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Immobilized isocyanates for derivatization of amines for chiral recognition in liquid chromatography with UV detection

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Abstract: Solid-phase derivatization reagents containing a 3,5-dinitrophenyl moiety for the derivatization of amines are described. The reagents are useful for Pirkle-type recognition of stereochemical composition of amines and related nucleophiles. The ability to stabilize 3,5-dinitrophenylisocyanate by covalent immobilization on a polymeric support varied greatly with the support chosen. The kinetics of the activated dinitrophenylcarbamate and the use of this reagent for enhanced recognition of optical purity is shown and compared to previous findings with a similar polymeric activated ester.

Keywords: *Liquid chromatography; solid-phase derivatization; amines; Pirkle phases; isocyanates; enantiomers.*

Introduction

The enantiomeric composition of chiral pharmaceuticals is rapidly becoming of critical interest from a pharmacokinetic, as well as a regulatory, point of view. For many years the behaviour of different enantiomers in a biological system was assumed to be similar since few methods had been developed which could clearly determine, in a quantitative manner, the amount of each enantiomer present. It became clear after such tragedies as the thalidomide disaster [1], that equal biological behaviour of enantiomers could not be assumed and must be proven.

Over the past decade several chromatographic methods have been established which allow for resolution of optical isomers [2, 3]. These methods are of two major types: direct and indirect recognition. Direct recognition relies on separation of the enantiomers via *in situ* formation of diastereomeric complexes on a chiral stationary phase. Resolution is dependent on the difference in stability of the transient diastereomers. This method does not require derivatization, although derivatives are often formed to enhance interactions with the stationary phase and increase detectability of the analyte. Indirect resolution requires derivatization with a chiral reagent of high optical purity to form permanent diasteromers, which are then separated on an achiral stationary phase. The possibility of different rates of reaction of the chiral analyte with the chiral analytical reagent always exists. This phenomenon, known as kinetic resolution, will lead to incorrect enantiomeric ratios unless reactions proceed to a high degree of completion. The optical purity of the analytical reagent must be very high, greater than 99.0%, or incorrect ratios will again be obtained. This method is generally less expensive to perform, since chiral stationary phases are not required for separation. However, these methods may be more difficult to validate.

Over the past decade this laboratory has been involved in the development of polymeric reagents which are capable of performing derivatization of chiral and achiral species under a variety of solvent conditions. The final analytical species are the same as those obtained by solution-phase derivatizations, but are obtained in a more facile and automatable fashion. These reagents were based on 200–400 US mesh (30–70 μm) polystyrene-divinylbenzene (STY-DVB, see Appendix for Glossary of Terms) beads of varying crosslinkage and porosity. These polymeric beads have been attached to an *o*-nitrobenzophenol (P-BP) and a hydroxybenzotriazole (P-HoBTA)

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which act as weak leashes for analytical derivatization reagents. These have proven useful for conversion of amines, amino acids and amino alcohols to chromatographically stable amides [4]. The final derivatives have enhanced chromatographic properties and lower detection limits over the original analyte. The physical properties of the solid support and the chemical properties of the activated leash have been optimized, to prepare a reagent which allows for room temperature conversion of most amines, alcohols, phenols and thiols to 3,5-dinitrophenyl derivatives [5]. In a previous publication a novel reagent based on the *o*-nitrobenzophenol microporous polymer, which imparted a FMOC-L-proline label to chiral amines was reported [6]. This reagent was capable of performing chiral HPLC analysis of pharmaceuticals in urine, in which only the pH and organic content of the urine needed to be adjusted. The diastereomers formed in the precolumn, on-line reactor were easily separated via reversed-phase stationary phases and possessed favourable fluorescent properties. The method was accurate and quite simple in practice, and for the handful of analytes investigated, no kinetic resolution was detected. We are currently automating this method to allow for more precise quantification and higher sample throughput [7].

