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4-(6-Methoxy-2-naphthyl)-2-butyl chloroformate enantiomers: New reagents for the enantiospecific analysis of amino compounds in biogenic matrices

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Abstract

(+)- and (−)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C) were prepared from the prochiral non-steroidal anti-inflammatory agent nabumetone with the aim of developing easily detectable chloroformate reagents for the enantiospecific HPLC analysis of amino compounds in biogenic matrices on achiral stationary phases. The new reagents were tested in the derivatization of β-adrenoceptor antagonists and anti-arrhythmic agents and allowed derivatization in the presence of water. (+)- and (−)-NAB-C were compared with other reagents with a 6-methoxy-2-naphthyl moiety as a chromophore. The reagents were suitable for the analysis of nanogram amounts of, for example, metoprolol enantiomers in plasma, a prerequisite for application in pharmaco- or toxicokinetic studies.

Keywords: Amines; Amino alcohols; Biological matrices; Derivatization; Enantiospecific HPLC; Fluorescent detection; (+)- and (−)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C)

1. Introduction

An important number of chiral biogenic as well as xenobiotic compounds with therapeutic or toxic impact are characterized by a primary or secondary amino moiety, e.g. amino acids, adrenoceptor agonists and antagonists, and anti-arrhythmic agents. Regardless of the chromatographic methods based on chiral stationary phases many different types of chiral derivatizing agents have been applied to the enantiospecific analysis of these substances (see Ref. [1] for an overview).

Primary and secondary amines readily react with activated carboxylic acids, e.g. α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl, Mosher's acid chloride) [2], (*R,R*)-*O,O*-diacetyltartaric acid anhydride (DATAAN) [3], *N*-pentafluorobenzoyl-(*S*)-(−)-prolyl 1-imidazolide [4], with sulphonic acid derivatives, e.g. (1*S*)-(+)-

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camphor-10-sulphonyl chloride [5], or with isocyanates, e.g. 1-phenylethylisocyanate (PEIC) [6]. The selective derivatization of primary amines is feasible with *o*-phthaldialdehyde and a chiral thiol [7]. However, often these adducts are not very stable and require on-line derivatization of the respective substrate [8]. Derivatization with isothiocyanates, e.g. 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) [9], yielding stable thioureas, or with chloroformates, e.g. (−)-menthyl chloroformate (MCF) [10,11] and 1-(9-fluorenyl)ethyl chloroformate (FLEC) [12], yielding carbamates, may also be used for the derivatization of primary and secondary amines. Both reagent types are applicable in the presence of water, a fact that is greatly appreciated in drug analysis with biogenic matrices.

Up to now, chiral chloroformates have received minor attention in the analysis of biogenic compounds and xenobiotics compared with different acylating agents, isocyanates and isothiocyanates. They can be readily prepared with a chiral alcohol and phosgene or one of its less toxic and less volatile substitutes (e.g. triphosgene [13,14]). In general, chloroformates are versatile chiral derivatizing agents (CDAs), applicable to the derivatization of primary and secondary amines [11,12, 15–17], amino acids [12], and amino alcohols [12,18–24]. Although a variety of functional groups may be subject to chloroformate derivatization, in many cases the choice of reaction conditions allows selective and rapid formation of carbamates from primary and secondary amines.

The application of chiral chloroformates to the liquid chromatographic separation of drugs and other amino compounds of interest—especially from biogenic fluids—has been limited to basically two reagents: MCF [10,11] and FLEC [12]. Both reagents are commercially available. MCF exhibits neither relevant UV absorbance nor any fluorescence. In contrast, FLEC—closely related to the more frequently used but achiral 9-(1-fluorenyl)methyl chloroformate (FMOC-Cl) [25–27]—is highly fluorescent (excitation about 265 nm, emission about 345 nm). FLEC was introduced by Einarsson et al. [12] for the derivatization of amino acids, imino acids, primary and secondary amines as well as *β*-blocking agents.

Though the application of chloroformate CDAs is favorable in the derivatization of primary and secondary amines, the present assays are primarily based on MCF—lacking any strong chromophore—and FLEC, which is highly fluorescent and also highly expensive. Therefore, the development of easily detectable chloroformate reagents with favorable chromatographic properties would be welcome. In previous studies several reagents derived from the 2-arylpropionic acid *S*-(+)-naproxen—with a 6-methoxy-2-naphthyl chromophore–have been developed and applied in this laboratory [28–33] as well as reagents from various other 2-arylpropionic acids [29,34–37].

It was the aim of the present study to prepare and test new easily-detectable chloroformate reagents derived from nabumetone (4-(6-methoxy-2-naphthyl)-2-butanone), a fluorescent prochiral ketone. The new reagents should combine the chromophoric properties of the previously developed chloroformate derived from *S*-(+)-naproxen (NAP-C) [30] with better resolution on achiral HPLC stationary phases. The new CDAs are compared with other reagents derived from naproxen and, finally, their applicability to bioanalytical purposes is shown.

2. Experimental

2.1. Chemicals and solvents

All chemicals were of analytical grade or HPLC grade—with respect to the HPLC mobile phases—and obtained from Merck (Darmstadt) unless stated otherwise. Water was obtained from a Millipore Milli-Q water purification system (Millipore, Eschborn, Germany). Phosgene (20% solution in toluene) was purchased from Fluka (Neu-Ulm, Germany). Triethylamine (>99%, gold label) and triphosgene (98%) were obtained from Aldrich (Steinheim, Germany) and *trans*-4-hydroxy-L-proline was from Sigma (Munich, Germany).

(*S*)-(+)-Naproxen (ee = 98.8%) was kindly provided by Grünenthal (Stolberg, Germany) and nabumetone by Smith Kline & Beecham (Munich, Germany). Reference samples of the various

drugs were supplied as follows: flecainide acetate and its enantiomers, Kettelhack Riker (Borken, Germany); propafenone hydrochloride and its enantiomers, Knoll (Ludwigshafen, Germany); tocainide hydrochloride, metoprolol tartrate and alprenolol hydrochloride and their enantiomers, Astra Chemicals (Wedel, Germany); mexiletine hydrochloride and its enantiomers, Boehringer Ingelheim (Ingelheim, Germany); acebutolol hydrochloride and diacetolol hydrochloride, Procter & Gamble (Weiterstadt, Germany); propranolol hydrochloride and its enantiomers, Zeneca (Plankstadt, Germany); oxprenolol and its enantiomers, Ciba-Geigy (Wehr, Germany); carvedilol and its enantiomers, Boehringer Mannheim (Mannheim, Germany).

2.2. Physicochemical characterization

Melting points were obtained using a Büchi apparatus, Model SMP 20 (Büchi, Flawil, Switzerland) and are uncorrected. Fluorescence spectra were partly recorded on a Perkin-Elmer fluorescence spectrometer Model 650-10 S (Perkin-Elmer, Überlingen, Germany) and a Knauer TY recorder (Knauer, Berlin, Germany). In general, UV and fluorescence data of most of the compounds, especially the diastereomeric derivatives of xenobiotic compounds, were obtained after chromatographic separation in the scan mode of the respective HPLC detector unit. Infrared spectra were obtained from a Perkin-Elmer 1420 ratio-recording photometer (Perkin-Elmer Überlingen, Germany) using KBr pellets for solid compounds (≈ 300 mg KBr per 1 mg compound). Oily compounds were directly determined as a liquid film between KBr windows. Wavenumber designations in cm^{-1} describe characteristic band positions of the particular compound. Rotational indices were determined on a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Überlingen, Germany) at a wavelength of 589 nm. Samples were dissolved in anhydrous methylene chloride and were kept at 20°C before being transferred to a 2 ml thermostatted microcuvette (length 9.998 cm).

Electron impact mass specta were recorded either on a Finnigan MAT 212 mass spectrometer (Finnigan, Bremen, Germany) by courtesy of Professor Dr. H. Linde (Department of Pharmaceutical Chemistry, J.W. Goethe-University, Frankfurt, Germany) or on a Finnigan MAT 711 mass spectrometer by courtesy of Professor Dr. R. Tacke (Department of Inorganic Chemistry, Karlsruhe University, Karlsruhe, Germany). ^{1}H-NMR spectra were recorded on a Bruker AC 300 spectrometer (Bruker, Karlsruhe, Germany) at the Department of Pharmaceutical Chemistry (Professor Dr. H. Linde) or on a Bruker WH 270 spectrometer at the Department of Organic Chemistry (Professor Dr. C. Griesinger, J.W. Goethe-University, Frankfurt, Germany). Samples were prepared by dissolving the compounds in deuterated chloroform or DMSO-d_6. Chemical shifts were reported in ppm (δ scale) downfield from internal tetramethylsilane standard.

