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# Chiral separation of the $\beta_2$-sympathomimetic fenoterol by HPLC and capillary zone electrophoresis for pharmacokinetic studies

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**ABSTRACT: The development of methods for the separation of the enantiomers of fenoterol by chiral HPLC and capillary zone electrophoresis (CZE) is described. For the HPLC separation precolumn fluorescence derivatization with naphthyl isocyanate was applied. The resulting urea derivatives were resolved on a cellulose tris(3,5-dimethylphenylcarbamate)-coated silica gel column employing a column switching procedure. Detection was carried out fluorimetrically with a detection limit in the low ng/mL range. The method was adapted to the determination of fenoterol enantiomers in rat heart perfusates using liquid–liquid extraction. As an alternative a CE method was used for the direct separation of fenoterol enantiomers comparing different cyclodextrin derivatives as chiral selectors. Copyright © 2010 John Wiley & Sons, Ltd.**

**Keywords**: fenoterol; precolumn derivatization; 1-naphthyl isocyanate; *S*-(+)-1-(1-naphthyl)-ethylisocyanate; enantioselective HPLC; Chiracel OD-RH, chiral CE; cyclodextrins; column switching

## Introduction

Fenoterol, 5-[1-hydroxy-2-[[2-(4-hydroxyphenyl)-1-methylethyl]-amino]ethyl]-1,3-benzenediol (Fig. 1) is $\beta_2$-adrenoreceptor agonist widely used in the treatment of asthma and tocolysis. Since this drug is a racemate and consists of *SS*-(+)-fenoterol and the pharmacologically active *RR*-(–)-fenoterol, there is increasing interest in characterizing the pharmacodynamic and pharmacokinetic properties of the enantiomers (Agranat *et al.*, 2002). Our knowledge on the specific properties of fenoterol enantiomers is limited due to the difficulty of measuring the enantiomers of this drug in body fluids at therapeutic concentrations. Thus, our aim was to develop a method for enantioselective determination of fenoterol in crystalloid perfusates for application in experiments with isolated perfused organs. In view of the possible importance of $\beta_2$-adrenoreceptor antagonist in the treatment of heart failure (Xiao *et al.*, 2004), the method was intended to be used to study cardiac uptake kinetics and inotropic effects of fenoterol in the isolated perfused rat heart.

Wainer's group (Beigi *et al.*, 2006) perfused isolated rat cardiomyocytes treated with the enantiomers of fenoterol and observed that *RR*(–)-fenoterol has an higher efficacy in inducing a positive inotropic effect .

Generally, chiral separations can be carried out by (a) direct separation of the enantiomers using chiral stationary phases or chiral mobile phase additives or (b) indirect separation by conversion of the enantiomers to diastereomers using a chiral reagent followed by chromatography on an achiral column (Gübitz and Schmid 2001).

Direct chiral HPLC separation of fenoterol was achieved on an amylose tris (3,5-dimethylphenylcarbamate) (Chiralpack AD) column (Beigi *et al.*, 2006) and on a vancomycin phase (Chirobiotic V, application guide, Advanced Separation Technologies, Whippany, NJ, USA). Chiral CE separations of fenoterol were described using cyclodextrin derivatives (Tanaka *et al.*, 1996; de Boer *et al.*, 1999; Wang *et al.*, 2001) and AGP (Tanaka and Terabe 1997) as electrolyte additives. However, these methods were not sensitive enough for determining fenoterol in biological samples. For enhancement in detection sensitivity the introduction of a fluorophore is a useful approach.

A series of chiral fluorescence derivatization reagents have been described in the literature (Toyooka, 1996). A widely used reagent represents *S*-(+)-1-(1-)naphthylethyl isocyanate [*S*(+)NEIC], which was applied among others to the chiral separation of $\beta$-blockers (Gübitz and Mihellyes 1984; Jira *et al.*, 1991; Laethem *et al.*, 1993; Belas *et al.*, 1995) mexiletine (Freitag *et al.*,

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**Abbreviations used:** DEHP, di(2-ethylhexyl)phosphate; DM-$\beta$-CD, dimethyl-$\beta$-CD; HP-$\beta$-CDNIC, hydroxymethyl-$\beta$-CD; *S*(+)NEIC, *S*-(+)-1-(1-)naphthylethyl isocyanate; TM-$\beta$-CD, trimethyl-$\beta$-CD.