This paper describes the use of a polymeric hydroxybenzotriazole ester (P-HoBTA–DNB, I, Fig. 1) to form separable derivatives of chiral alcohols as their 3,5-dinitrobenzoate (DNB) esters. A similar reagent (P-HoBTA–DNPC, II, Fig. 1) leashed a 3,5-dinitrophenyl iso-

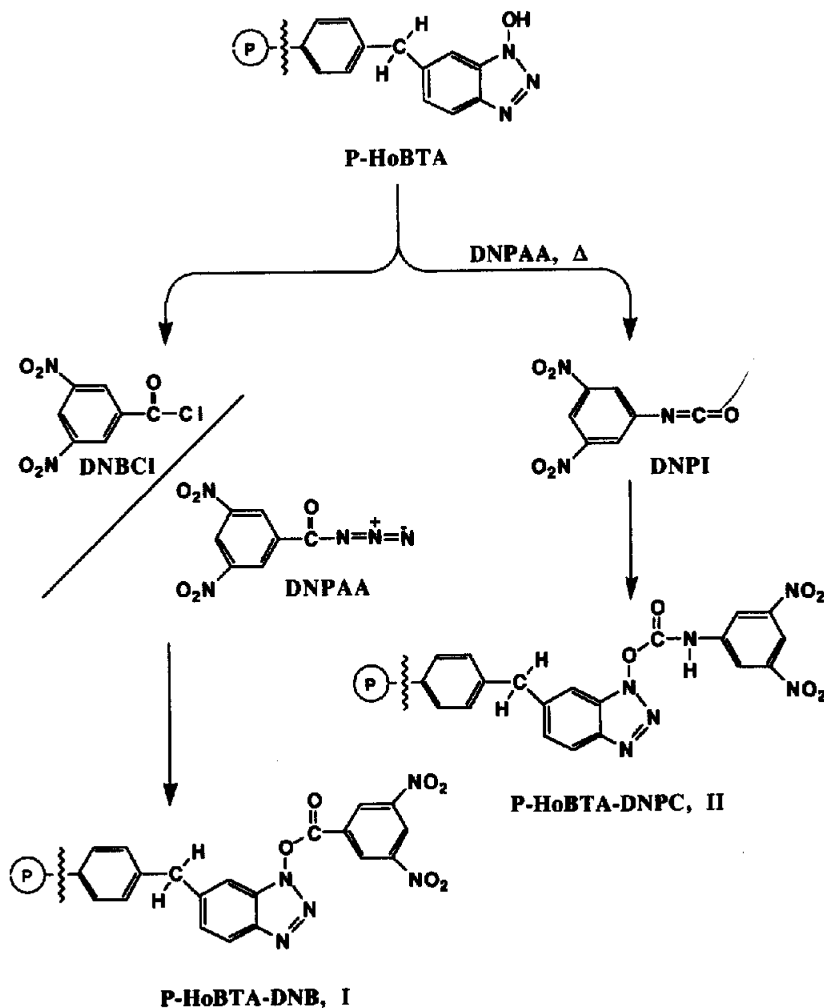

Figure 1
Solid-phase reagent structures. From the intermediate hydroxybenzotriazole, both the dinitrobenzoate (I) and the dinitrophenyl carbamate (II) were prepared. The dinitrobenzoate (I) can be prepared from the acid chloride or the acyl azide.

cyanate (DNPI) moiety to produce ureas and carbamates of chiral amines and alcohols, respectively. This is significant, in that DNPI is not commercially available and must be produced *in situ*, due to its tendency to dimerize to produce an insoluble bis-dinitrophenylurea. Perrin has used an imidazole-blocked version of the activated 3,5-dinitrophenyl carbamate (ICDNA) to perform derivatizations of alcohols and ureas, but these reagents were still based on solution-phase principles involving a large excess of the analytical reagent present in the final solution [8]. Research has been performed on the synthesis and use of an activated carbamate based on succinimido-blocked α-phenethylamine [9]. This optically active solution-phase reagent gave the expected urea diastereomers when reacted with a chiral amine. It was novel, but not useful, since it was less versatile than the commercially available phenethylisocyanate (PEI). Large amounts of unreacted isocyanate still had to be removed from solution by reaction with excess taurine. Since DNPI is not commercially available, we have chosen to immobilize it as an activated carbamate to our polymeric leashes, thus allowing only the amount that reacts with the analyte to be used. This is a more economical approach and in theory should yield cleaner chromatograms.