2.3. Preparation of racemic 4-(6-methoxy-2-naphthyl)-2-butanol (NAB-OH)

4-(6-Methoxy-2-naphthyl)-2-butanone (nabumetone, 2 mmol) was dissolved in anhydrous diethyl ether (20 ml) and slowly added, with constant stirring, to a mixture of lithium aluminium hydride (0.55 mmol) in diethyl ether. The course of the reaction was monitored by TLC on silica gel plates. Aluminium complexes were hydrolyzed by adding water and dilute hydrochloric acid (≈ 6 M). The organic phase was separated in a funnel and the aqueous phase was extracted three times with 10 ml of diethyl ether. The combined ether layers were dried over sodium sulphate and subsequently evaporated to dryness. Racemic NAB-OH was stored in a desiccator and used without further purification.

2.4. Derivatization with phosgene

All synthetic steps were carried out under a well-ventilated hood. Phosgene-contaminated vapors were collected in a trap cooled with liquid nitrogen and carefully detoxified by adding dilute methanolic sodium hydroxide prior to disposal.

A solution of the alcohol (0.5 mmol) in 10 ml of dry toluene containing triethylamine (0.5 mmol) was placed in a tapered flask closed with a

silicon-rubber septum. The solution was cooled and kept at 0°C. 1 ml ($\approx$2 mmol) of a phosgene solution (20% in dry toluene) was added with a syringe under constant stirring. The reaction mixture was agitated with a magnetic stirrer for an additional 4 h. Precipitating salts were filtered off and discarded. The filtrate was concentrated in vacuo yielding the oily chloroformate. Derivatization of the alcohol with triphosgene, a solid substitute of phosgene, was also tested, yielding the chloroformate derived from NAB-OH.

2.5. Enantiomeric resolution of racemic 4-(6-methoxy-2-naphthyl)-2-butanol (NAB-OH)

Racemic NAB-OH (0.5 mmol) was dissolved in dry methylene chloride (20 ml) and absolute pyridine (0.55 mmol) was added. Then an excess of (1*S*)-(−)-camphanoyl chloride (1.0 mmol) was added in small portions under constant stirring. The solution was stirred at ambient temperature overnight. Excess pyridine was removed by washing three times with 1 M hydrochloric acid in a separatory funnel, a treatment that simultaneously hydrolyzed underivatized camphanoyl chloride. The organic phase was washed with water, saturated sodium bicarbonate solution, and again with water until neutral. The organic phase was separated, dried over magnesium sulphate and evaporated to dryness, yielding a yellowish solid residue of the camphanoyl ester (0.47 mmol). The crude product was purified via flash chromatography.

2.6. Flash chromatography

1 g of the crude camphanoyl ester was dissolved in a small amount of methylene chloride ($\approx$ 10 ml). The flash column (50 × 4 cm i.d.) was filled with 200 g of silica gel 60, particle size 40–63 μm (230–400 mesh), and the eluent consisted of methylene chloride–methanol (99.5:0.5, v/v). The flow rate was 45 ml min^{-1} at a pressure of 4×10^4 Pa.

2.7. Analytical HPLC and enantiomeric purity of underivatized NAB-OH

The analytical scale chromatographic system consisted of a Merck Hitachi L-6200A intelligent pump (Merck, Darmstadt, Germany), an AS-4000 intelligent autosampler with 200 μl injection loop, an L-4500 diode-array detector with a D-6000 interface connected to a personal computer with a Model D-6500 chromatography data station software and a DAD system manager.

The DAD set-up was: spectral bandwidth, 7 nm; wavelength range, 200–400 nm; spectral interval, 400 ms. The monitoring wavelength was set to 230 nm. Peaks were quantified based on area calculations. Determination of the enantiomeric purity of resolved NAB-OH was performed on a Chiralcel OD-H column (J.T. Baker, Gross-Gerau, Germany) with a mobile phase of *n*-hexane–2-propanol (90:10, v/v) at a flow rate of 0.9 ml min^{-1}.

2.8. Analytical HPLC and diastereomeric purity of NAB-OH camphanoyl esters

Determination of the diastereomeric purity of the camphanoyl ester was performed on a (*R*)-DNBPG covalent column (J.T. Baker, Gross-Gerau, Germany) with a mobile phase of *n*-hexane–2-propanol (70:30, v/v) at a flow rate of 1.0 ml min^{-1}.

2.9. Preparative-scale HPLC

The preparative chromatographic system consisted of a Gilson Model 303 pump (Abimed, Langenfeld, Germany), a Gilson Model 201-202 controller, a Gilson Model 201 fraction collector, a Gilson model 804 manometric module, and a Rheodyne injection valve with 20 ml loop (Rheodyne, Cotati, CA). The separation process was monitored with a Knauer UV/Vis filter photometer (Knauer, Berlin, Germany) at a fixed wavelength of 220 nm and a Knauer TY strip chart recorder.

The sorbent was packed into stainless-steel Prepbar® cartridges, 250 × 50 mm i.d. (Merck, Darmstadt, Germany). The preparative resolution of the diastereomeric camphanoyl ester was performed on (*R*)-DNBPG covalent material with a mobile phase of *n*-hexane–2-propanol (70:30, v/v) at a flow rate of 65 ml min^{-1}. Parts of the solvents were recycled to avoid unnecessary waste of resources.

2.10. Cleavage of the camphanoyl esters

The diastereomerically pure camphanoyl esters of NAB-OH (5 mmol) were dissolved in dry diethyl ether (30 ml). The solution was added dropwise to 2.5 mmol of lithium aluminium hydride in diethyl ether kept at 0°C under argon. The liquids were stirred at ambient temperature for 60 min. Consequently, the reaction mixture was kept in an ice-bath prior to hydrolysis. Complexes were hydrolyzed by adding a small amount of aqueous ammonium chloride solution (10 g per 100 ml). While collecting the ether layers the aqueous phase was extracted three times with diethyl ether. The combined organic phases were dried over magnesium sulphate and afterwards evaporated to dryness. The raw product was subject to flash chromatography as described for camphanoyl esters, yielding the pure NAB-OH enantiomers.

2.11. Derivatization of amino substrates with NAB-C

20 μl aliquots of the substrate stock solutions (1 mM in methanol or water) were pipetted into a screw-capped tube and diluted with 50 μl borate buffer pH 8 (Merck, Darmstadt, Germany). 50 μl of the chloroformate reagent (18 mM in acetone) was added and the solution was swirl-mixed. The mixture was kept at ambient temperature for 0.5 h. The reaction was stopped by addition of 100 μl *trans*-4-hydroxy-L-proline (10 mM) in water. After 2 min an aliquot of the solution was injected directly onto the column for reversed-phase analysis. Injection on normal-phase columns required an additional extraction step: 2 ml of methylene chloride was added and the solution was swirl-mixed for 30 s. The aqueous phase was discarded and the organic solvent was evaporated to dryness in vacuo. The residue was reconstituted in 100 μl mobile phase and an aliquot injected onto the HPLC column.

2.12. Chromatographic behaviour of the derivatives

The HPLC separation of the diastereomeric products was performed either on a reversed-phase system or on a silica-gel stationary phase. Stainless-steel columns (250 × 4 mm i.d.) were packed with Zorbax ODS material (particle size, 5 μm; Bischoff, Leonberg, Germany) for reversed-phase separations or Zorbax-Sil (particle size, 5 μm; Bischoff) for normal-phase separations. The respective mobile phases consisted of acetonitrile–water or *n*-hexane–2-propanol in varying compositions. Further details with regard to mobile phase composition are given in Section 3. The detection wavelengths were set to 230 nm (UV detection), or 270 nm (excitation) and 365 nm (emission) for fluorescent detection.

2.13. Comparison of various CDAs for the derivatization of amino compounds

Stock solutions of the reagents (NAP-chloride, NAP-isocyanate, NAP-isothiocyanate, NAP-chloroformate, and NAB-chloroformate) were prepared in toluene (10 mM). Racemic alprenolol hydrochloride was dissolved in methanol, yielding a final concentration of 1 mM. 20 μl aliquots of alprenolol solution were evaporated to dryness and recovered in 100 μl of anhydrous toluene–triethylamine (100:1, v/v). After addition of 50 μl of reagent solution the mixtures were allowed to stand at ambient temperature for 1 h, except those containing NAP-isothiocyanate as reagent. They were kept at 50°C for a 60 min period. Solutions were evaporated to dryness, reconstituted in mobile phase and aliquots were analyzed by HPLC. The reaction conditions were the respective standard conditions and were not optimized for every single reagent.

