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Direct optical resolution of *trans*-dihydrodiol enantiomers of fjord-region polycyclic aromatic hydrocarbons by high-performance liquid chromatography on a modified cellulose phase

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Abstract

Enantioselective separation of *trans*-dihydrodiol metabolites of a series of fjord-region polycyclic aromatic hydrocarbons (PAHs), such as benzo[*c*]phenanthrene and dibenzo[*a,l*]pyrene, was evaluated by HPLC using commercially available cellulose-based CSPs as chiral columns. A baseline separation ($R_s \geq 1.6$) with sharp, well-defined peaks of individual enantiomers was attained using cellulose–tris-(*N*-3,5-dimethylphenylcarbamate) and *n*-heptane–ethanol (9:1, v/v) as mobile phase. These chromatographic conditions permit a direct, simple and rapid (mostly within 30 min) enantiomeric resolution of PAH dihydrodiols. CD spectra were obtained for all optically pure enantiomers and absolute structure assignment was made for the hitherto unknown (+)- and (−)-enantiomers of naphtho[1,2-*a*]pyrene-9,10- and naphtho[1,2-*e*]pyrene-11,12-dihydrodiol. The method appears to have great potential to be applied (i) in metabolism studies of fjord-region PAHs and (ii) for optical resolution of *trans*-dihydrodiol metabolites on a preparative scale.

Keywords: Enantiomer separation; Circular dichroism; Chiral stationary phases, LC; Absolute stereochemistry; Polynuclear aromatic hydrocarbons; Dihydrodiol metabolites

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a class of chemical carcinogens occurring widespread as environmental pollutants [1]. Biological effects of PAHs including mutagenicity and carcinogenicity require metabolic activation to electrophilic reactive metabolites, in particular dihydrodiol epoxides, which are formed in a sequence of reactions catalyzed by cytochromes P450 and microsomal epoxide hydrolase [2–4]. PAH metabolism is initiated by the formation of arene oxides, which proceeds with a high degree of stereoselectivity due to the stereohetereotopic interactions of the PAH and the cytochrome P450 attacking the planar PAH molecule at specific sites of prochiral nature. The intermediate arene oxides undergo subsequent hydrolysis by microsomal epoxide hydrolase in a regio- and enantioselective manner to produce enantiomerically enriched *trans*-dihydrodiols. If this reaction sequence occurs at an angular benzo ring of a given PAH, further

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stereoselective oxidation of the adjacent double bond by cytochrome P450 enzymes leads to different stereoisomers of the chemically highly reactive bay- or fjord-region dihydrodiol epoxides [2] which are thought to play a major role in PAH carcinogenesis due to their ability to form covalent DNA adducts [5] (bay- and fjord-regions are sterically crowded areas in a PAH molecule due to an angular annealing of benzene rings; cf. Fig. 1).

Chromatographic techniques allowing separation of dihydrodiol enantiomers are of ongoing interest, in particular for recent studies devoted to determine the stereoselectivity involved in PAH metabolism by human cytochromes P450 and microsomal epoxide hydrolase [6–8], as well as for the synthesis of optically active dihydrodiol epoxides for which the enantiomerically pure dihydrodiols are needed as

