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Solid-phase reagent containing the 3,5-dinitrophenyl tag for the improved derivatization of chiral and achiral amines, amino alcohols and amino acids in high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A solid-phase reaction technique is described for improved derivatization of aliphatic amines, amino alcohols and amino acids. A polymeric activated ester is used for the immobilization of the 3,5-dinitrobenzoyl group, which can then be used for derivatizations of strong or weak nucleophiles, while avoiding solution-phase derivatization conditions. The reagent is easily prepared and can be regenerated after use to attain its original reactivity. The resulting chromatograms are free of system peaks due to excess derivatizing reagent, and sample handling is kept to a minimum. The reagent can be used in conjunction with both reversed- and normal-phase chromatography and can be used for off-line gas chromatographic or high-performance liquid chromatographic (HPLC) derivatizations. In addition, the reagent can be used on-line for derivatization in HPLC. Since the labelling reagent is a strong π-acid, chiral substrates can be derivatized and separated on a Pirkle-type π-donor column. The confirmation and quantitation of amphetamine in urine was accomplished using a polymer containing two labelling moieties, *p*-nitrobenzoyl and 3,5-dinitrobenzoyl. The derivatization and separation of chiral and achiral amines, amino alcohols and amino acids is described.

INTRODUCTION

Amine-containing compounds are used in the synthesis of a majority of chiral and achiral pharmaceutically important compounds. The precursors and final products must be chemically and optically pure to assure high yield and purity of the final product. However, the analysis of aliphatic amines and amino acids is complicated by ionization of the amino functionality and poor chromatographic behavior on silica-based supports. The usual methodology for trace analysis of amines involves a homogeneous solution-based derivatization procedure, whereby a chromophore or electrophore is introduced, thus increasing the chromatographic performance, detectability and, at times, the volatility of the analyte [1]. Such derivatization procedures can be unwieldy, requiring time and effort and introducing many steps where analyte losses can occur. The resulting chromatograms are further complicated by excess or hydrolyzed derivatization reagents. Without suitable sample treatment

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after the derivatization step, these reagents can contaminate the system, interfere with ultimate quantitation, and reduce the efficacy and lifetime of the chromatographic column. Such arguments are applicable to gas chromatography (GC), as well as to high-performance liquid chromatography (HPLC).

Over the past decade, solid-phase reagents have become increasingly popular for the facile conversion of analytes to more detectable species. These methods have largely been enzyme based, whereby an enzyme–substrate reaction produces an electrochemically or optically detectable species [2,3], or based on organic chemistry, whereby an immobilized activated site is reacted with a sufficiently electrophilic or nucleophilic substrate [4–7]. The advantages of performing these solid-phase reactions have been well covered in the literature [8,9]. Some of the more notable advantages include:

(1) Only the amount of reagent that reacts with the analyte is used. This yields chromatograms which are free of excess derivatizing reagent and allows the same batch of polymer to be used many times before efficacy of derivatization becomes a concern.

(2) More selective conversion of the analyte to the desired derivatives, yielding: (a) less complex chromatograms with higher signal-to-noise ratios and (b) functional group discrimination which is often not possible in solution phase derivatization without the use of blocking groups.

(3) Improved stability of the solid-phase reagents over time compared with the analogous solution-phase reagent.

(4) Trace analysis of analytes is facilitated by high local concentrations of the derivatizing reagent. These concentrations in solution would likely exceed the solubility of the derivatization reagent.

(5) Solid-phase derivatizations often allow for a higher percentage of conversions [10].

(6) The use of co-immobilized reagents allows for the quantitation and confirmation of analyte presence within a single chromatographic run. This methodology has been proven invalid for solution-phase derivatization, due to kinetic effects and the possibility of cross-reactions between the derivatization reagents in solution.

The resolution of optical isomers by HPLC is recognized as being the most sensitive technique available for unequivocal determination of enantiomeric composition [11]. The importance of this technique cannot be understated, in that about 25% of all pharmaceuticals dispensed between 1959 and 1980 contained at least one chiral center and the pharmacological activity of the enantiomers is often very different [12,13]. One need only be reminded of the thalidomide tragedy to understand the importance of absolute knowledge of enantiomeric excess for any given pharmaceutical [14]. Chiral molecules that lack chromophoric ligands generally require derivatization for visualization by UV–fluorescence detection. For physical resolution of chiral molecules, at least three points of interaction between the chiral analyte and the chiral selector are necessary, while one of these interactions must be steric in nature [15]. Thus, derivatization, which enhances the detectability of the analyte, can also be used to introduce a specific site for interaction with the brush-type Pirkle chiral columns. The usual treatment is to introduce a π-donor (naphthyl) or π-acceptor (3,5-dinitrophenyl) moiety to the molecule being analyzed [16–18]. Columns are commercially available, designed specifically to be used for such derivatized analytes.