2.14. Enantiospecific determination of metoprolol in plasma

Racemic metoprolol tartrate (MW 684.8 g mol^{-1}) and racemic flecainide acetate (MW 474.4 g mol^{-1}) were dissolved in water at a concentration equivalent to 200 μg ml^{-1} free base per enantiomer. (+)-NAB-chloroformate was dissolved in dry acetonitrile at a concentration of 100 μg ml^{-1}. Stock solutions were stored at 8°C prior to use. Standard solutions were prepared prior to use by diluting equivalents of the respec-

tive stock solutions with water, yielding final concentrations of 2 μg ml^{-1} and 200 ng ml^{-1} metoprolol enantiomer and 2 μg ml^{-1} flecainide enantiomer respectively.

1 ml of human plasma was pipetted into a screw-capped glass tube, spiked with 50 μl of internal standard solution and alkalinized with 1 ml of pH 11 borate buffer. The liquid was thoroughly swirl-mixed before 5 ml of diisopropyl ether was added. The tubes were agitated on a reciprocating shaker for 15 min and centrifuged at 4000 rev min^{-1} (− 10°C). The aqueous layer was frozen in a dry ice–acetone bath and discarded. The organic layer was transferred to glass tubes and evaporated in vacuo.

Extracts were reconstituted in 100 μl of 0.01 M hydrochloric acid and adjusted to pH 11 by adding 200 μl of borate buffer. 50 μl of reagent solution—(+)-NAB-chloroformate in acetonitrile—was added. The liquids were swirl-mixed and allowed to react protected from direct light for 1 h. The derivatives were extracted by adding 3 ml of methylene chloride and swirl-mixing for 30 s. The aqueous phase was discarded. The organic layer was evaporated to dryness in vacuo. The residues were dissolved in 100 μl of *n*-hexane–methanol (100:6, v/v) and 20 μl aliquots were analyzed by HPLC.

The chromatographic system consisted of a Jasco PU-980 HPLC pump (Jasco, Groß-Zimmern, Germany), a programmable Jasco UV-975 UV/Vis detector, a Jasco AS-950 autosampler with tray cooling unit and 100 μl injection loop, a Shimadzu RF 551 fluorescence monitor (Shimadzu, Duisburg, Germany), a data acquisition box (Nuclear Interface, Münster, Germany), and a computerized HPLC management system operating with NINA® software, Version 4.0, Rev. 1 (Nuclear Interface, Münster, Germany).

A stainless-steel column packed with bare silica gel (Nucleosil 120; particle size, 3 μm; 125 mm × 3 mm i.d.) (Bischoff, Leonberg, Germany) was used as stationary phase protected by a guard column filled with Ultrasep ES 100 (particle size, 6 μm; 20 mm × 4 mm i.d.) (Bischoff, Leonberg, Germany). The mobile phase consisted of *n*-hexane–methanol (100:0.4, v/v) which was delivered at a flow rate of 1.0 ml min^{-1}. Detection wavelengths were set at 230 nm (UV) and excitation 270 nm/emission 350 nm (fluorescence). The sample injected onto the HPLC column amounted to 20 μl.

2.15. Derivatization time course and yield

Working solution aliquots of 100 ng metoprolol per enantiomer (50 μl) and 50 μl of internal standard solution were diluted in 200 μl of borate buffer pH 11. 50 μl of reagent solution was added and the mixture was thoroughly swirl-mixed. Reaction was stopped after given periods of time by adding 200 μl of 1 M hydrochloric acid. The samples were worked up in the usual way and HPLC-analyzed. Peak areas of the analytes and internal standard were plotted against time.

2.16. Stability of the derivatization products

Samples of 100 ng of (R)-(+)-metoprolol of known enantiomeric purity were spiked to drug-free plasma, extracted, derivatized and analyzed as described above. The derivatized samples were recovered in *n*-hexane–methanol and aliquots were injected into the HPLC system. The samples were re-analyzed after overnight storage in the cooled tray of the autosampler.

2.17. Assay validation

Overall recovery of metoprolol enantiomers was determined by spiking drug-free plasma with concentrations equal to 10 and 100 ng ml^{-1}, followed by extraction, derivatization and HPLC analysis as described above. Peak heights of the respective compounds were compared with those of the same concentrations evaporated to dryness and immediately recovered in derivatization media. Calibration curves were established by analyzing spiked plasma samples at six different concentrations before every analytical run. Concentration of metoprolol enantiomers was plotted against the peak area ratio of the respective metoprolol enantiomer and the internal standard. The data were analyzed by linear regression analysis. Intraday variability was tested by analyzing replicate samples of spiked human plasma at concen-

trations of 2, 10 and 100 ng metoprolol enantiomer ml^{-1} ($n = 6$). Spiked plasma samples at concentrations of 2, 10 and 100 ng metoprolol enantiomer ml^{-1} were evaluated on three different days to establish interday variability.

2.18. Blood sampling

A single dose of 100 mg of immediate release racemic metoprolol tartrate (Beloc®) was given to a healthy male volunteer (age, 30 years; body weight, 63 kg) having fasted overnight. Blood samples were drawn from an intravenous catheter in the forearm and collected in heparinized plastic tubes before and 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4.5, 5, 6, 7.5, 9, 12, and 24 h after drug administration. The samples were centrifuged immediately and the plasma stored at −20°C until analysed.

3. Results

3.1. Enantiomeric resolution of racemic 4-(6-methoxy-2-naphthyl)-2-butanol (NAB-OH)

3.1.1. Analytical HPLC and determination of the enantiomeric purity of NAB-OH

Various chiral stationary phases (CSPs) were tested for the analytical resolution of NAB-OH enantiomers. These tests were for screening preparative HPLC separations as well as for the determination of the optical purity of NAB-OH enantiomers. Included were different phase types, e.g. cellulose triacetate, bonded β-cyclodextrin, "brush"-type (Pirkle) columns, and stationary phases based on derivatized carbohydrates.

Close to baseline resolution was achieved on a Chiralcel OD-H column within 10 min. Reversed-phase separations on Chiralcel OD-R and OJ columns were also feasible but required retention times of about 30 min with a loss of resolution quality. However, none of these columns was available in dimensions suitable for the resolution of 2–3 g NAB-OH.

3.1.2. Derivatization with camphanoyl chloride

Since underivatized NAB-OH could not be resolved on the available preparative CSPs and derivatization with other CDAs, e.g. MTPA-Cl, failed to resolve racemic NAB-OH into its enantiomers—either by liquid chromatography or by crystallization—the alcohol was coupled to (−)-camphanoyl chloride. The diastereomeric camphanoyl esters were well resolved on different HPLC columns, including silica gel, octadecylsilyl, cellulosetriacetate and dinitrobenzoylphenylglycine-based stationary phases. However, the analytical resolutions of NAB-OH camphanoyl esters on achiral normal- and reversed-phase columns were not transferable to separations on a preparative scale. Resolution diminished on the available preparative columns leaving the diastereomers barely separated. Best results were obtained from a chiral (*R*)-*N*-3,5-(dinitrobenzoyl)phenylglycine stationary phase with the chiral selector covalently bonded to silica gel.

On a preparative scale the diastereomers were resolved within 60 min. The timing of repetitive injections allowed the separation of two 100 mg samples within 1 h. Solvents were recycled while no peaks eluted. A diastereomeric purity of 99.7% was achieved for the first eluting compound—(+)-NAB-OH camphanoyl ester—and a purity of 98.9% for the second eluting compound after evaporation of the solvent and recrystallization from 2-propanol. The purified camphanoyl esters were cleaved with lithium aluminum hydride, yielding NAB-OH enantiomers after flash chromatography. The enantiomeric excesses were ee = 99.3% for (+)-NAB-OH and ee = 95.6% for (−)-NAB-OH. Specific rotations were $[\alpha]_D^{20} = +11.8°$ and $-12.6°$ respectively. Derivatization of the resolved alcohols led to chloroformates with an ee > 99% for (+)-NAB-C and an ee = 98.5% for (−)-NAB-C.

Fig. 1 illustrates the preparative pathway leading to purified NAB-OH enantiomers.

3.2. Physicochemical characterization

3.2.1. 4-(6-Methoxy-2-naphthyl)-2-butanol (NAB-OH)

Molecular formula, $C_{15}H_{18}O_2$; molecular weight ($g\ mol^{-1}$), 230.29; melting point, 94–95°C; optical rotation (°), $[\alpha]_D^{20} = +11.8$ and -12.6 (CH_2Cl_2, C = 1); IR (cm^{-1}), 3350 (OH); mass

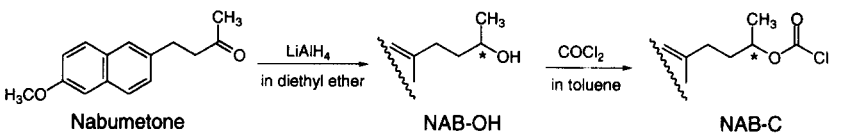

Fig. 1. Synthetic pathway leading to chloroformates derived from nabumetone, (+)- and (−)-NAB-C.

spectrometry (*m/z*), 230.1 (M^+, 74.1%), 172.1 ($M^+ - CH_2CH(CH_3)OH$, 100%); ^{1}H-NMR (δ, ppm), 1.16–1.18 (d, 3H, <CH–C<u>H</u>$_3$), 1.40 (s, 1H, –OH), 1.72–1.81 (m, 2H, –CH$_2$C<u>H</u>$_2$–CH<), 2.67–2.87 (m, 2H, aryl–C<u>H</u>$_2$), 3.73–3.81 (m, 1H, <C<u>H</u>–CH$_3$), 3.83 (s, 3H, H_3CO–aryl), 7.03–7.66 (m, 6H, aromatic protons; yield (%), 89.