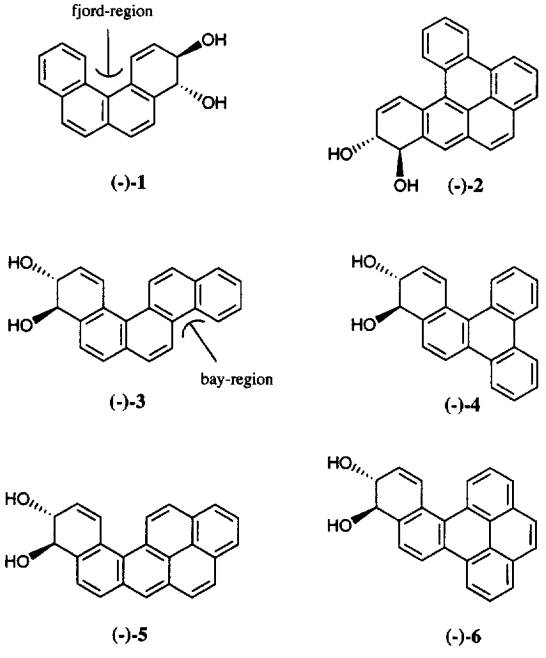

Fig. 1. Structures of polycyclic aromatic hydrocarbon *trans*-dihydrodiols which have been investigated for enantiomeric resolution by CSP-HPLC on a Chiralcel OD-H phase. Only the absolute structure of the (−)-(*R,R*)-enantiomers are shown for benzo[*c*]phenanthrene-3,4-dihydrodiol **1**, dibenzo[*a,l*]pyrene-11,12-dihydrodiol **2**, benzo[*c*]chrysene-9,10-dihydrodiol **3**, benzo-[*g*]chrysene-11,12-dihydrodiol **4**, naphtho[1,2-*a*]pyrene-9,10-dihydrodiol **5** and naphtho[1,2-*e*]pyrene-11,12-dihydrodiol **6**; the sterically hindered fjord- and bay-region are indicated in the structures of dihydrodiols **1** and **3**, respectively.

chiral precursors [9]. The more classical method entailing normal-phase high-performance liquid chromatography (HPLC) separation of diastereomeric bis-ester derivatives, e.g., obtained by reacting the dihydrodiol with (−)-menthoxyacetyl chloride [10] or Mosher's reagent [11], has increasingly been replaced in recent years by the convenient direct resolution of dihydrodiol enantiomers with HPLC on chiral stationary phases (CSPs) [7,12,13]. Among the commercially available CSPs Pirkle's phases based on (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine [(*R*)-DNBPG] or (*S*)-*N*-(3,5-dinitrobenzoyl)leucine either ionically or covalently bonded to γ-aminopropyl-silanized silica have been extensively applied for the resolution of K-region as well as non-K-region dihydrodiol racemates of various PAHs including phenanthrene [14], chrysene [14], benz[*a*]anthracene [14–17], benzo[*a*]pyrene [17], dibenz[*a,h*]anthracene [18] and other oxygenated metabolites of PAHs [19]. An alternative CSP, based also predominantly on π-donor–acceptor interactions, has been developed by Gil-Av and coworkers [20] using (*R*)-(−)-2-(2,4,5,7-tetranitrofluoren-9-ylideneaminooxy)-propionic acid (TAPA) as chiral selector and has been applied for the separation of several dihydrodiols of benz[*a*]anthracene and benzo[*a*]pyrene [21]. Recently, Funk et al. [22] have further explored scope and limitations of this type of CSP. They demonstrated a broad applicability of TAPA and related CSPs for the optical resolution of PAH dihydrodiols and reported in particular the enantiomeric resolution of non-K-region dihydrodiols of dibenz[*a,h*]anthracene, whose direct separation provided major problems with Pirkle's CSPs [22].

Interestingly, enantiomeric resolution of PAH dihydrodiols with a double bond in the fjord-region like benzo[*c*]phenanthrene-3,4-dihydrodiol either failed using TAPA or related CSPs [22] or required partial hydrogenation to achieve an efficient separation of the resulting two tetrahydrodiol enantiomers on (*R*)-DNBPG [23]. In order to overcome these problems, commercially available modified cellulose CSPs, known for the high resolving power of racemates have now been investigated for their ability to resolve PAH dihydrodiols. The study focused on PAH dihydrodiols with a fjord-region double bond, namely benzo[*c*]phenanthrene-3,4-dihydrodiol (±)-**1**, dibenzo[*a,l*]pyrene-11,12-di-

hydrodiol (±)-**2**, benzo[*c*]chrysene-9,10- (±)-**3** and benzo[*g*]chrysene-11,12-dihydrodiol (±)-**4** and hitherto unknown naphtho[1,2-*a*]pyrene-9,10- (±)-**5** and naphtho[1,2-*e*]pyrene-11,12-dihydrodiol (±)-**6** (Fig. 1). The results indicate that cellulose–tris-(*N*-3,5-dimethyl-phenylcarbamate) is the chiral selector of choice allowing a baseline separation of all six enantiomeric pairs of PAH dihydrodiols tested in this study.

2. Experimental

2.1. Chemicals

Racemic benzo[*c*]phenanthrene-3,4-dihydrodiol [8,11], benzo[*g*]chrysene-11,12-dihydrodiol [24,25], benzo[*c*]chrysene-9,10-dihydrodiol [26], and dibenzo[*a,l*]pyrene-11,12-dihydrodiol [27] and the corresponding optically active (*S,S*)-(+)- and (*R,R*)-(−)-enantiomers [9,11,24,26] used as reference materials were synthesized as previously reported. Synthesis of racemic naphtho[1,2-*a*]pyrene-9,10- and naphtho[1,2-*e*]pyrene-11,12-dihydrodiols will be published separately. Solvents of analytical grade were purchased from Merck (Darmstadt, Germany). All solvents were stored over molecular sieves (4 Å) and the mixtures prepared were filtered through a PTFE membrane with 1.5-μm pores before usage.

