyl)-α-phenylglycine as chiral selector

Typical data obtained with plasma extracts obtained on the BioFocus are presented in Fig. 6. Analysis of the extract of blank plasma revealed a clean electropherogram (Fig. 6A), whereas the extract of blank plasma spiked with 60 μg/ml ABZSO provided two almost completely resolved ABZSO peaks (Rs = 1.02), the peak height of the firstly detected enantiomer being somewhat lower compared to that of the secondly detected enantiomer (Fig. 6B). Data obtained with an extract of a patient plasma that was determined to contain 5.61 μg/ml ABZSO by achiral HPLC [14] are depicted in panel C and an electropherogram obtained after combining the two ABZSO containing extracts (1:5, v/v) is shown in Fig. 6D. The latter electropherogram and spectral analysis of all data (for an example see insert of Fig. 5C) suggested the unambiguous monitoring of ABZSO and $ABZSO_2$ in the patient plasma extract. Furthermore, an enantiomeric peak height ratio of 2.57 was observed. The amount of the firstly detected ABZSO enantiomer is thereby shown to be significantly higher compared to the enantiomer with a higher effective mobility. This stereoselectivity in the metabolism of ABZ is well known and was thus employed for assignment of the two enantiomers. According to the HPLC literature [3–6], (+)-ABZSO is reported to be the major ABZSO enantiomer in human plasma. Data obtained with plasma extracts on the ABI

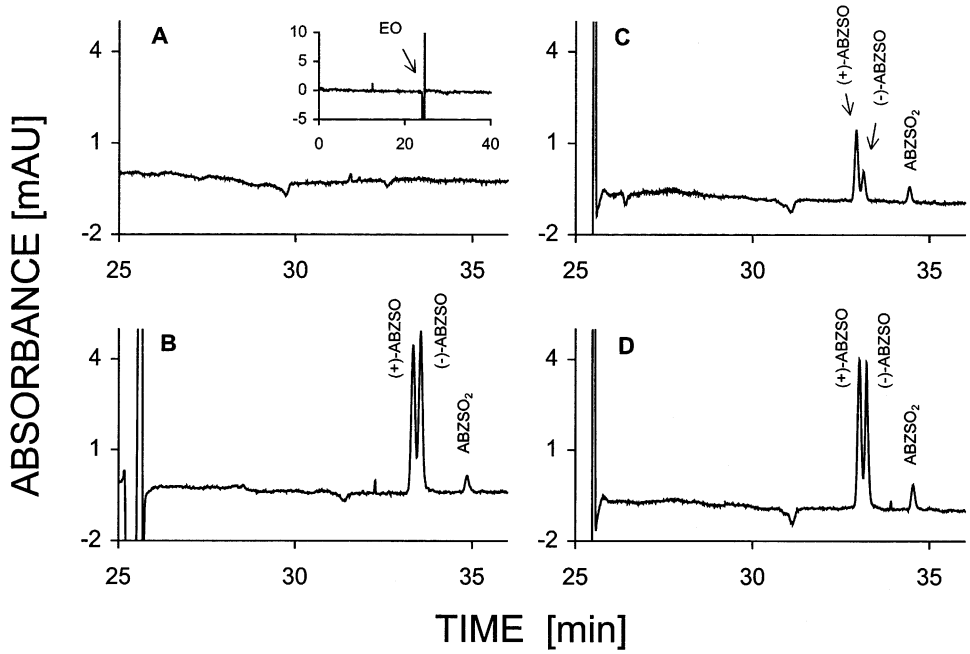


Fig. 6. Chiral electropherograms obtained on the BioFocus for analysis of (A) blank plasma extract, (B) extract of blank plasma fortified with 60 μg/ml ABZSO, (C) extract of a patient plasma containing 5.61 μg/ml ABZSO, and (D) 1:5 (v/v) combination of the extracts of (B) and (C). The applied voltage (current) was 20 kV (19 μA). Samples were injected at (A), (C), and (D) 10 psi s, and (B) 5 psi s. The insert of panel A shows the complete run with the EO marker. Other conditions as for Fig. 5C.

270A-HT revealed the same results. Due to the lower chiral resolution obtained, however, the proper recognition of the stereoselectivity was less obvious (data not shown).