Experimental

Materials

Achiral reversed-phase separations were performed on 3 μm, 50 × 4.6 mm i.d. Rainin Microsorb™ C18 columns (Rainin Instruments, Inc., Berkeley, CA). Polymers used were generously donated from Bio-Rad, Inc. (Bio-Rad Laboratories, Richmond, CA) and Rohm and Haas (Rohm and Haas, Spring House, PA). 3,5-Dinitrobenzoyl chloride (DNBCl) and other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). LC solvents were obtained from EM Science, Inc., as their Omnisolv™ grade (EM Science, Gibbstown, NJ). All solvents were filtered through an off-line, 0.45 μm filter (Supelco Corp., Bellefonte, PA, Nylon 66), a 0.2 μm PTFE membrane (Whatman Inc., Clifton, NJ, on-line degasser/filtration) and were blanketed with Helium (2 psig) during use. Direct chiral analyses were performed on a 250 × 4.6 mm i.d. 5 μm LC-(*R*)-naphthyl urea column (Supelco).

Apparatus

The LC system for off-line derivatizations consisted of a Waters Model 6000A pump (Waters Chromatography Division, Millipore Corp., Milford, MA), a Rheodyne Model 7125 injection valve (Rheodyne, Inc., Cotati, CA) and a UV Monitor III (LDC Analytical, division of Thermo Instruments Corp., Riviera Beach, FL) fixed wavelength UV detector. Data acquisition was performed using Rainin Dynamax™ DA through a Macintosh Plus™ personal computer (Apple Computers, Cupertino, CA).

Synthesis of polymeric reagents

Polymeric leash. The polymeric 1-hydroxybenzotriazole (P-HoBTA, Fig. 1) was synthesized according to a literature procedure [10], and was characterized as in a previous publication [4].

Attachment of analytical label. The attachment of the isocyanate was attempted through two procedures. The first involved synthesis of imidazole-*N*-carbonic-acid-3,5-dinitroanilide (ICDNA), which was then allowed to react with the polymeric leash. A 200 mg mass of P-HoBTA was added to 10 ml of a 1:1 solution of CaH_2-dried DMF–THF containing 42 mg of ICDNA. The molar ratio of labelling reagent to P-HoBTA was 1.5:1. The solution was stirred at RT for 60 min, washed with 4 × 10 ml of 40°C dry dichloromethane (DCM) and dried under high vacuum for 60 min. The ICDNA was synthesized according to a literature procedure [11].

A more fruitful approach involved the synthesis of the acyl azide of 3,5-dinitrobenzoic acid [12]. The 3,5-dinitrophenylacylazide (DNPAA) was thermally decomposed to produce DNPI *in situ*, which was then allowed to react with the polymeric leash. A 71 mg mass (3.0 meq) of DNPAA in 15 ml CaH_2-dried toluene was decomposed for 30 min to generate free DNPI in solution. This solution was cooled to 20°C using an ice bath, added to 200 mg of the P-HoBTA and then allowed to warm to room temperature over 60 min with stirring. The polymer was washed over sintered glass with 4 × 10 ml 40°C DCM and dried under high vacuum for 60 min.

Determination of polymeric loading

The amount of analytical reagent per gram of dry polymer was determined via a controlled

hydrolysis procedure. Using conditions which had been optimized for the saponification of an analogous activated ester [5], the hydrolysis product, 3,5-dinitroaniline (DNA), was tested for stability. A stock solution of DNA was prepared and a 1 ml aliquot was added to 5 ml of the hydrolysis solvent (1 N KOH in DIOX–DMSO–H_2O; 30:20:50). This was heated to reflux for 30 min, neutralized, diluted to 25 ml and injected into the RP-HPLC. Neither DNA nor DNPU were present, indicating the hydrolysis procedure was too harsh. A room temperature experiment was performed and near quantitative recovery of the DNA was found. An accurately weighed sample of the labelled polymer was hydrolysed at room temperature and analysed via comparison to an external standard calibration plot of DNA. The analysis was performed in triplicate.