2.2. Columns

Two modified cellulose phases were tested as CSP: cellulose–tris-(4-methoxybenzoate) coated on 10 μm silica gel particles known as Chiralcel OJ (Baker, Gross-Gerau, Germany) and cellulose–tris-(*N*-3,5-dimethyl-phenylcarbamate) adsorbed on 5 μm silica gel particles known as Chiralcel OD-H (Baker). CSP-packed columns of 250×4.6 mm size were used equipped with pre-columns of 50 mm length.

2.3. Chromatographic conditions

Separations were carried out isocratically at 30°C. The mobile phases consisted of *n*-heptane with various portions of 2-propanol or ethanol as polar modifiers. Flow-rates were 1.0 and 0.5 ml min^{-1} for Chiralcel OJ and OD-H, respectively, resulting in pressures of 4.7 and 4.0 MPa with a mixture of *n*-heptane–ethanol (9:1, v/v) as mobile phase. Stock solutions were prepared by dissolving 1.0 mg of the *trans*-dihydrodiol in a mixture (1 ml) of dichloromethane–ethyl acetate (1:1, v/v) and subsequently diluted by 10-fold with the solvent mixture used as mobile phase. For HPLC runs a 5-μl aliquot of the stock solution was loaded on the column. Elution of the individual dihydrodiol enantiomers was monitored with a UV detector operating at 240 nm.

2.4. Circular dichroism (CD) spectroscopy

Fractions of several HPLC runs were collected and concentrated to obtain enantiomerically pure samples of *trans*-dihydrodiols. CD spectra of individual enantiomers were recorded in a range of 210 to 450 nm. The resolution was set to 0.5 nm and four separate spectra of each sample were accumulated.

2.5. Instrumentation

A Dionex HPLC system (Dionex, Idstein, Germany) was used consisting of a GP40 pump, an AS3500 autosampler with a column oven, and a Jasco UV970 UV detector (Jasco, Gross-Umstadt, Germany). The variable-wavelength UV detector was equipped with a 16-μl tapered flow cell and operated at 240 nm, a wavelength representing an absorption maximum of the pyrene chromophore. Data were collected and processed with Dionex PeakNet Software V4.10. The signs of optical rotation were measured in *n*-heptane–ethanol (9:1, v/v) using an IBZ Chiralyser optical rotation detector (IBZ-Messtechnik, Hannover, Germany). CD spectra of samples dissolved in *n*-heptane–ethanol (9:1, v/v) were taken at room temperature in a cylindric quartz cell of 5 mm path length (Hellma, Jena, Germany) using a Jasco J710 spectropolarimeter equipped with a Model J715 software data processor. CD spectra are expressed by elipticity in millidegrees.

3. Results and discussion

In this study it is demonstrated for the first time

that modified cellulose as a chiral selector – termed as type II CSPs by Wainer [28] – permits the direct separation of enantiomeric pairs of PAH dihydrodiol metabolites by CSP-HPLC. In previous reports various type I CSPs such as (*R*)-DNBPG and (*S*)-DNBL (Pirkle's chiral columns) or TAPA and related chiral support materials [22] have been successfully applied for the enantiomeric resolution of various oxygenated metabolites of PAH including monools [17], dihydrodiols [7,12,22], dihydrodiol epoxides [29] and arene oxides [7,19], but the direct separation of PAH dihydrodiol enantiomers with a double bond in the fjord-region appeared to be difficult on type I CSPs [23] or not possible at all [22]. Now it has been found for a series of six PAH dihydrodiols with this structural requirement (structures shown in Fig. 1) that the pairs of enantiomers can be resolved by CSP-HPLC on a Chiralcel OD-H column as illustrated in Fig. 2. The chromatographic data of all dihydrodiol enantiomers are compiled in Table 1.