3.2.2. 4-(6-Methoxy-2-naphthyl)-2-butyl chloroformate (NAB-C)

Molecular formula, $C_{16}H_{17}O_3Cl$; molecular weight (g mol^{-1}), 292.75; melting point, not determined (oily compound); optical rotation (°), $[\alpha]_D^{20} = +30.9$ and -30.9 (CH_2Cl_2, C = 1); IR (cm^{-1}), 1773 (C=O); mass spectrometry (*m/z*), 292.1 (M^+, 1.15%), 171.1 ($M^+ - CH_2CH(CH_3)OCOCl$, 100%); ^{1}H-NMR (δ, ppm), 1.40–1.42 (d, 3H, <CH–C<u>H</u>$_3$), 1.89–2.21 (2m, 2H, –CH$_2$–C<u>H</u>$_2$–CH<), 2.73–2.93 (m, 2H, aryl–C<u>H</u>$_2$–), 3.91 (s, 3H, H_3CO–aryl), 4.94–5.05 (m, 1H, <C<u>H</u>–CH$_3$), 7.12–7.72 (m, 6H, aromatic protons); yield (%), 91.

3.3. Chromophoric properties of reagents and derivatives

The spectra of the respective reagents were recorded in the scan mode of the HPLC fluorescence or UV monitor. The solvent was acetonitrile or the eluent used for chromatographic separation of the derivatives. NAB-C enantiomers have the following excitation and emission optima: $\lambda_{ex} = 262$ nm; $\lambda_{em} = 353$ nm. UV maxima at 226 nm, usually the wavelengths taken for optimum excitation in fluorescence detection, could not be employed due to a rising baseline and a significantly increasing noise level. UV absorption was monitored at 230 nm, the UV maximum.

3.4. Derivatization of amino substrates with NAB-C

Derivatizations with NAB-C were usually performed in borate buffer pH 8 with the reagent dissolved in acetonitrile. Reactions could be stopped by adding a solution of hydroxyproline to the reaction mixture. Extraction of the diastereomeric carbamate derivatives into methylene chloride was necessary for normal-phase HPLC investigations. Derivatization under anhydrous conditions, e.g. in toluene with TEA as a proton scavenger, was also feasible. Exact derivatization conditions for the reaction of NAB-C with metoprolol enantiomers after extraction from plasma are described later.

3.5. Chromatographic behaviour of the derivatives

Carbamate derivatives with NAB-C were separated on reversed chromatographic phases (Table 1) as well as on normal-phase columns (Table 2), where the diastereomeric derivatives of β-adrenoceptor antagonists and anti-arrhythmic agents were usually better resolved.

3.6. Comparison of various CDAs for the derivatization of amino compounds

To compare the characteristics of different CDAs deriving from (*S*)-(+)-naproxen and nabumetone the chiral β-adrenoceptor antagonist alprenolol was taken as the model substrate. The following CDAs were included in the survey (Fig. 2): the acid chloride (NAP-Cl), the isocyanate (NAP-IC), the isothiocyanate (NAP-IT), the chloroformate (NAP-C)—all deriving from (*S*)-(+)-naproxen—and the chloroformate deriving from nabumetone (NAB-C). Toluene was the solvent for all reactions, containing

Table 1
HPLC separation parameters of the diastereomeric carbamates of β-adrenoceptor antagonists and anti-arrhythmic agents after derivatization with NAB-C on reversed-phase columns. Column A: Zorbax ODS 5 μm, 250 mm × 4.0 mm i.d.; Column B: Jasco Sep Sil C_{18} 5 μm, 250 mm × 3.0 mm i.d.; mobile phase (MP): acetonitrile–water (v/v); flow rate: 0.8 ml min^{-1}; n.d. = not determined; 1st = substrate configuration of the first-eluting diastereomer after derivatization with (−)-NAB-C

Substrate	k'_1	k'_2	α	R	1st	Column	MP
Atenolol	8.2	8.8	1.07	1.0	(*S*)-(−)	A	50:50
Diacetolol	12.0	12.8	1.07	0.8	(*S*)-(−)	A	50:50
Metoprolol	20.1	21.1	1.05	0.6	(*S*)-(−)	B	50:50
Acebutolol	19.9	21.0	1.06	1.1	(*S*)-(−)	B	45:55
Propranolol	7.2	8.3	1.05	0.4	(*S*)-(−)	A	80:20
Alprenolol	9.2	9.5	1.03	0.4	(*S*)-(−)	A	80:20
Tocainide	12.7	15.3	1.20	1.8	(*S*)-(+)	A	53:47
Mexiletine	26.0	27.8	1.07	1.8	(*S*)-(+)	A	65:35
Propafenone	36.2	36.8	1.02	n.d.	n.d.	A	65:35

0.1% triethylamine as a proton scavenger. All derivatizations were performed at ambient temperature except for the thiourea formation where an elevated temperature of 50°C was necessary.

Table 3 summarizes separation parameters. Mobile phases were delivered at a flow rate of 1.0 ml min^{-1}. Mobile phase compositions were *n*-hexane–2-propanol 100:1 (v/v), for derivatizations with NAP-IT, NAP-C, and NAB-C, 100:2 for NAP-Cl, and 100:4 for NAP-IC with respect to normal-phase HPLC and acetonitrile–water, 75:25 (v/v) for NAP-IT, NAP-C, and NAB-C, 70:30 for NAP-IC, and 65:35 for NAP-Cl. The elution order of the diastereomers is depicted in Fig. 3, where the respective chromatograms of normal- and reversed-phase separations are given.

Abnormal peak shapes were observed with the diastereomeric amides deriving from alprenolol and NAP-Cl, especially on normal-phase HPLC columns. Derivatizations with the isocyanate NAP-IC did not lead to defined diastereomeric products but to two pairs of derivatives which were resolved by normal-phase HPLC.

3.7. Enantiospecific determination of metoprolol in plasma

3.7.1. Derivatization procedures

For metoprolol enantiomers derivatized with NAB-C (solution in acetonitrile) in borate buffer pH 11 peak areas reached maximum values about 40 min after reaction onset (see Fig. 4). No significant differences between both enantiomers were detected, indicating the absence of kinetic resolution. The derivatization time was prolonged to 60 min in the analysis of metoprolol from plasma to ensure complete derivatization of the substrate. Employing an achiral assay, no metoprolol peaks were detected for 100 ng amounts of the enantiomers of the β-adrenoceptor antagonist after a 40 min period. The derivatization yield therefore exceeds 95%.

3.7.2. Racemization and stability

Racemization was studied with (*S*)-(−)-metoprolol of known enantiomeric purity (ee = 96.4%). The value determined after derivatization with (+)-NAB-C was 96.5% and is thus in the expected range, indicating that no racemization occurred during analysis. Values obtained from samples analyzed after overnight storage in the cooled tray of the autosampler were of the same magnitude within the limits of statistical error.

3.7.3. Recovery

Plasma samples were buffered to pH 11 and metoprolol enantiomers were extracted into diisopropyl ether (DIP). Extractions with other solvents, e.g. methylene chloride or *n*-hexane containing small amounts of different aliphatic alcohols, were not successful. Either recovery of metoprolol was too low or matrix compounds interfering with the HPLC analysis of metoprolol derivatives were co-extracted. Recovery–ex-

Table 2
HPLC separation parameters of the diastereomeric carbamates of β-adrenoceptor antagonists and anti-arrhythmic agents after derivatization with (−)-NAB-C; column: Zorbax-Sil 5 μm, 250 mm × 4.0 mm i.d., mobile phase (MP): *n*-hexane–2-propanol; flow rate: 1.5 ml min^{-1}; compositions (v/v): MP 1, 100:1.0; MP2, 100:1.5; MP3, 100:5.0; MP4, 100:0.25; 1st = substrate configuration of the first-eluting diastereomer

Substrate	k'_1	k'_2	α	R	1st	MP
Alprenolol	3.2	3.9	1.22	2.4	(*R*)-(+)	1
Propranolol	5.1	6.3	1.24	3.4	(*R*)-(+)	1
Metoprolol	9.5	11.1	1.17	2.7	(*R*)-(+)	2
Acebutolol	22.3	23.4	1.05	<0.2	(*R*)-(+)	3
Tocainide	11.0	13.0	1.18	2.6	(*R*)-(−)	2
Flecainide	11.3	14.3	1.27	3.0	(*S*)-(+)	2
Mexiletine	1.1	1.3	1.18	0.8	(*R*)-(−)	2
Propafenone	13.4	14.2	1.06	0.8	(*R*)-(−)	2

pressed as overall recovery after derivatization with the chloroformate deriving from nabumetone (NAB-C)—was determined at two concentrations, 10 and 100 ng enantiomer ml^{-1} ($n = 6$). Overall recovery was 82.3 ± 6.3% for (*S*)-(−)-metoprolol and 81.9 ± 5.9% for (*R*)-(+)-metoprolol at 100 ng ml^{-1} and 84.2 ± 8.7% for (*S*)-(−) and 82.9 ± 7.5% for (*R*)-(+) at 10 ng ml^{-1}. No significant difference between the two enantiomers could be observed.