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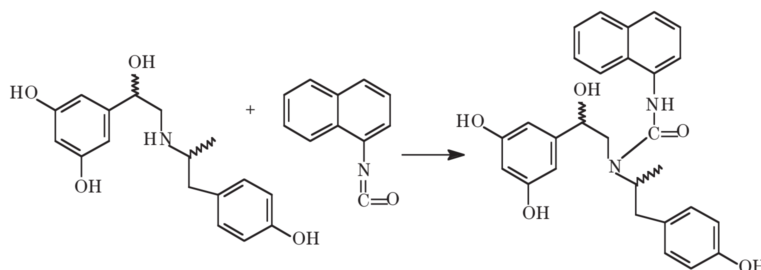


**Figure 1.** Structure of fenoterol and derivatization reaction.

1993) and mefloquine (Souri *et al.*, 1997). Achiral derivatization is mainly used for enhancing detection selectivity and sensitivity. Achiral 1-naphthyl isocyanate (NIC) was introduced as a fluorescence derivatization reagent by Wintersteiger *et al.* (Wintersteiger and Wenningerweinzierl, 1981; Wintersteiger *et al.*, 1982) and applied in the sequel by several authors. In a previous paper we described an enantioselective HPLC method for sympathomimetics as their fluorescent urea derivatives after reaction of NIC with their amine functions on a cellulose tris(3,5-dimethylphenylcarbamate) coated silica gel column (Chiracel OD-RH) (Ullrich *et al.*, 2001). With respect to developing a sensitive method for the determination of fenoterol in biological samples, we checked (a) the indirect separation on an RP column after chiral derivatization by *S*-(+)NEIC, (b) the achiral derivatization by NIC (Fig. 1) followed by chiral separation on a Chiracel OD-RH column and (c) the direct chiral separation by CZE comparing different CD derivatives.

## Experimental

### Materials and Reagents

Potassium dihydrogen phosphate, acetonitrile and di(2-ethylhexyl)phosphate (DEHP) were from Merck (Darmstadt, Germany); 1-naphthyl isocyanate and *S*-(+)-1-(1-naphthyl)ethyl isocyanate were from Aldrich (Deisenhofen, Germany); and $\beta$-CD, $\gamma$-CD, dimethyl-$\beta$-CD (DM-$\beta$-CD) trimethyl-$\beta$-CD (TM-$\beta$-CD) and hydroxymethyl-$\beta$-CD (HP-$\beta$-CD) were obtained from Cyclolab (Budapest, Hungary). Racemic fenoterol hydrobromide was from Sigma (Deisenhofen, Germany). Phosphoric acid (85%) was from Laborchemie (Apolda, Germany). The water used in the experiments was purified using Seralpur Pro 90 CN (SERAL, Germany). All chemicals were used without further purification procedures.

Aqueous Krebs Henseleit perfusion buffer was prepared by mixing 50 mL of a sodium chloride solution (107 g/L) with 50 mL of a solution of potassium chloride (5.96 g/L), sodium hydrogencarbonate (32.26 g/L), magnesium chloride hexahydrate (3.25 g/L) and with 50 mL of a calcium chloride bihydrate solution (5.17 g/L) and with 650 mL water.