3.1. Separation of enantiomers

Two commercially available modified cellulose-based CSPs and mixtures of *n*-heptane with different alcohols as mobile phases have been tested to optimize the partial enantiomeric resolution of dihydrodiol (±)-**1** initially observed on cellulose-tris-(4-methoxybenzoate) with *n*-heptane–2-propanol (65:35, v/v). (±)-**1** is the prototypic dihydrodiol possessing a double bond in the fjord-region and was selected, therefore, as the model compound. An optimal separation of (+)- and (−)-**1** (α=1.21; R_s =2.4) was achieved with a chromatographic system consisting of a Chiralcel OD-H column and a mixture of *n*-heptane–ethanol (9:1, v/v) as mobile phase (Fig. 2A). For all other dihydodiols investigated a similar good separation of enantiomeric pairs ($\alpha \geq 1.10$; $R_s \geq 1.6$) was observed (Table 1). Optical resolutions take less than 30 min for (±)-**1**, (±)-**3**, (±)-**5** and (±)-**6**, whereas separations of (±)-**2** and (±)-**4** was achieved within 50 min under the conditions used. All separations were performed at 30°C which is in the temperature range allowing an optimum resolution on cellulose-type CSPs [31]. Peak symmetry was always found to be better than 1.7 (calculated as ratio of peak width at 10% peak height).

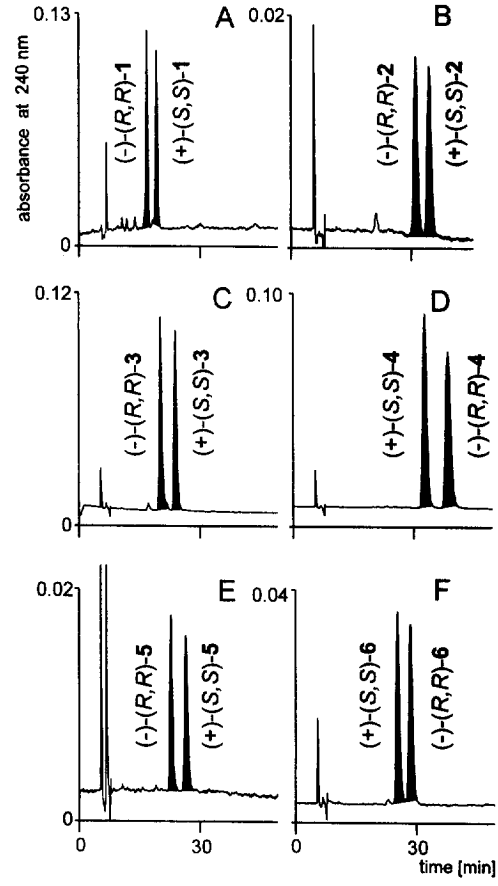

Fig. 2. Enantiomeric separation of *trans*-dihydrodiols of polycyclic aromatic hydrocarbons by CSP-HPLC on a Chiracel OD-H phase using a mixture of *n*-heptane–ethanol (9:1, v/v) as eluent at a flow-rate of 0.5 ml min^{-1}; benzo[*c*]phenanthrene-3,4-dihydrodiol **A**, dibenzo[*a,l*]pyrene-11,12-dihydrodiol **B**; benzo[*c*]chrysene-9,10-dihydrodiol **C**; benzo[*g*]chrysene-11,12-dihydrodiol **D**; naphtho[1,2-*a*]pyrene-9,10-dihydrodiol **E** and naphtho[1,2-*e*]pyrene-11,12-dihydrodiol **F**.

Under the standard conditions used the k' values of the hexacyclic dihydrodiol enantiomers of **2**, **5** and **6** are of comparable size and are larger than those of the smaller tetra-**1** and pentacyclic dihydrodiol **3** suggesting that a hydrophobic interaction of the solute and the OD-H phase significantly influences the retention time in addition to the chiral recognition mechanism, but under the non-aqueous conditions (heptane/alcohol) used here this interaction should be in principal of minor importance. However, it is worthwhile to note that the pentacyclic dihydrodiol **4**, for which both enantiomers

Table 1
Chromatographic parameters for the enantiomeric separation of PAH *trans*-dihydrodiols on a cellulose–tris(*N*-3,5-dimethylphenylcarbamate) chiral stationary phase[a]

Dihydrodiol	Enantiomer 1[b] k_1'[c]	Enantiomer 2[b] k_2'[c]	α[d]	R_s[e]
(±)-**1**	1.61 (*R,R*)	1.97 (*S,S*)	1.22	2.4
(±)-**2**	3.21 (*R,R*)	3.53 (*S,S*)	1.10	1.6
(±)-**3**	2.17 (*R,R*)	2.72 (*S,S*)	1.26	3.0
(±)-**4**	3.54 (*S,S*)	4.35 (*R,R*)	1.23	2.6
(±)-**5**	2.58 (*R,R*)[f]	3.18 (*S,S*)	1.23	2.7
(±)-**6**	2.97 (*S,S*)[f]	3.50 (*R,R*)	1.18	2.0