We have synthesized two polymers which contain activated ester moieties. The

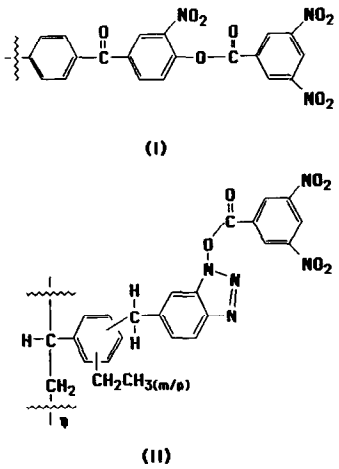

Fig. 1. Structure of polymeric reagents. (I) Polymeric benzophenone–DNB, (II) polymeric benzotriazole–DNB.

first polymer (I, Fig. 1) was prepared from 200–400 mesh polystyrene beads cross-linked with 4% divinylbenzene (DVB). We modified the polymer to obtain an *o*-nitrophenol to which various acid chloride–chloroformate moieties could be attached. The second polymer (II, Fig. 1) was based on ethylvinylbenzene (EVB) cross-linked with DVB, in which a hydroxybenzotriazole functionality had been introduced. The two polymers showed remarkably different reaction kinetics due to their different activated linkages and polymeric supports. These reagents could be used off-line or on-line, pre-column, for real- or delayed-time derivatizations of sufficiently nucleophilic analytes, such as amines and amine analogues. The derivatives had enhanced sensitivity to absorbance detection due to the addition of a chromophoric label. The dinitrophenyl derivatives were amenable to reductive electrochemical detection, as well as photochemical dissociation and reduction of the cleaved aromatic nitro groups to nitrite, which could then be detected electrochemical in an oxidative mode [19]. Thus, three possible modes of detection were possible; a significant advantage in confirmation of the analyte in a complex matrix over detection of the underivatized analyte. Other detector labels (fluorenyl, *o*-acetylsalicyloyl and *p*-nitrobenzoyl) can be attached to the polymeric reagents to yield different chromatographic and detector selectivity to the derivatized analyte.

These different polymers can be mixed to form a known amount of each label per gram of the mixed polymer. Derivatization of a single analyte produced a characteristic profile of derivatives having known retention times and detector responses. This efficiently solved the problem of co-elution of a matrix component with the analyte of interest since the likelihood of matrix co-elution with both derivatives was minimal [20].

In this paper, the simultaneous determination of amphetamine in urine using a 1:1 molar mix of immobilized *p*-nitrobenzoyl and 3,5-dinitrobenzoyl (DNB) moieties is shown. Both derivatives eluted well-resolved from the matrix, without any extraction/matrix work-up involved. The simultaneous analysis and confirmation

within a 5-min chromatographic run exemplifies the utility of solid-phase derivatization methodology for analyses in complex matrices.

EXPERIMENTAL

Materials

A LiChrosorb 5-μm particle size, C_{18} column (EM Science, Cherry Hill, NJ, U.S.A.), 250 mm × 4.6 mm I.D., was used for the hydrolysis analysis and analysis of achiral derivatives. For chiral separations Supelcosil LC-(*R*)-Naphthyl Urea/LC-(*S*)-Naphthyl Urea, 250 mm × 4.6 mm I.D. columns (Supelco, Bellefonte, PA, U.S.A.) were used. 3,5-Dinitrobenzoyl chloride (DNBCl) and other reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.). Amino acids and amino acid methyl esters were purchased from Sigma (St. Louis, MO, U.S.A.). The dinitrobenzamide (DNBa) standards were synthesized following literature procedures [21,22]. Polymeric supports were purchased from Fluka (polystyrene cross-linked with 4% DVB, 200–400 mesh), and from Waters (Milford, MA, U.S.A.; Porapak Q; 57% DVB, 38.6% EVB, *meta/para* ratio 2.5:1, 100–120 mesh) [23]. Amphetamine sulphate and norephedrine HCl were donated by Thomas Doyle (FDA, Washington DC, U.S.A.). HPLC solvents were obtained from EM Science, as their Omnisolv grade. All solvents were filtered through a 0.45-μm membrane (PTFE, Millipore, Bedford, MA, U.S.A.; Nylon 66, Supelco) and degassed under vacuum before use.