3.7.4. Linearity

Calibration was performed before every analytical run over a concentration range from 2–100 ng metoprolol enantiomer per ml (see Fig. 5). Peak area ratios of metoprolol enantiomers versus internal standard—(*R*)-(−)-flecainide—were plotted against drug concentration. Calibration curves were linear over the investigated range with a slope of 0.03071 ± 0.00133 and an intercept of 0.00611 ± 0.01825 for the (*S*)-(−)-enantiomer and a slope of 0.03149 ± 0.0006 and an intercept of −0.01649 ± 0.01614 for the (*R*)-(+)-enantiomer ($n = 6$). Correlation coefficients were > 0.999 for both enantiomers. The limit of quantitation was 2 ng ml^{-1}, the lowest concentration of the calibration curve. The limit of detection obtained at a signal-to-noise ratio > 3 was 0.9 ng ml^{-1}.

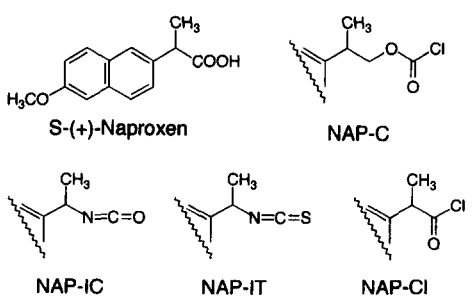

Fig. 2. Chiral derivatizing agents derived from the 2-arylpropionic acid *S*-(+)-naproxen.

3.7.5. Intra-day and inter-day variability

Within-day accuracy and precision were determined by analyzing six replicate samples each for high, intermediate, and low concentrations. Values for between-day variability were established by evaluating the recalculated values of the calibration curves ($n = 6$). Table 4 summarizes the results for both metoprolol enantiomers.

3.7.6. Investigation of metoprolol plasma levels

A single oral dose of 100 mg rac-metoprolol tartrate (Beloc®) was given to a healthy male volunteer having fasted overnight. The person had a light breakfast about 1.5 h after drug intake. Plasma samples were obtained from an intravenous catheter. Fig. 6 depicts the chromatographic separation of metoprolol enantiomers recovered from plasma and derivatized with NAB-C. 45 min after intake the drug yielded maximum plasma concentrations with $C_{max} = 69.4$ ng ml^{-1} for (*S*)-(−)-metoprolol and 67.2 ng ml^{-1} for (*R*)-(+)-metoprolol. A second, but smaller, peak in the plasma concentration–time

Table 3
Separation parameters of the diastereomeric derivatives obtained from the β-adrenoceptor antagonist alprenolol after derivatization with reagents derived from naproxen and nabumetone. n.r. = not resolved

Reagent	Normal-phase				Reversed-phase			
	k'_1	k'_2	α	R	k'_1	k'_2	α	R
NAP-Cl	4.1	4.7	1.14	1.1	12.5	13.7	1.09	0.6
NAP-IC	5.3	6.3	1.18	2.0	5.8	6.4	1.10	0.6
NAP-IT	5.1	5.3	1.04	0.6	6.5	8.2	1.25	2.7
NAP-C	5.7	6.3	1.10	1.4	9.7	9.7	1.00	n.r.
(+)-NAB-C	4.0	4.7	1.17	2.0	13.8	14.6	1.06	0.8

curve occurred 4.5 h after drug intake for both enantiomers. Plasma levels were significantly higher for the (*S*)-(−)-enantiomer (Fig. 7).

4. Discussion

(*S*)-(+)-Naproxen has been the starting material for fluorescent CDAs in previous investigations. To yield chloroformates with the same chromophore and different side-chain arrangements, the non-steroidal anti-inflammatory drug 4-(6-methoxy-2-naphthyl)-2-butanone (nabumetone) was included in the present study.

Since preparation of CDAs in an analytically-oriented laboratory requires simple preparative steps, the alcohol precursors of the chloroformates were prepared by reduction of the prochiral ketone with lithium aluminium hydride (LAH) in anhydrous media. The corresponding racemic alcohol had to be resolved into its enantiomers. A clean-up of the reactive chloroformate was not possible; therefore, the racemic alcohol precursor was subject to optical resolution.

Enantiomeric separation of racemic alcohols may generally be achieved either directly via preparative liquid chromatography on CSPs (see Ref. [38] for a review) or indirectly via chiral derivatization, with for example an activated carboxylic acid, separation of the diastereomers and subsequent cleavage of the derivatives yielding the enantiopure compound. Separation of diastereomers is feasible by fractional crystallization [39], enantioselective enzymatic cleavage [40,41], or preparative liquid chromatography on achiral stationary phases. Since preliminary derivatization studies with for example MTPA-Cl or camphanoyl chloride and fractional crystallization failed to resolve NAB-OH enantiomers, and usually high losses have to be expected with this technique, where a high enantiomeric purity is required, preparative HPLC was chosen as suitable means of resolution. A total of nine CSPs of different types e.g. β-cyclodextrin, cellulose triacetate, cellulose and amylose derivatives, and amino acid derivatives, were included in the screening. Close to baseline resolution was achieved for NAB-OH enantiomers either rapidly in the normal-phase mode on a Chiralcel OD-H column or in the reversed-phase mode on Chiralcel OD-R and OJ material with longer retention times and an inferior resolution compared to Chiralcel OD-H. However, none of the stationary phases was available in a size suitable for the separation of milligram to gram amounts of the alcohol.

Derivatization with (1*S*)-(−)-camphanoyl chloride yielded the diastereomeric esters of NAB-OH which were readily resolved on the majority of the tested stationary phases. Finally, a chiral Pirkle-type stationary phase [42], covalently bonded (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine, was selected since it yielded the best preparative resolution.

HPLC on a preparative scale may comprise specific problems: poor solubility of the substance in a mobile phase may be one of them. NAB-OH camphanoyl esters were not soluble in small amounts of *n*-hexane–2-propanol or the alcoholic component. To reduce sample volume and the