Derivatization conditions for model analytes

The derivatization conditions for similar reagents had recently been optimized [5]. Only time was varied here under ambient temperature conditions. A 200 ppm solution of *n*-butylamine was prepared in acetonitrile (ACN). For derivatization of *n*-butanol in ACN, five equivalents of triethylamine (TEA) were added. Aliquots (50 μl) of the analyte solution were added to the polymeric reagent (20–100 mg), allowed to remain in contact with the support for 30 s intervals, and then eluted to a 1 ml volume with ACN or dimethylformamide (DMF). The aliquot was then diluted with varying amounts of H_2O to match the mobile phase strength. Reactions were performed in off-line derivatization cartridges prepared from glass, disposable 1 ml pipettes, which had a tissue plug at the bottom to filter the polymer from the eluted derivatives. Positive pneumatic pressure was used to elute the derivatives from the off-line reactor. The spent polymer was then washed with hot 1:1 dioxane (DIOX)–ACN, dried, tagged and reused. Analysis of each derivatization was performed on a C18 reversed-phase support at a composition of 55% ACN–H_2O at 0.8 ml min^{-1}. Per cent conversions were based on external standard calibration plots, using authentic standards of the expected derivatives, which had been synthesized and characterized for structure and purity [4]. The per cent conversions were based on the analyte concentration; the polymeric reagent being in large excess.

Sample preparation and derivatization conditions

Since the samples were analysed via normal phase Pirkle-type chromatography, the sample preparation and derivatization conditions varied somewhat from those used above. Since many of the pharmaceuticals investigated were obtained as hydrochlorides, the salts needed to be neutralized prior to analysis. These samples were dissolved in aqueous ACN containing KOH or borate buffer (pH 9–10). After addition to the polymer, the aliquot was eluted with 1 ml of HEX–THF (3:1). The off-line reactor contained 100–150 mg Na_2SO_4 below the polymeric bed to remove water from the eluted derivatives. This minimized elution from the polymer of the bis-DNPU formed and yielded a dry solution, which was compatible with the mobile phase used for chiral resolution.

Results and Discussion

The work performed here was prompted by previous successes in chiral analyses using derivatives formed via solid-phase derivatization. The activated dinitrophenylbenzoate reagents (Fig. 1, **I**) were stable compounds, which reacted efficiently under mild conditions to yield the expected derivatives. The analogous dinitrophenylcarbamate reagent was prepared (Fig. 1, **II**), but found to have limited utility because of stability of the reagent. It was; however, useful for the analysis of chiral amines and amino alcohols, generating the expected derivatives.

Preparation of the analytical reagent

The activated dinitrobenzoate reagent (P-HoBTA–DNB, **I**, Fig. 1) was easily prepared through pyridine-catalysed acylation of the polymeric hydroxybenzotriazole. This was a stable reagent when kept desiccated at room temperature.

The activated dinitrophenylcarbamate reagent (P-HoBTA–DNPC, **II**, Fig. 1) was not as easily prepared. We had at first thought the reagent would be more readily prepared than the ester, since it should be less reactive. The lone pare of electrons on the anilide nitrogen add a resonance form in which the carbonyl carbon is less electropositive.

It became apparent that the ICDNA route to the activated carbamate was not a judicious choice. This reagent was not as reactive as the

free isocyanate and the leaving group, imidazole, could react again with the newly formed polymeric carbamate. The major product of the reaction was the bis-dinitrophenyl urea. Since the benzotriazole was a better stabilized leaving group than the imidazole, thermodynamically, this was not an appropriate method of immobilization.

The immobilization of 3,5-dinitrophenyl isocyanate (DNPI) onto our activated supports was possible only through the decomposition of 3,5-dinitrophenyl acyl azide (DNPAA) in toluene (Fig. 1). DNPAA undergoes rearrangement when heated, evolving nitrogen gas to form the isocyanate. This stock solution of DNPI was reported several years ago for the synthesis of chiral stationary phases and/or π-acid derivatives [12], and more recently for the simultaneous extraction and derivatization of phenylpropanolamine from canine plasma [13].

The polymer appeared to catalyse the hydrolysis of the DNPI. Addition of the toluene solution of DNPI to the polymer immediately produced the bright yellow colour of dinitroaniline (DNA). Proton donation by the P-HoBTA or moisture in the polymer may have been the cause of hydrolysis. Aqueous acid catalyses the decomposition of isocyanates to amines or in this case, anilines. Under these conditions, DNA can further react with the DNPI present to produce the bis-DNPU. Nevertheless, immobilization was moderately successful; a controlled hydrolysis yielded a loading of 0.14 meq g^{-1}. Since the polymer had a known hydroxybenzotriazole concentration of 0.74 meq g^{-1}, only 20% of the sites contained the DNPI label.