[a] Operating conditions of the Chiralcel OD-H column (250×4.6 mm): *n*-heptane–ethanol (9:1, v/v) as mobile phase at a flow-rate of 0.5 ml min^{-1}.
[b] Enantiomers are designated as 1 and 2 according to their elution order.
[c] k_1' and k_2' are the capacity factors of the eluting enantiomeric dihydrodiols [$k'=(t_R/t_0)-1$], where t_R is the retention time of the eluting enantiomer and the dead time (t_0=7.20 min) was estimated with 1,3,5-tri-*tert.*-butylbenzene [30,31].
[d] The selectivity α is defined as the ratio of the capacity factors of the two enantiomeric solutes (k_2'/k_1').
[e] The resolution $R_s=2(t_{R2}-t_{R1})/(W_2+W_1)$, where W is the peak width at base.
[f] Absolute configuration of enantiomers are assigned on the basis of CD Cotton effects and the sign of the optical rotation of the other dihydrodiols of known absolute structures.

have an even larger k' value than the hexacyclic enantiomeric pairs, whereas the chiral recognition by the OD-H phase is not significantly altered, as indicated by an only small increase of the selectivity α (Table 1). This suggests that the geometry of the aromatic moiety, is an important factor determining the interaction of the PAH dihydrodiols with the modified cellulose phase.

3.2. Elution order

For the enantiomeric pairs of the benzo[*c*]phenanthrene dihydrodiol **1**, the dibenzo[*a,l*]pyrene dihydrodiol **2** and the benzochrysene dihydrodiols **3** and **4**, the elution order of the enantiomers (Fig. 2 Table 1) was determined by co-chromatography with optically active reference compounds whose absolute structures have previously been elucidated unequivocally by the exciton chirality method [9,11,24,26]. From these co-chromatography experiments it was concluded that the first eluting peak contains the (−)-(*R,R*)-enantiomer (Fig. 2A Fig. 2B Fig. 2C) except for the resolution of (±)-**4**, which surprisingly showed an inverse elution order of the enantiomers (Fig. 2D). Since the absolute structures of the optically active dihydrodiols of **5** and **6** were hitherto unknown, the sign of the optical rotation was measured for individual enantiomers and used to assign the (*R,R*)-configuration to both (−)-enantiomers. This was possible due to the fact that a parallel exists between the sign of optical rotation and absolute structure of PAH dihydrodiols [2,10,12]. This assignment was further supported by comparing the Cotton effects in the CD spectra (Fig. 3) of the entire series of closely related PAH dihydrodiols studied herein. The CD spectra of the dihydrodiols are as yet not

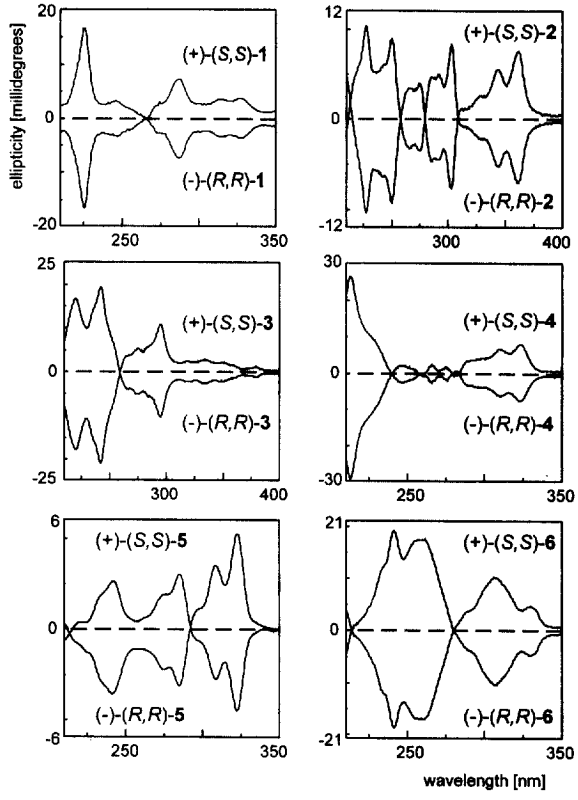

Fig. 3. Circular dichroism spectra of the enantiomers of benzo[*c*]phenanthrene-3,4-dihydrodiol **1**, dibenzo[*a,l*]pyrene-11,12-dihydrodiol **2**, benzo[*c*]chrysene-9,10-dihydrodiol **3**, benzo[*g*]chrysene-11,12-dihydrodiol **4**, naphtho[1,2-*a*]pyrene-9,10-dihydrodiol **5** and naphtho[1,2-*e*]pyrene-11,12-dihydrodiol **6**. Samples were prepared from collected fractions of several CSP-HPLC runs and, to improve the signal-to-noise ratio, four CD spectra were accumulated for individual enantiomers.

known except for those of (+)- and (−)-**1** [23] and may be useful for analyses of the stereoselectivity with which these dihydrodiols are formed by different metabolizing systems.