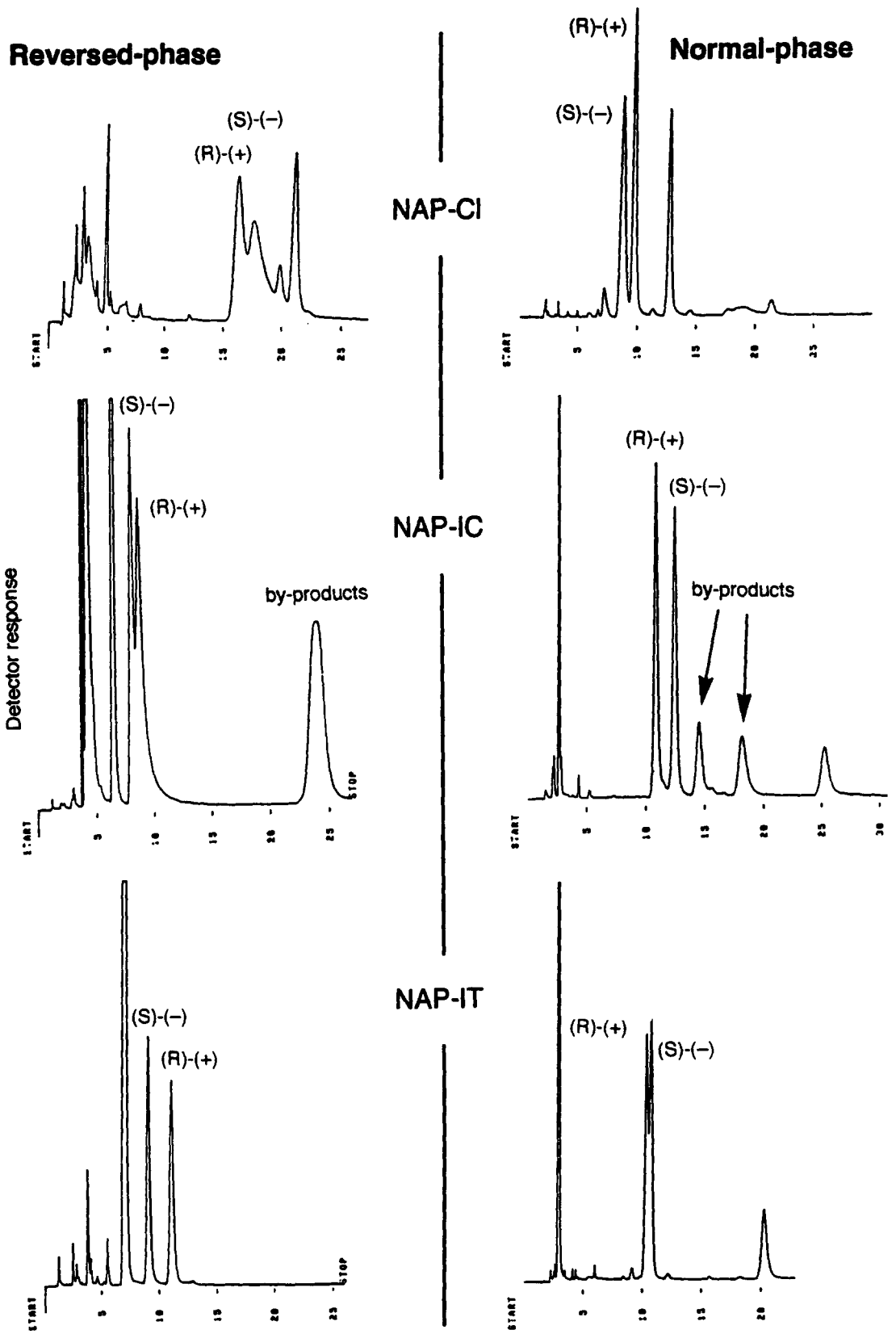
Reversed-phase
Normal-phase
(S)-(−)
(R)-(+)
NAP-CI
(R)-(+)
(S)-(−)
(S)-(−)
(R)-(+)
by-products
NAP-IC
Detector response
(R)-(+)
(S)-(−)
by-products
(S)-(−)
(R)-(+)
NAP-IT
(R)-(+)
(S)-(−)

Fig. 3.

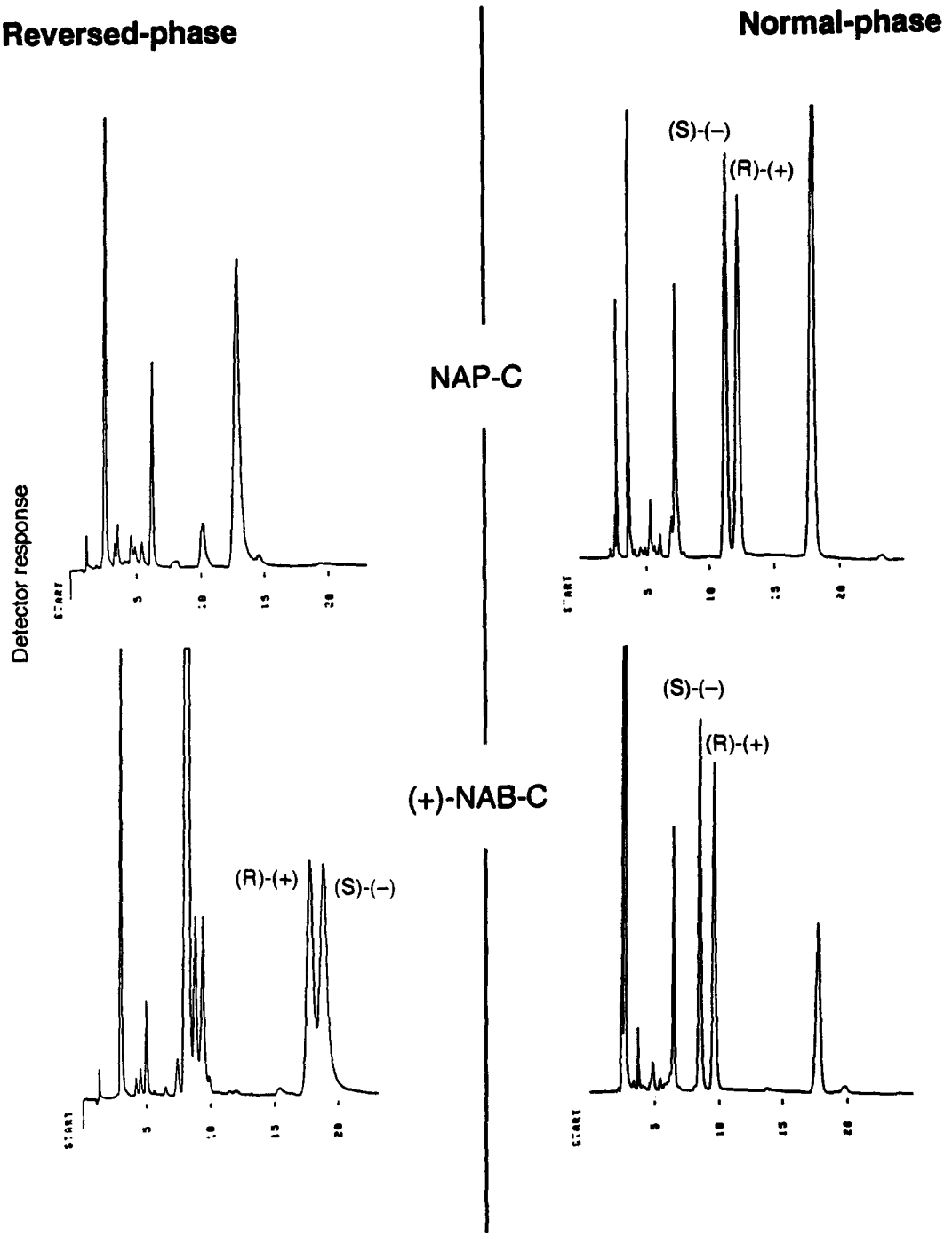

Fig. 3. Normal- and reversed-phase separations of the diastereomeric derivatives of alprenolol with CDAs derived from (*S*)-(+)-naproxen and nabumetone. Reversed-phase chromatograms are given in the left-hand column, normal-phase chromatograms in the right-hand column. Separation parameters: normal-phase: column, Zorbax-Sil 5 μm, 250 mm × 4.0 mm i.d.; mobile phase, *n*-hexane–2-propanol; flow rate, 1.0 ml min^{-1}; reversed-phase: column, Zorbax ODS 5 μm, 250 mm × 4.0 mm i.d.; mobile phase, acetonitrile–water; flow rate, 1.0 ml min^{-1}; detection, UV 230 nm. Mobile phase compositions were *n*-hexane–2-propanol 100:1 (v/v) for derivatizations with NAP-IT, NAP-C, and NAB-C, 100:2 for NAP-Cl, and 100:4 for NAP-IC with respect to normal-phase HPLC and acetonitrile–water 75:25 (v/v) for NAP-IT, NAP-C, and NAB-C, 70:30 for NAP-IC, and 65:35 for NAP-Cl.

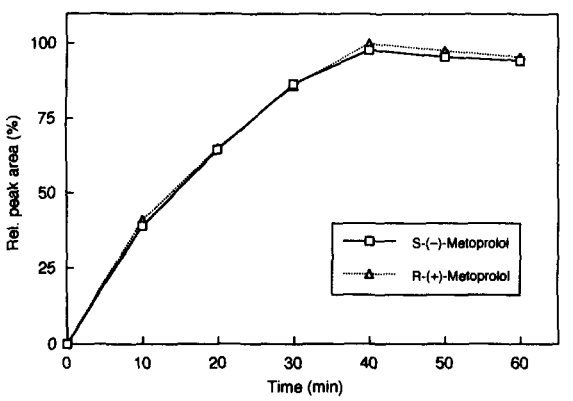

Fig. 4. Derivatization–time course for the reaction of metoprolol enantiomers with (+)-NAB-C in borate buffer pH 11 at ambient temperature.

subsequent peak broadening, minor amounts of methylene chloride were added. To obtain pure fractions of the individual diastereomers, precise timing of the fraction collector and repetitive separation of the second—and more impure—fraction was necessary.

Recrystallization of the recovered fractions led to products of high diastereomeric purity. Since the camphanoyl moiety is a good leaving group and may be substituted by hydroxyl when alkaline cleavage is applied, hydride reduction was chosen to avoid racemization during work-up. Thus, NAB-OH enantiomers of a high enantiomeric purity were prepared. A related procedure has been described for the preparation of 1-(9-fluorenyl)ethanol enantiomers, the precursors of FLEC [12].