Chiral analysis of ABZSO enantiomers in extracts of human plasma was found to be reproducible. For the example presented in Fig. 6B, RSD values ($n = 4$) for peak detection time, peak height/detection time and peak area/detection time of (+)-ABZSO were determined to be 1.61, 15.93 and 17.80%. Corresponding values for (−)-ABZSO were 1.61, 16.06 and 15.47%. RSD values of ratios of peak heights and areas were calculated to be 0.70 and 3.20%, respectively. Thus, based upon these data, the developed assay appears to be suitable to provide accurate enantiomeric ratios. Due to the strong absorption of the chiral selector (BGE has a yellow color), the sensitivity of the method, however, was found to be poor. For extraction from 1 ml of plasma fortified with 1 μg/ml ABZSO, the two enantiomers could not be detected any more (detection limit per enantiomer was about 1–2 μg/ml). Although some sensitivity enhancement is obtained via extraction from larger amounts of plasma (Fig. 6C), a more sensitive assay is required for analysis of patient samples containing subμg/ml ABZSO levels.

3.5. Separation of albendazole sulfoxide enantiomers using a plug of finite length of (R)-DNBPG migrating through the sample

With the goal of increasing the assay sensitivity for ABZSO enantiomers, alternative approaches avoiding the presence of the chiral selector during analyte detection, namely partial filling of the capillary with the chiral selector and a migrating plug–plug separation mode during which the plug of chiral selector is overtaking the plug of analyte, were considered. At pH 9 and higher, ABZSO and the chiral selector are both negatively charged, a constellation, which does not lend itself for a partial filling technique. Employing the Bio-

Focus with an untreated fused-silica capillary of 50 cm total length (46.4 cm to the detector) and a BGE comprising 30 mM $Na_2B_4O_7$ (pH 9.2) and no chiral selector (electroosmotic mobility of 5.27×10^{-8} $m^2/V\,s$), ABZSO and (R)-DNBPG were injected as sample and determined to have effective mobilities of -0.60×10^{-8} and -2.22×10^{-8} $m^2/V\,s$, respectively. Furthermore, ABZSO in presence of 50 mM (R)-DNBPG was determined to migrate with a higher mobility (e.g. about -0.98×10^{-8} $m^2/V\,s$ at pH 9.0). The chiral selector has a much higher mobility and a plug of (R)-DNBPG could thus migrate easily through a sample plug. Exploitation of such a configuration requires reversed polarity and a capillary with minimized electroosmosis. Using 3 mM spermine (for dynamic capillary coating with spermine and other polyamines refer to [34]) as buffer additive, electroosmosis could be effectively reduced about 100-fold, which was found to be sufficient for analysis of ABZSO in the reversed polarity mode using a BGE composed of 30 mM $Na_2B_4O_7$ and 3 mM spermine (pH 9.2). Using a 50 μm i.d. capillary of 45.4 cm effective (50 cm total) length, ABZSO and (R)-DNBPG were detected after 53.83 and 8.48 min, respectively, this corresponding to effective mobilities of -0.35×10^{-8} and -2.23×10^{-8} $m^2/V\,s$, respectively, in an electroosmosis free environment. Considering the pH dependence of the ABZSO effective mobility around pH 9.2, these values agree well with those obtained without the presence of spermine (see above).