Kinetics and stability of activated dinitrophenyl carbamate

The P-HoBTA–DNPC produced a 24% yield of *n*-butyl-dinitrophenylurea when reacted with a 200 ppm solution of *n*-butylamine in ACN for 60 s at room temperature. Thus, the reagent was considerably less reactive than the P-HoBTA–DNB, which reacted nearly quantitatively under the same conditions in roughly 6 s. This may have been due to the relatively low loading of DNPI. The per cent derivatization did not increase with the addition of an organic base catalyst. In fact, addition of TEA only complicated factors by increasing the hydrolysis and subsequent dimerization. Decreased reactivity was not the main problem associated with this reagent.

The main problem encountered was the instability of the final reagent. Even the use of high vacuum and desiccation did not prevent the formation of large peaks in the reagent blank. At first it was thought the addition of the isocyanate to the hydroxybenzotriazole was reversible. This is a well documented phenomenon with imidazole-activated ureas which are similar in structure [14]. In light of these latest results it is unlikely that this is a major pathway.

Recent work with a different polymeric support has solved many of these problems. The data presented above were derived from a highly crosslinked, high surface area, STY-DVB support. We had initially chosen this support based on the high reactivity of chemical functionalities which were immobilized on the surface of the pore volumes [5]. These reagents reacted nearly as fast as the solution-phase reagents, since the immobilized reagent was present on the surface of the pores, and thus, readily accessible to analytes in solution. More recently was realized the optimal mixture of surface area, pore size and crosslinking which protect the reagent from hydrolysis and stabilized the reactive center through hydrophobic shielding. This understanding has allowed the use of the P-HoBTA–DNB and the P-HoBTA–DNPC for on-line conversion of chiral amines to separable derivatives for Pirkle-type HPLC assays.

Derivatization of amines for chiral recognition

Several phenethylamine analogues were investigated. Amphetamine, methamphetamine, norephedrine and pseudoephedrine were derivatized and chromatographed on two π-base, Pirkle-type stationary phases. The dinitrophenylurea derivatives of amphetamine and methamphetamine were readily separated and resolved in under 20 min (Fig. 2).

When preparing the DNPI stock solution, if the DNPAA was not allowed to decompose completely, the remaining acyl azide in solution reacted with the polymer to yield the active ester (Fig. 1). This led to a 'mixed-bed reactor' effect which we have described elsewhere [4, 15]. In effect, both the activated ester and carbamate were present on the same polymeric bed to react with the incoming