### Instrumentation

**CZE.** All experiments were performed with an Agilent HP$^3$D CE (Agilent Technologies, Waldbronn, Germany) and fused-silica capillary (Supelco, Bellefonte, CA, USA) of 50 µm i.d. with a total length of 64.5 cm (effective length 55 cm). Detection was performed using the diode array detector at 200 nm with a bandwidth of 20 nm. All separations were performed with the capillary thermostated at 20°C and 30 kV applied as a positive potential to the inlet vial. New capillaries were conditioned by successively washing for 5 min each with 0.1 M phosphoric acid and water, followed by a 10 min flush with the background electrolyte (BGE) prior to use. Between separations, the capillary was conditioned with BGE for 5 min. All samples were filtered through 0.45 µm nylon membrane syringe filter (Schleicher, Schüll, Dassel, Germany), and introduced into the capillary at a pressure of 50 mbar for 3 s.

**HPLC equipment.** The analyses were performed on a Spectra-Physics system (pump P2000, UV–vis detector UV3000/FOCUS/SM5000, fluorescence detector FL2000, autosampler AS 1000, column switching system, Rheodyne injector 7125, Cotati, U.S.A.) connected to a 100 µL external loop, completed with a L-6200 pump (Merck, Darmstadt, Germany). For optical rotation measurements a LaChrom HPLC system ( Merck, Darmstadt, Germany) consisting of a diode array detector (L-7450) and a pump (L-7100) in connection with a Chiralyser polarimeter (IBZ Messtechnik, Hannover, Germany) was used. A Jetstream 2 column thermostat (WO Industrial Electronics, Austria) was employed for thermoregulation.

### Derivatization of the Pure Substance for Method Development

A slightly modified procedure developed by Gübitz and Mihellyes (1984) for derivatization of $\beta$-blocking agents with *S*-(+)NEIC was adapted to the derivatization of fenoterol hydrobromide with NIC and *S*-(+)NEIC, respectively. A 2 mg aliquot of the salt was dissolved in a freshly prepared solution of 0.85 µL 1-NIC or *S*-(+)NEIC and 1 mL dry chloroform and dimethyl formamide (8:2) and 3 µL triethylamine were added. After a reaction time of 15 min in an ultrasonic bath 20 µL of the reaction mixture were injected.

The structure of the NIC derivative was confirmed by MS.

### Separation of Diastereomeric *S*(+)NEIC Fenoterol Derivatives

Column: LiChrospher $RP_{18}$, 125 × 0.4 cm; 5 µm (Merck, Darmstadt, Germany); mobile phase, MeOH–water (55:45); UV detection, 220 nm; fluorescence detection, $\lambda_{ex}/\lambda_{em}$ 228/322 nm. The flow rate was 1.5 mL/min, and the injection volume was 20 µL.

### Separation of NIC Fenoterol Derivatives

**Achiral separation.** Column: LiChrospher $RP_8$, 125 × 0.4 cm; 5 µm (Merck,Darmstadt,Germany); mobile phase, MeOH–water (55:45); UV detection, 220 nm; fluorescence detection, $\lambda_{ex}/\lambda_{em}$ 232/388 nm. The flow rate was 1.5 mL/min, and the injection volume was 20 µL.

**Chiral separation.** Column: OD-RH 150 × 4.6 cm (Daicel, Tokio, Japan); mobile phase, MeCN/3.5 mM phosphoric acid pH 2.6 (60:40, v/v). The flow rate was 0.5 mL/min, and the injection volume was 20 µL.

### Column Switching

A 20 µL aliquot of the reaction mixture were injected on column 1 (RP-8). Elution of the fenoterol NIC derivative was done by dual pump 1 and monitored by UV detection (Fig. 2A). Meanwhile column 2 (Chiralcel OD-RH) was conditioned using pump 2 (the flow rate was 0.5 mL/min each). The derivative was transferred by switching to column 2 between 3 and 3.6 min (Fig. 2B). Column 1 was washed with MeCN (Fig. 2A). UV detection, 220 nm; fluorescence detection, $\lambda_{ex}/\lambda_{em}$ 232/388 nm.

**Mobile phases: MeCN–aqueous phosphoric acid (3.5 mM) mixtures.** Column 1: LiChrospher RP-Select B (125 × 0.4 cm i.d.; 5 µm). Precolumn 1: LiChrospher 100 $RP_{18}$ (5 µm).