The plug–plug experiments were performed with the BGE composed of 30 mM $Na_2B_4O_7$ and 3 mM spermine (pH 9.2). The catholyte used to produce the migrating plug of chiral selector comprised 30 mM $Na_2B_4O_7$, 50 mM (R)-DNBPG and 3 mM spermine (pH 9.0). In all experiments, the sample was initially placed between the BGE and this catholyte. Upon application of power, the chiral selector begins to penetrate into and then gradually migrates through the sample zone, thereby providing a temporary medium for differential migration of the enantiomers. Analysis of ABZSO in this discontinuous buffer system led to the data presented in Fig. 7A. After 8.38 min, a sharp transition produced by the advancing front of (R)-DNBPG was monitored. Then, within the plateau region of the chiral selector, $ABZSO_2$ and the enantiomers of ABZSO ($Rs = 0.97$) were monitored (insert of Fig. 7A), the detection sequence being reversed compared to that observed using normal polarity and a strong electroosmotic flow towards the cathode (Fig. 5). Limiting the duration of the (R)-DNBPG supply from the cathode (experimentally achieved by a brief power interruption at a specified time point during which the (R)-DNBPG containing catholyte is exchanged with BGE), resulted in conditions with an analytical reagent zone of finite length and provided electropherograms in which the absorbance of the (R)-DNBPG plateau decreased gradually to the level of the BGE. Depending on the (R)-DNBPG amount supplied, ABZSO enantiomers were detected within the (R)-DNBPG change (Fig. 7B and C) or thereafter (Fig. 7D). Unfortunately, zones of ABZSO enantiomers became substantially broader while the chiral selector was gradually leaving the sample and assay sensitivity was thereby not improved. Experiments performed with a BGE and catholyte comprising 100 mM borate (instead of 30 mM) and otherwise identical conditions provided elongated runs with complete ABZSO enantiomeric resolution (detection after about 35 min and $Rs = 1.51$ with uninterrupted (R)-DNBPG delivery). Using a (R)-DNBPG plug resulted in drawn out ABZSO peaks as well (data not shown).

The appearance of the (R)-DNBPG front and thereby also the run time was found to be dependent on the amount of sample injected. Without sample and with a 10 psi s injection, the transitions were monitored after 7.8 and 10.9 min, respectively. Furthermore, analysis of an extract of a patient plasma comprising 5.21 μg/ml ABZSO revealed (+)-ABZSO/(−)-ABZSO peak height ratios of 2.73 in both, the plug-plug configuration and the conventional approach. It was interesting to find that the discontinuous configuration of Fig. 7A provided somewhat improved enantiomeric resolution compared to that observed for the conditions of Fig. 5A. The determined Rs value was almost the same as that obtained in the longer capillary (Fig. 5C). The running time, however, was about half of that

observed for the conditions of Fig. 5C. A difference in pH is believed to be the major reason for the observed increase in resolution. Using the BioFocus in the normal configuration (Fig. 5A), enantiomeric separation performed at pH 9.25 was indeed found to provide increased resolution as well (Rs = 0.51 compared to Rs = 0.40 at pH 9.0).

4. Conclusions

To our knowledge, this is the first account dealing with the electrokinetic separation of the enantiomers of a sulfoxide with a sulfur stereogenic center. Using aqueous or nonaqueous borate based BGEs at pH 9.3 and an air thermostated capillary operated at power levels of about 0.2 to 0.4 W/m (estimated intracapillary temperature of 34–37 °C), ABZSO enantiomers could not be separated via use of common neutral CDs. The same was found to be true with an aqueous phosphate buffer at pH 7.1 employing negatively charged carboxymethyl-β-CD as chiral selector. Configurations with dual and ternary CD mixtures and operational conditions at lower temperatures were not investigated. With the Pirkle-type (R)-DNBPG chiral selector, however, ABZSO enantiomers could be separated within a borate BGE at a pH 9.0–9.5 and detected by UV absorbance at 295 nm. Having untreated fused-silica capillaries and 50 mM (R)-DNBPG, enantiomeric resolution was found to be dependent on capillary i.d., capillary length and operational temperature. A resolution of unity was obtained under various operational conditions. Optimized