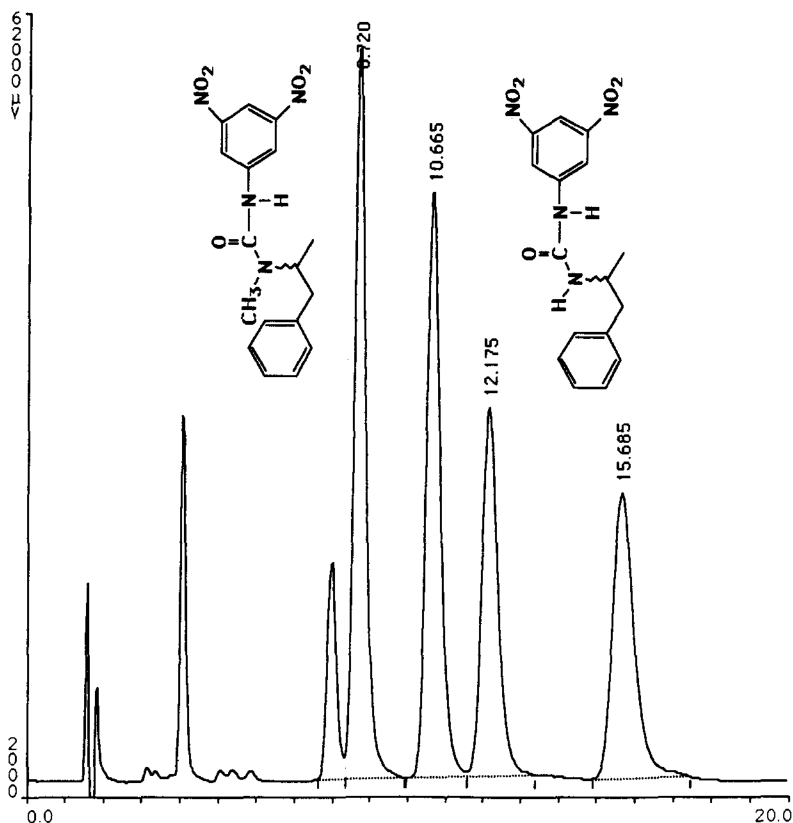

Figure 2
Chiral resolution of amphetamine and methamphetamine. Racemic amphetamine and methamphetamine were prepared in 0.5 M borate buffer (pH 9.0) containing 50% ACN. Conditions: derivatization — 50 μl × 200 ppm each × 25 mg P-HoBTA-DNPC, 60 s at room temperature, elute to 1 ml with THF–HEX (1:3); separation — HEX–EtOH–ACN (930:70:5) at 2.0 ml min^{-1} on 250 × 4.6 mm i.d., LC-(*R*)-naphthylurea, 5 μl × 0.004 AUFS at 254 nm.

nucleophile. Although in this case it was accidental, we have deliberately mixed two or more polymers containing different detector sensitive labels for improved identification of analytes in complex matrices [15]. Thus, when a solution of 2-aminoctane was derivatized, both the amide and the urea derivative were formed. The resolution of the two derivatives was excellent and served as a confirmation of the optical ratio observed (Fig. 3). The optical ratio was, as expected for the racemate, 1:1 by area for the amide and urea derivatives. There was no change in the elution order of enantiomers with the different detector labels.

Derivatization of amino alcohols for chiral recognition

The analysis of pseudoephedrine was simplified by selective reaction of the amino functionality only; the benzylic hydroxyl group was not acylated. These amino alcohols separated poorly as the amide, but quite effectively as the urea. Sudafed™ and Actifed™ both were found to contain *d*-pseudoephedrine, with no traces of *l*-pseudoephedrine (Fig. 4). The 1-[1*R*,2*R*]-pseudoephedrine obtained from Sigma was found to contain 2.26 ± 0.2% of the minor enantiomer. The extra hydrogen bonding possible with the urea functionality was responsible for the enhanced recognition vs the dinitrobenzamide derivative. A Dexitrim™ tablet and an Entex LA™ tablet were analysed for phenylpropanolamine (*d,l*-norephedrine). Both contain the same active ingredient, at the same concentration but the former is sold as an over-the-counter appetite suppressant and the latter as a prescription-strength long-acting decongestant. The enantiomers resolved well in under 10 min (Fig. 5).

Derivatization of alcohols

It was hoped to convert chiral alcohols to separable derivatives using the dinitrobenzoate activated ester. The 3,5-dinitrobenzoate esters were formed in high yield under mild con-