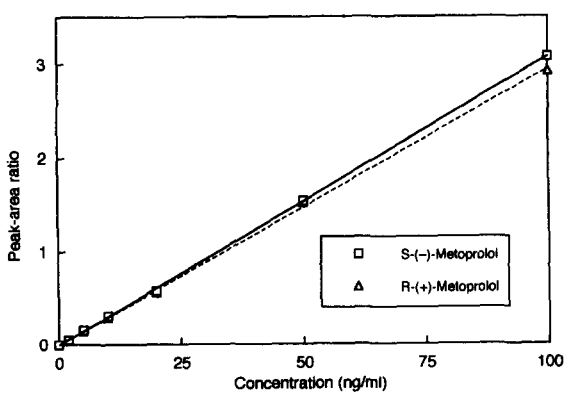

Fig. 5. Calibration curve for the analysis of metoprolol enantiomers after extraction from plasma and derivatization with (+)-NAB-C. (*R*)-Flecainide served as an internal standard.

The separation process made both optical isomers of NAB-OH available so that both enantiomers of the respective chloroformate CDA were prepared—a favorable situation in the analysis of small amounts of one enantiomer in the presence of large amounts of its counterpart; the elution order of potential derivatives can easily be reversed by simply choosing the other CDA enantiomer.

In summary, a combination of chiral derivatization and subsequent preparative HPLC was necessary to resolve rac-NAB-OH into its enantiomers, representing a vivid example of the power and importance of CDAs in liquid chromatography.

The standard approach to chloroformate synthesis is the direct reaction of an alcohol precursor with phosgene [43]. However, phosgene may be substituted by the less volatile and therefore less toxic triphosgene. Whenever chloroformates of a very high chemical purity are required on a small scale phosgene is preferred since it can easily be removed from the reaction mixture.

4.1. Analytical screening of (+)- and (−)-NAB-C

Detectability is a very important factor in the analysis of xenobiotics from biogenic matrices. The newly-developed reagents for the derivatization of amino compounds were outlined for reagent-oriented detection. They all have in common the 6-methoxy-2-naphthyl moiety, which combines high UV absorption ($\varepsilon = 100\,000$ at $\lambda =$ 230 nm for naproxen [44]) with an intrinsic fluorescence the intensity of which is significantly higher compared to that of a simple naphthyl fluorophore [45].

Maximum UV absorption is found around 230 nm with a shoulder at 270 nm. However, the UV maximum cannot be used for fluorescence excitation with conventional variable wavelength fluorescence monitors, due to an increasing noise level. HPLC detectors with cut-off filters may be an alternative. The fluorescence intensity achieved with both chloroformates (+)- and (−)-NAB-C and the respective carbamate derivatives was com-

Table 4
Within-day and between-day assay precision and accuracy for the analysis of (*S*)-(−)- and (*R*)-(+)-metoprolol from plasma

Metoprolol enantiomer	Conc. added (ng ml^{-1})	Within-day assays				Between-day assays			
		Conc. found ($n = 6$) (mean ± SD)		RSD (%)	RE (%)	Conc. found ($n = 6$) (mean ± SD)		RSD (%)	RE (%)
(*S*)-(−)	2	2.25	±0.32	14.2	+12.5	1.61	±0.21	13.0	−19.5
	10	9.97	±1.02	10.2	−0.3	9.20	±1.3	14.1	−8.0
	100	98.1	±12.3	12.5	−1.9	99.0	±1.9	1.9	−1.0
(*R*)-(+)	2	1.98	±0.18	9.0	−1.0	2.1	±0.36	16.9	+5.0
	10	9.03	±0.84	9.3	−9.7	8.7	±1.0	11.5	−13.0
	100	99.0	±12.9	13.0	−1.0	99.8	±2.3	2.3	−0.2

parable with those known from other naproxen-derived CDAs. Compared to the fluorescence yields seen with the fluorenyl moiety in FLEC or benzoxazole derivatives—e.g. flunoxaprofen-, benoxaprofen-derived reagents—the fluorescence of the 6-methoxy-2-naphthyl moiety is less prominent, yet strong enough to significantly enhance detectability of derivatized compounds.

4.2. Chromatographic behaviour of the derivatives

β-Adrenoceptor antagonists and anti-arrhythmic agents are model compounds for drugs with a primary or secondary amino moiety. They are usually applied as racemic mixtures, although in many cases stereoselective pharmacokinetics and pharmacodynamics have been observed. Therefore, selected β-adrenoceptor antagonists and anti-arrhythmic agents were included in the present investigations as substrates for the derivatization with isothiocyanates and chloroformates.

No resolution was obtained from the derivatives of the chloroformate derived from (*S*)-(+)-naproxen (NAP-C) on reversed-phase systems. Under normal-phase conditions NAP-C derivatives were less resolved than the carbamates derived from NAB-C, the CDA derived from nabumetone. The reason is obviously the larger distance between both chiral centers in the case of NAP-C derivatives [46,47]. In the carbamate derived from NAP-C six bonds separate both stereocenters compared to five in the derivatives of NAB-C and NAP-IT. An additional aspect, less conformational rigidity [48] in the case of NAB-C derivatives—an ethylene moiety separates the naphthyl moiety from the chiral center—compared to NAP-IT, may explain the superiority of the isothiocyanate to the chloroformates in the reversed-phase separations of the respective derivatives.

Both chloroformates had to be compared to FLEC. FLEC may be superior in the reversed-phase separation of propranolol and analogs. However, the primary amine tocainide is far better resolved after derivatization with NAB-C, the chloroformate derived from nabumetone [12]. NAB-C is especially suitable for normal-phase separations where most derivatives show selectivities (α) > 1.18 and are baseline-resolved. Equivalent separations for FLEC have not been published so far.

4.3. Comparison of various CDAs for the derivatization of amino compounds

Several CDAs derived from (*S*)-(+)-naproxen or related compounds with a 6-methoxy-2-naphthyl chromophore are available to date, most of them designed for the derivatization of primary and secondary amines. Spahn [28] introduced the acyl chloride derived from naproxen (NAP-Cl), and the isocyanate analog was synthesized and tested by Martin and co-workers [29,45]. The preparation and application of the respective

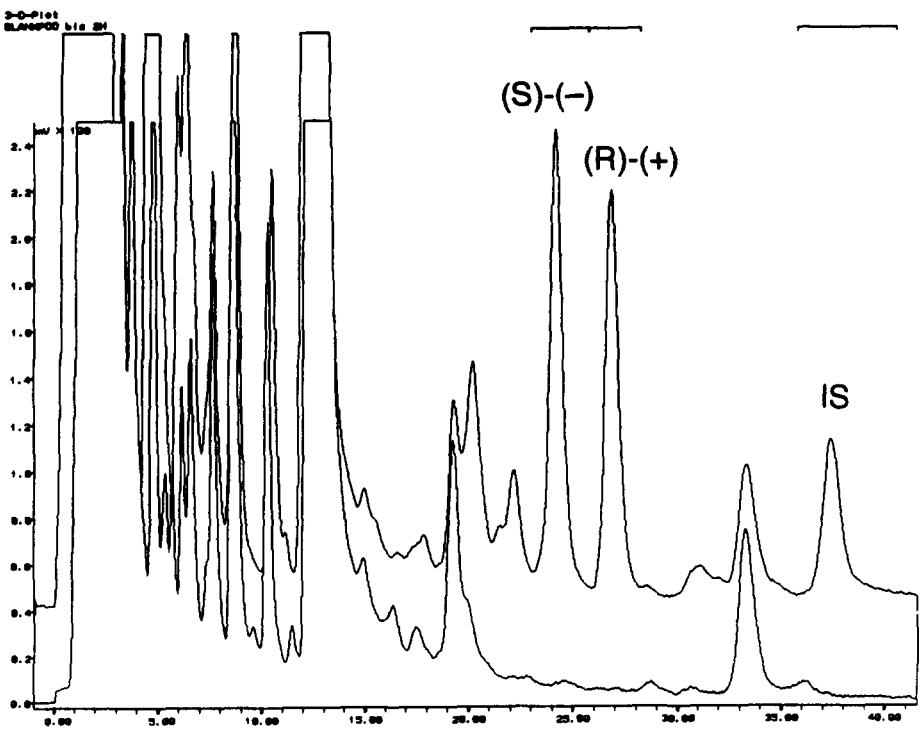

Fig. 6. Normal-phase HPLC separation of the NAB-C derivatives of metoprolol enantiomers with (*R*)-(–)-flecainide as internal standard. The β-adrenoceptor antagonist was recovered from a plasma sample collected 2 h after peroral intake of 100 mg of rac-metoprolol tartrate. The lower trace depicts a chromatogram obtained from drug-free plasma. (Column: Nucleosil 120 3 μm, 125 mm × 3 mm i.d.; mobile phase: *n*-hexane–methanol 100:0.4 (v/v); flow rate: 1.0 ml min^{-1}; detection: fluorescence (excitation 270 nm, emission 350 nm)).

isothiocyanate (NAP-IT) and chloroformate (NAP-C) have been described previously [30].