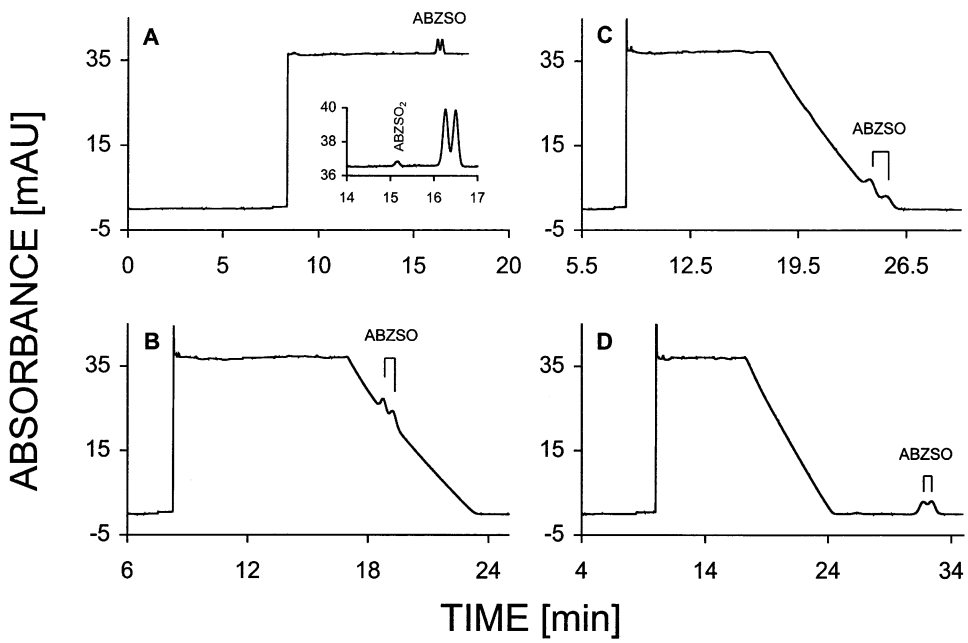


Fig. 7. Electropherograms obtained on the BioFocus with a plug of (R)-DNBPG migrating through a plug of ABZSO in a configuration with minimized electroosmosis and reversed polarity using a 50 μm i.d. capillary of 45.4 cm effective (50 cm total) length. Data presented correspond to those registered (A) for continuous delivery of chiral selector from the cathodic electrode chamber, and (B)–(D) for discontinuation of (R)-DNBPG delivery after (B) 6.0 min, (C) 5.5 min and (D) 4.0 min of electrophoresis time. In all cases, a sample of 200 μg/ml ABZSO dissolved in acetonitrile was injected at 5 psi s and the voltage applied was −20 kV. Initial currents were about 35 μA and currents during ABZSO detection were about (A) 30, (B) 16, (C) 15, and (D) 15 μA. The BGE was composed of 30 mM $Na_2B_4O_7$ and 3 mM spermine (pH 9.2) and the catholyte used to produce the migrating plug of chiral selector comprised 30 mM $Na_2B_4O_7$, 50 mM (R)-DNBPG and 3 mM spermine (pH 9.0). The insert of panel A shows a part of the same data on expanded x- and y-axes scales.

separation was noted for pH 9.25 and the lowest temperature setting. No chiral separation was observed at intracapillary temperatures above about 45 °C. Preliminary data indicate that the same approach could be employed for analysis of OxBZ enantiomers. Analysis of plasma extracts of patients under ABZ pharmacotherapy confirmed the known enantioselectivity in the sulfoxidation of ABZ with the (+)-ABZSO being the predominant enantiomer in blood. With use of 2 ml of plasma, enantiomers present at >1 μg/ml could be detected only, a limitation which is based upon the absorbance of the chiral selector. (R)-DNBPG and ABZSO are negatively charged at pH 9.0 and higher (effective mobilities of -2.2×10^{-8} and about -0.5×10^{-8} $m^2/V\,s$ (pH dependent), respectively), which prevents the application of a partial filling technique. Effective mobility of ABZSO in presence of (R)-DNBPG was noted to be about -1.0×10^{-8} $m^2/V\,s$ (pH dependent). The use of a plug of (R)-DNBPG migrating across the sample plug in an electroosmosis free environment (dynamic coating of capillary walls with spermine) did provide chiral separation but did not lead to increased sensitivity. Other approaches, including the use of fluorescence detection and capillary electrochromatography, will have to be tested to obtain the enantiomer sensitivity of about 0.05 μg/ml that is required for monitoring the enantiomeric ratio of a large number of patient samples.

5. Note

After acceptance of our manuscript, a paper of Paias et al. describing the enantioselective analysis of ABZSO in cerebrospinal fluid appeared (F.O. Paias, V.L. Lanchote, O.M. Takayanagui, P.S. Bonato, Electrophoresis 22 (2001) 3263–3269). In that work, ABZSO enantiomers are being separated in presence of sulfated beta-cyclodextrin.

Acknowledgements

The authors thank C. Lanz for his assistance in abolishing electroosmosis with spermine. This work was funded by the Swiss National Science Foundation.

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