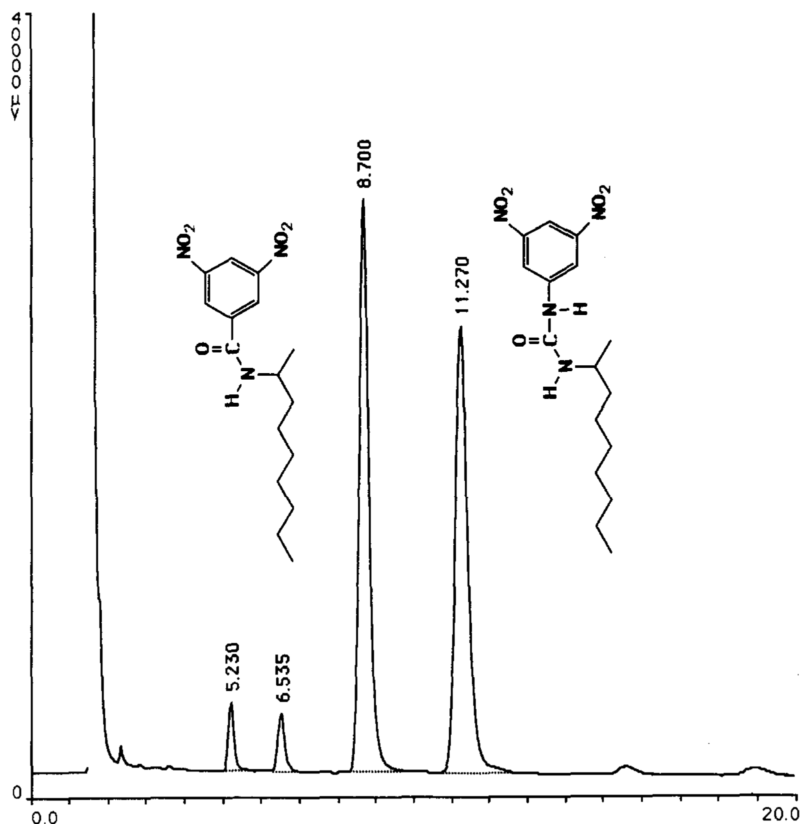

Figure 3
Chromatography of amide and urea derivatives. The enantiomers of (±)-2-aminooctane were derivatized with the P-HoBTA-DNPC. A small amount of the dinitrobenzoate label was attached to the polymer from reaction with the dinitrophenylacyl azide causing the 'mixed-bed derivatization' effect. This chromatogram clearly shows the difference in recognition between the two derivatives. Conditions: derivatization — 50 μl × 200 ppm amine in ACN × 25 mg P-HoBTA-DNPC, 60 s at room temperature, elute to 1 ml with THF-HEX (1:3); separation — HEX-EtOH-DCM (90:5:5) at 2.0 ml min^{-1} on 250 × 4.6 mm i.d., LC-(*R*)-naphthylurea, 5 μl × 0.004 AUFS at 254 nm.

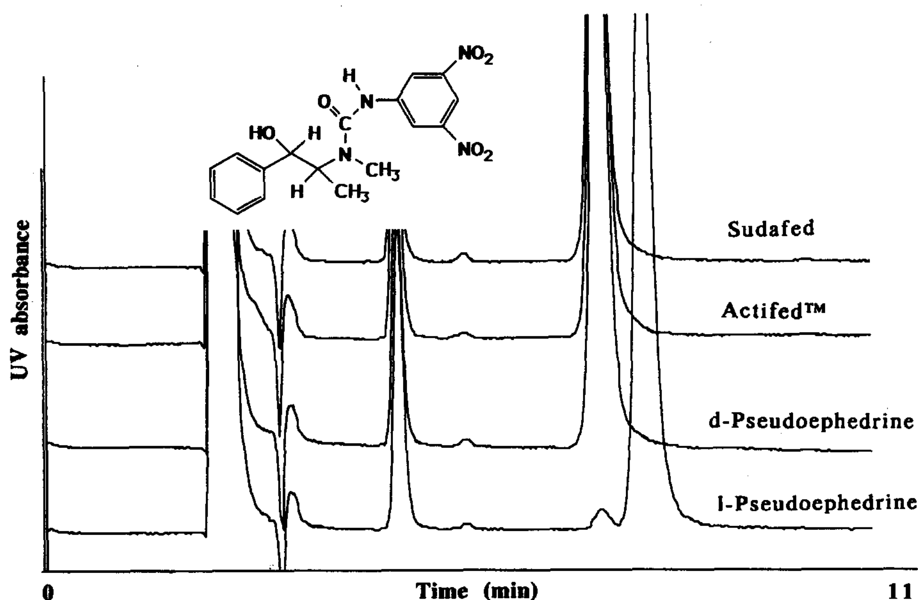

Figure 4
Chiral analysis of anorectics. Resolution of DNPU derivatives of Dexitrim™ (an anorectic, top trace) and Entex LA™ (a decongestant, bottom trace). Conditions: derivatization — 50 μl of 1 mg ml^{-1} in 0.5 M borate buffer (pH 9.0)-50% ACN vs 25 mg P-HoBTA-DNPC, 60 s at room temperature, elute to 1 ml with DCM: separation — HEX-IPA-MeOH (83:12:5) at 2 ml min^{-1} on 250 × 4.6 mm i.d., LC-(*R*)-naphthylurea, 5 μl × 0.004 AUFS at 254 nm.