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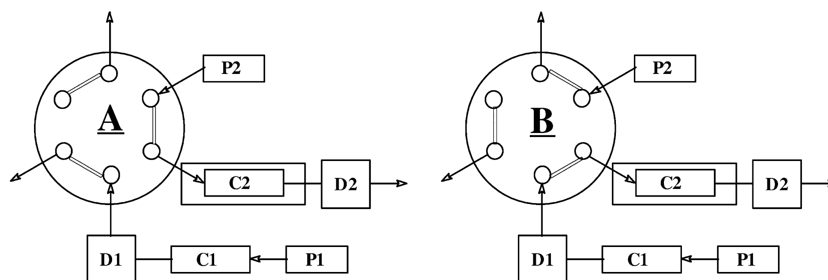


**Figure 2.** Column switching schedule for the chromatographic separation of the fenoterol enantiomers as NIC derivatives. P1, gradient pump 1—flow rate, 0.5 mL/min; mobile phase, MeCN–3.5 mM phosphoric acid, pH 2.6 (60:40, v/v), or MeCN. P2, pump 2—flow rate, 0.5 mL/min; mobile phase, MeCN–3.5 mM phosphoric acid pH 2.6 (60:40, v/v). C1, column 1—$RP_8$, 125 × 4.6 cm. C2, column 2—OD-RH 150 × 4.6 cm. D1, UV detector. D2, fluorescence detector. Switching schedule: 0–3.0 min position A; 3.0–3.6 min position B (transfer of the fenoterol zone to column C2); 3.6–16 min position A (washing of column C1 with MeCN).

Column 2: Chiralcel OD-RH (15 × 0.46 cm i.d.; 5 μm). Precolumn 2: LiChrospher 100 $RP_{18}$ (5 μm). Thermostat, 25°C. UV detection, 220 nm. FL detection, $\lambda_{ex}/\lambda_{em}$: 232/388 nm (for fenoterol derivates).

### Liquid–Liquid Extraction

Aqueous Krebs Henseleit perfusion buffer samples (0.45 mL, spiked with 10 μM fenoterol) were extracted with 1 mM DEHP in 1.1 mL methyl *tert*-butyl ether–ethyl acetate (1:1) and centrifugated for 15 min at 15,000 rpm. The organic phase was transferred to another vial to which 10 μL of a 0.1 M NIC solution was added. The solution was vortexed for 10 min and kept for 15 min in an ultrasonic bath. After evaporation the residue was dissolved in 200 μL mobile phase; 20 μL of the solution was injected.

## Results and Discussion

### CE Separations

Several authors have described CE methods for direct chiral separation of fenoterol (Tanaka *et al.*, 1996; Tanaka and Terabe, 1997; de Boer *et al.*, 1999). Among others, cyclodextrin derivatives such as HP-$\beta$-CD in a PEG 2000 gel (de Boer *et al.*, 1999) or $\beta$-CD-sulfobutylether and $\beta$-CD-phosphate (Tanaka *et al.*, 1996) were used.

We compared $\beta$-CD, g-CD, DM-$\beta$-CD, TM- $\beta$-CD and HP-$\beta$-CD for their ability to separate fenoterol enantiomers under different conditions. No separation was observed using g-CD and DM-$\beta$-CD. While $\beta$-CD showed a partial resolution, baseline resolutions were obtained with HP-$\beta$-CD and TM-$\beta$-CD. The influence of pH, ionic strength and selector concentration was investigated. It turned out that TM-$\beta$-CD showed the highest enantioselectivity for fenoterol using 50 mM phosphate buffer, pH 3.0, as electrolyte (Fig. 3). Although the CE approach represents a very simple and rapid method, the sensitivity was found to be not sufficient for determination of fenoterol enantiomers in biological samples. A LIF detector was not available.