The chiral recognition of cellulose carbamate-based CSPs appears to result from a combination of various attractive interaction forces including hydrogen bonding, dipole–dipole interactions, and charge transfer complex ($\pi-\pi$) formations with the polar carbamate moiety (Ref. [32] and references cited therein). Among these interaction forces hydrogen bonding to the carbamate group plays a predominant role for the chiral recognition as pointed out by Okamoto et al. [33] using nuclear magnetic resonance (NMR) spectroscopy to characterize the interactions of enantiomeric *trans*-stilbene oxides with soluble cellulose–tris(4-trimethylsilylphenylcarbamate). In the present study, a closely related series of non-K-region PAH dihydrodiols having all a fjord-region double bond and adopting preferentially a conformation in less polar solvents with pseudo-diequatorially oriented hydroxyl groups are investigated [24,27]. The similar selectivity observed for all these PAH dihydrodiols (α ranging from 1.10–1.26; Table 1) suggests that the interaction of both or at least one of the hydroxyl groups with the carbamate moiety is important for the chiral resolution.

In addition, the sterical interaction dictated by an adopted helical conformation seems to play a major role in the chiral recognition mechanisms of modified cellulose phases ([34] and references cited therein). This conformation involves the formation of a chiral helical groove with rigid steric requirements ([34] and references cited therein) and may explain why an inverse elution order was noticed herein for the enantiomeric pair of dihydrodiol **4**. It could well be that the shape of the triphenylene residue in **4** allow an optimal steric fit into the helical groove, as indicated by the significantly higher capacity factors of both enantiomers (Table 1). This assumed exceptional steric fit into the helical groove may also be responsible for changing the strength of the interaction with the carbamate group of the two enantiomers leading to the inverse elution order. In line with this explanation, the same change in elution order was also observed for the enantiomers of the hexacyclic dihydrodiol **6** possessing a structure to which dihydrodiol **4**, is completely superimposable. However, dihydrodiol **6** is formally derived from **4** by annealing an additional benzo ring at the bay-region of **4**, which may result in a reduced steric fit into the helical groove and explain the smaller capacity factors of (+)- and (−)-**6** compared to those of the enantiomers of **4** (Table 1). It is of interest to note that for the isomeric hexacyclic naphthopyrene dihydrodiol **5** the first eluting enantiomer has again (*R*,*R*)-configuration. Taken the present results together, it appears that the OD-H phase permits an excellent enantiomeric separation of PAH dihydrodiols, but could not be used to certainly assign the absolute structure of dihydrodiols on the basis of the elution order as this has been demonstrated much more strictly for one of Pirkle's phases, namely for (*R*)-DNBPG [17].

4. Conclusions

The selection of a column for the chiral separation of a racemic mixture is as yet done empirically, and for the use of several modified cellulose phases some useful guidelines are available [35]. In the present study the high chiral recognition ability of the OD-H phase is further demonstrated by the enantiomeric resolution of PAH dihydrodiol metabolites and the method appears to offer a powerful tool for investigations of the enantioselectivity involved in PAH metabolism. In fact, this method has already been successfully applied in an accompanying study for the analysis of the enantiomeric composition of benzo[*c*]phenanthrene-3,4-dihydrodiol formed in different V79-derived cell lines expressing distinct human cytochrome P450 enzymes [8]. Furthermore, preliminary results with benzo[*a*]pyrene-7,8-dihydrodiol (Lansiedel et al., unpublished) and K-region benzo[*c*]phenanthrene-5,6-dihydrodiol [8] indicate applicability of the method for the chiral resolution of other PAH dihydrodiols with different structural features. Finally, the CSP-HPLC conditions described herein are thought to be suitable for scale up and thus for a preparative separation of enantiomeric pairs of dihydrodiols needed for the synthesis of their corresponding optically active fjord-region dihydrodiol epoxides to investigate their mutagenic and carcinogenic properties.

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