Consequently, one may ask which is the most favorable CDA and which one should be chosen in a specific situation? A comparison of the chromatographic properties of the diastereomeric derivatives of a single substrate, the β-adrenoceptor antagonist alprenolol, under different chromatographic conditions (normal- and reversed-phase) is certainly limited, yet may provide valuable information.

The acid chloride derived from (*S*)-(+)-naproxen—NAP-Cl—provides sufficient selectivity (α) on normal- and reversed-phase systems. However, deformed peak shapes were noticed on both types of stationary phase. Extensive peak tailing on the reversed-phase system will certainly prohibit quantitative measurement of the diastereomeric ratios. Even on the normal-phase system both diastereomeric amides showed different peak shapes, although the peak areas were of equivalent sizes. Furthermore, by-products of the highly reactive reagent that may interfere with potential analytes were detected especially in the normal-phase chromatogram.

Although reversed-phase separations of NAP-IC derivatives have been reported for various substrates [29,45], the urea derivatives of alprenolol enantiomers were baseline-resolved only on a silica gel column. A disadvantage in the analysis of alkanolamines—e.g. β-adrenoceptor antagonists—may be the high reactivity of the reagent. Isocyanates may react not only with amines but also with alcohols at ambient temperature, yielding the mono- and bi-derivatives of the respective alkanolamines with variable composition.

The best reversed-phase separation of alprenolol was achieved with the NAP-IT derivatives. A selectivity (α) of 1.25 and a resolution

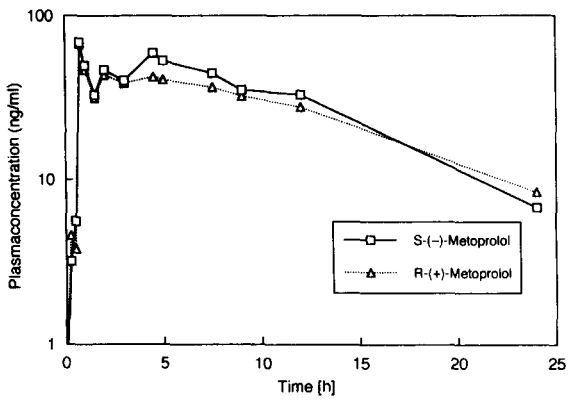

Fig. 7. Plasma concentration–time curve of metoprolol enantiomers in a volunteer after a single oral dose of 100 mg of rac-metoprolol tartrate.

factor of 2.5 allowed baseline resolution within 12 min. In contrast with NAP-IC derivatives a defined pair of products was yielded in spite of a slightly elevated temperature (50°C) during reaction. Normal-phase separations were less successful than with the isocyanate derivatives, probably due to weaker hydrogen bonding between the thiocarbonyl moiety of the derivatives and the stationary phase. Therefore, NAP-IT may be the preferred reagent in the selective derivatization of amino compounds when easy separation on reversed-phase columns, plus a high UV absorption is required. In addition, the isothiocyanate may be applied in the presence of water, like both chloroformates, a favourable property with respect to the development of bioanalytical methods.

Good normal-phase separations were obtained from the alprenolol derivatives of both chloroformate reagents. While NAP-C derivatives were not separated on a reversed-phase system, the carbamates derived from NAB-C and the β-adrenoceptor antagonist were at least partly resolved. The derivatives were easily detectable with a standard fluorescence monitor.

The chloroformate derived from nabumetone (NAB-C) is therefore especially recommended in the selective derivatization of amino compounds combined with fluorescence detection of the derivatives. Furthermore, the reagents and derivatives are characterized by different lipophilicities (the mobile phases were optimized to compare the resolution potential of each reagent with the derivatives eluting in a defined time window). Evaluation of the retention times of the derivatives and the corresponding mobile phases suggests the following rank order of lipophilicity:

$$\text{NAP} - \text{IC} \leqslant \text{NAP} - \text{Cl} < \text{NAP} - \text{IT} < \text{NAP} - \text{C} < \text{NAB} - \text{C}$$

Thus, the choice of the reagent may be based not only on the separability of the diastereomeric derivatives, but also on the physicochemical properties of the substrate and interfering matrix compounds, the preferred stationary phase, and the available detector.

4.4. Enantiospecific determination of metoprolol in plasma

To test the applicability of a selected CDA [(+)-NAB-C] to the bioanalysis of the xenobiotic metoprolol, (*R,S*)-(+)-1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-2-propanol was chosen as a model compound for a racemically applied drug with enantioselective pharmacokinetics [49]. The drug represents a rather lipophilic β_1-selective adrenoceptor antagonist applied in the treatment of coronary artery disease (CAD), tachyarrhythmia, and hypertension. Plasma concentrations of the drug enantiomers may vary widely due to genetically determined Cytochrome P_{450} polymorphism [50].

Metoprolol was first HPLC-analyzed in biogenic matrices by Hermansson and Von Bahr [51]. They used derivatization with the symmetric anhydrides of *tert*-butoxycarbonyl-L-leucine (BOC-L-leucine) and subsequent removal of the BOC moiety followed by reversed-phase separation of the diastereomeric derivatives. Derivatization with chiral isocyanates (see e.g. Refs. [52,53]) or isothiocyanates [54] and bioanalysis on different CSPs (see e.g. Refs. [55–60]) has been applied as well.

In the present study metoprolol enantiomers were derivatized in borate buffer pH 11, thus yielding a better linear correlation of the calibration curve than with derivatization at pH 8–9, the pH usually described in the literature [12]. Since the derivatization reaction was linear over a wide

concentration range and free from kinetic resolution and racemization, the diastereomeric carbamates derived from metoprolol enantiomers and (+)-NAB-C were well resolved on a silica gel stationary phase with (*R*)-(−)-flecainide as internal standard. Accuracy—expressed by the deviation from the nominal values—and reproducibility of the assay—expressed by intra- and inter-day variabilities—were within the limits given by the conference on *Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies* (Washington, DC, December 1990) [61]. Thus, this derivatization reaction was suitable for bioanalytical purposes.

Metoprolol exhibits a weak intrinsic fluorescence and may be monitored at the respective excitation and emission wavelengths [51,52,55,62]. A sensitive detection requires measurement at very short wavelengths. Hermansson and Von Bahr [51] used an excitation wavelength of 193 nm and achieved limits of quantitation below 1 ng ml^{-1} per enantiomer. However, operation at these wavelengths is feasible only with a special type of fluorimetric detector with cut-off filters and not with the usually applied variable wavelength fluorimetric detectors with monochromators. Pflugmann et al. [52] applied fluorescence detection at $\lambda_{ex} = 265$ nm and $\lambda_{em} = 313$ nm with a limit of detection of approximately 2 ng ml^{-1} after derivatization with the non-fluorescent PEIC. A limit of quantitation (LOQ) of 5 ng ml^{-1} was described for the naphthyl analog (NEIC) [53]. A study with the isothiocyanate GITC as derivatizing agent only gave a LOQ of 10 ng ml^{-1}. Limits of detection (LODs) with measurement of metoprolol fluorescence and direct separation on a CSP are usually about 4–5 ng ml^{-1} [55–57] at $\lambda_{ex} \approx 270$ nm and $\lambda_{em} \approx 310$ nm.

A LOQ of 2 ng metoprolol enantiomer per ml plasma and a LOD of 0.9 ng ml^{-1} for the assay appears very favourable. Since the derivatized samples were reconstituted in 100 μl of mobile phase with only 20 μl injected onto the HPLC column, at least 200 pg of metoprolol enantiomers is detectable. However, LOQ and LOD were both predominantly set by matrix effects and not determined at all by the fluorescence yield of the reagent. To improve the assay, further sample clean-up by pre-chromatographic removal of excess reagent and by-products, e.g. by solid-phase extraction of the derivatized sample [21], is therefore recommended.

In essence, CDAs derived from 2-arylpropionic acids and both the chloroformates derived from nabumetone, unlike many other reagents proposed for the derivatization of chiral xenobiotics, have confirmed their value in the enantiospecific analysis of compounds in biogenic matrices, e.g. plasma and urine (see Refs. [35,63–68,69] and the present study). Their application is predominantly directed to substrates with weak chromophoric properties and reagent-oriented detection, where they can help—and have helped—to lower detection limits in the enantiospecific determination of drugs in biological fluids.

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