### HPLC Separations

As an alternative we checked different HPLC methods for their applicability for the measurement of fenoterol enantiomers in biological samples. Although direct chiral separation of fenoterol by HPLC is possible, the formation of a fluorescent derivative was found to be necessary with respect to obtaining the required selectivity and sensitivity for pharmacokinetic investigations.

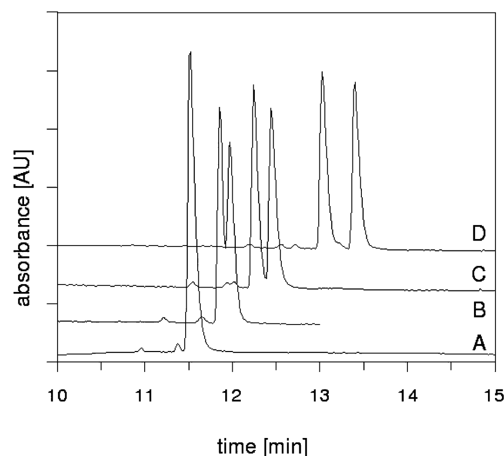


**Figure 3.** Chiral separation of fenoterol (0.1 mg/mL) by CZE. Chiral selector, 50 mM TM-$\beta$-CD; BGE, 75 mM phosphate buffer pH 3.0. Hydrodynamic injection, 30 kV, 20°C; detection, UV 200 nm. (A) Without CD; (B) 2.5 mM; (C) 7.5 mM; (D) 10 mM TM-$\beta$-CD.

In a previous paper we reported on the chiral separation of sympathomimetic drugs on a Chiracel OD-RH column after fluorescence derivatization with NIC. Figure 4 shows the chiral separation of fenoterol on the Chiracel OD-RH column. It was necessary to adapt the method to the determination of fenoterol enantiomers in biological matrices such as a rat heart perfusate. A column switching system was used (Fig. 2). Excess reagent and interfering matrix components were separated from the fenoterol derivative on a $RP_8$ column. The fenoterol derivative eluted between 3.0 and 3.6 min before the interfering components, controlled by UV detection. The fraction containing the fenoterol derivative was switched to the chiral column. As can be seen from Fig. 5, both enantiomers are well resolved on the chiral column without any interferences ($\alpha = 1.19$, Rs = 2.02). The enantiomer elution order was determined by polarimetric detection and was found to be *SS*-(+) before *RR*-(–).

As an alternative method we checked a chiral derivatization procedure using *S*-(+)NEIC as a chiral derivatization reagent. The diastereomeric derivatives were separated on an $RP_{18}$ column. However, as can be seen from Fig. 6, the sensitivity was significantly less compared with the NIC derivative.

For the application of the method to heart perfusates, liquid–liquid extraction of perfusates spiked with fenoterol was chosen.


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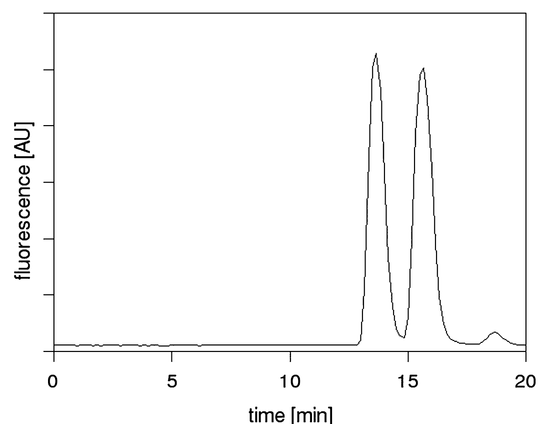


**Figure 4.** Chiral separation of NIC-fenoterol on the OD-RH column 150 × 4.6 cm; flow rate, 0.5 mL/min; mobile phase, MeCN–3.5 mM phosphoric acid pH 2.6 (60:40, v/v).