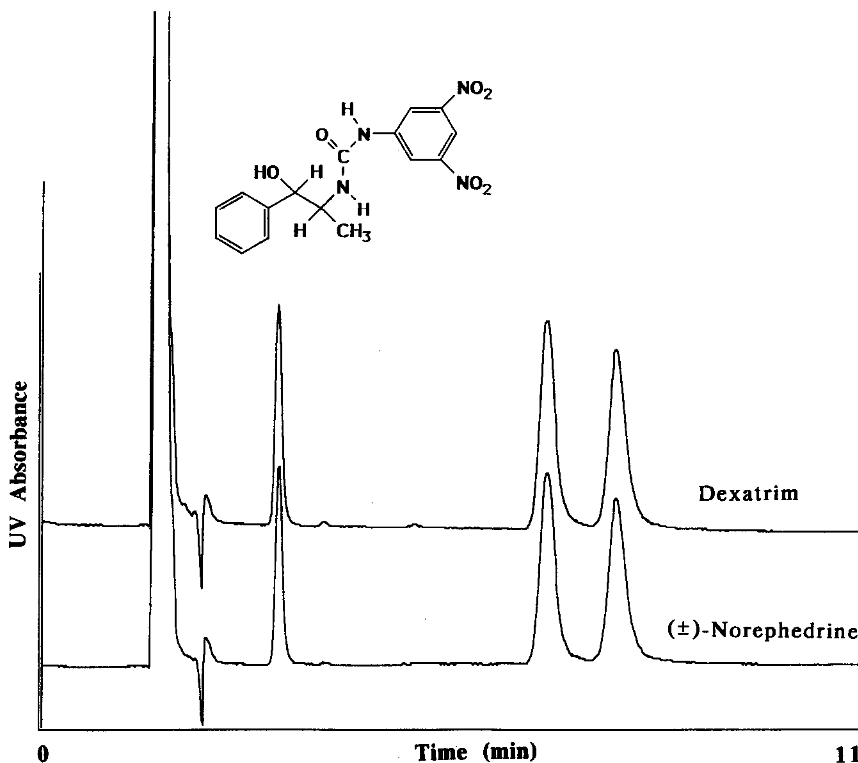

Figure 5
Analysis of decongestants. Sudafed™, Actifed™ and standards of *d* and *l*-pseudoephedrine were inspected for optical composition. Only the *l*-pseudoephedrine showed enantiomeric contamination, at a level of 2.26 ± 0.02% by area. Conditions are the same as those for Fig. 4.

ditions, but the derivatives did not resolve as well as the carbamate derivatives due to decreased hydrogen bonding interactions with the stationary phase. However, the activated carbamate did not derivatize alcohols in sufficient yields to be useful.

Conclusions

The immobilized hydroxybenzotriazole 3,5-dinitrophenyl carbamate provided a convenient route to modifying amines and amino alcohols, but not alcohols, for chiral recognition on Pirkle-type supports. The derivatives possessed excellent stability and chromatographic character, allowing for baseline resolution of enantiomers. The immobilized reagent was not ideal; however, due to its reduced shelf life. The instability of this reagent was due to the physical character of the solid support.

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Appendix — Glossary of Terms

ACN	acetonitrile
DCM	dichloromethane
DIOX	dioxane
DMF	dimethylformamide
DNBCl	dinitrobenzoyl chloride
DNPAA	3,5-dinitrophenylacylazide
DNPU	bis-3,5-dinitrophenyl urea
DVB	divinylbenzene
EVB	ethylvinylbenzene
FMOC	9-fluorenylchloroformate
HPLC	high-performance liquid chromatography
ICDNA	imidazole-*N*-carbonic acid-3,5-dinitroanilide
P-DMAP	polymeric dimethylaminopyridine
P-HoBTA–DNB	polymeric hydroxybenzotriazole–dinitrobenzoyl ester
P-HoBTA–DNPC	polymeric hydroxybenzotriazole–dinitrophenyl carbamate
STY	polystyrene
TEA	triethylamine
THF	tetrahydrofuran
UV	ultraviolet absorption detection