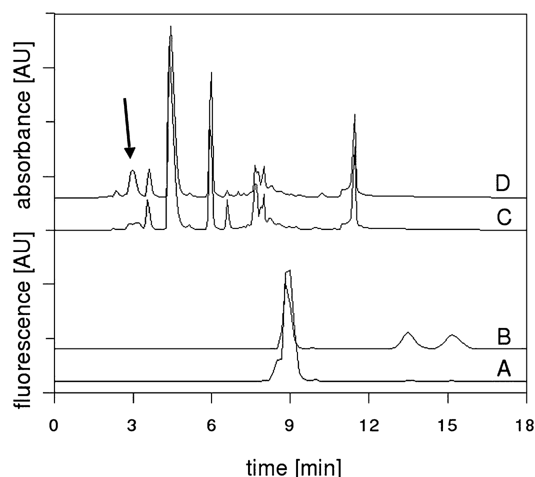


**Figure 5.** Chiral separation of the NIC-derivatives of fenoterol from a rat heart perfusate sample using column switching. (C) Blank; (D) sample on $RP_8$ column (arrow: fenoterol zone transferred to OD-RH-column); (A) blank; (B) fenoterol zone separated on OD-RH column; (C, D) UV-detection 220 nm; (A, B) fluorescence detection; $\lambda_{ex}/\lambda_{em}$ 232/388 nm.

As extraction solvents *tert*-butylmethyl ether and ethyl acetate with and without addition of the ion-pairing reagent (DEHP) were investigated. While with ethyl acetate without DEHP 65% was extracted, with *tert*-butylmethyl ether only 28% was obtained. It was found that ethyl acetate containing 1 mM DEHP resulted in a 95.6% recovery with an RSD of 3.4% ($n = 6$). The detection limit with fluorescence detection at a signal-to-noise ratio of 3:1 was 5 ng/mL heart perfusate. The linear range was between 5 and 50 ng/mL. Application of the method to isolated rat hearts regarding the different activities of the fenoterol enantiomers on heart is under investigation.

Our method could be also useful for other applications, such as, for example, the evaluation of enantioselective metabolism of fenoterol in perfused liver or placenta.

## Conclusions

Different methods for chiral separation of fenoterol were compared. Direct separation by CZE using different cyclodextrins was

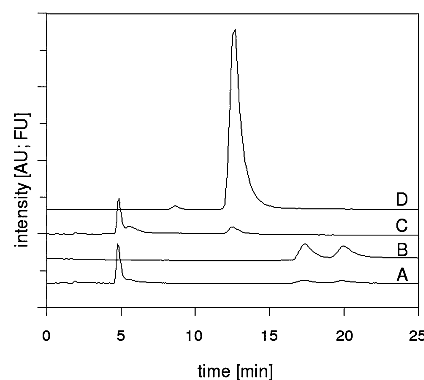


**Figure 6.** Comparison of the detection sensitivity of the diastereomeric *S*-(+)NEIC-fenoterol derivative and the NIC-fenoterol derivative, respectively, using UV detection (A, C) and fluorescence detection (B, D) on an $RP_{18}$ column. Mobile phase: MeOH–water (55:45, v/v); flow rate, 1.5 mL/min. UV detection, 220 nm; fluorescence detection, $\lambda_{ex}/\lambda_{em}$ 232/388 nm (NIC-derivative); $\lambda_{ex}/\lambda_{em}$ 228/332 nm [*S*-(+)NEIC-derivative].

found to be feasible, and TM-*β*-CD showed the best results. However, the sensitivity with respect to determining fenoterol in biological material was not sufficient. Therefore, as an alternative, HPLC techniques in combination with fluorescence detection were investigated. Achiral and chiral fluorescence derivatization with NIC and *S*-(+)NEIC, respectively, were compared and the sensitivity of the latter one was found to be less. In the case of NIC derivatization, a column switching system, combining an achiral and chiral column for removing interfering components, was developed.

The method was developed with respect to determining fenoterol in rat heart perfusates. It may be used for the enantioselective determination of fenoterol in various biological materials.

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