Apparatus

The HPLC system consisted of a Waters Model 6000A pump, a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) and a UV Monitor III (LDC Milton Roy, Riviera Beach, FL, U.S.A.). Data acquisition was performed using Rainin Dynamax DA (Rainin Instruments, Berkeley, CA, U.S.A.) through a Macintosh Plus personal computer (Apple Computers, Cupertino, CA, U.S.A.). At times, data was collected from a Hitachi D-2000 Chromatointegrator (Hitachi Instruments, Naka Works, Mito City, Japan).

Physical and spectral characterization of the derivatives, used to characterize the kinetics and thermodynamics of the polymeric reagent with various analyte classes, included a Varian Model XL-300 NMR Spectrometer (Varian, Palo Alto, CA, U.S.A.), a Perkin-Elmer Model 599B infrared spectrophotometer (Perkin-Elmer, Analytical Instruments, Norwalk, CT, U.S.A.), a Thomas Hoover capillary melting point apparatus (Arthur H. Thomas, Philadelphia, PA, U.S.A.), a Milton Roy Model Spectronic 1201 scanning UV–VIS spectrophotometer (Milton Roy, Analytical Products Division, Rochester, NY, U.S.A.) and a Nuclide magnetic sector mass spectrometer (Nuclide, State College, PA, U.S.A.). Elemental analyses of the polymeric reagents were performed at Galbraith Laboratories (Knoxville, TN, U.S.A.). Solvents removal under reduced pressure was performed on a Buchi RotoVap (VWR Scientific, Boston, MA, U.S.A.).

Synthesis of analytical standards of amines, amino alcohols and amino acids

All DNBa-standard derivatives were characterized via ^{1}H NMR and electron-impact mass spectrometry (EI–MS) and were determined to be the expected structures.

Amines. DNBa standards of primary and secondary amines were prepared via

solution-phase derivatization of the amine with pyridine-catalyzed DNBCl according to literature procedures [21].

Amino alcohols. Standards of primary and secondary amino alcohols were prepared via solution-phase derivatization of the amine functionality with triethylamine (TEA)-catalyzed DNBCl according to literature procedures [22].

Amino acids. For achiral, reversed-phase (RP) analysis of amino acids using solid-phase derivatization, only the amine functionality was modified. Synthesis of the derivatives required temporary protection of the carboxylic acid group, while the amine group was acylated with DNBCl. The standards were prepared following a modification of a literature procedure, in which the electrophilic reagent, trityl chloride, was replaced with DNBCl [24].

Amino acid methyl esters. The methyl esters of some of the amino acids used were purchased as the methyl ester hydrochlorides. Others were synthesized in our laboratory using the amino acid and dry methanol–HCl or BF_3–methanol (50:50, w/w) according to literature procedures [22].

Synthesis of the polymeric benzophenone and polymeric hydroxybenzotriazole

Polymeric benzophenone. Polystyrene–divinylbenzene was washed with acetonitrile (ACN) using a Soxhlet apparatus for 48 h to remove monomeric impurities which would leach from the polymer and complicate the chromatograms of the derivatives. The modification of the polystyrene followed a literature procedure [25]. The analytical label was covalently attached to the polymeric phenol intermediate, via the acid chloride, using pyridine as a nucleophilic catalyst [25].

Polymeric benzotriazole. The second polymeric reagent contained an immobilized hydroxybenzotriazole functionality. This polymeric hydroxybenzotriazole was synthesized from an EVB–DVB polymer following a literature procedure [26]. Addition of the analytical label to the polymeric hydroxybenzotriazole intermediate was identical to that of the polymeric benzophenone.

Physical characterization of the polymeric activated esters

Hydrolysis of polymeric benzophenone activated ester. A hydrolysis procedure was used to quantitatively cleave the analytical label from the polymer. The benzoic acid generated was quantitated via HPLC using external-standard calibration. A 200-mg amount, weighed to the nearest 0.1 mg, was hydrolyzed by suspension in 2 ml of 2 *M* KOH and 3 ml of dimethylformamide (DMF). The suspension was heated with stirring to 60°C for 30 min. The solution was then acidified (pH 3) using concentrated HCl, and the polymer was filtered directly into a 25-ml volumetric flask. The solution was made to the mark with ACN–water (30:70) and analyzed by RP-HPLC (Table I).

Hydrolysis of polymeric benzotriazole–DNB. The characterization of the benzotriazole labelled with *o*-acetylsalicyloyl chloride was via a similar hydrolysis procedure [6] (Table I).

Elemental analysis of polymeric benzophenone and polymeric benzotriazole. Elemental analyses of both polymers were performed at Galbraith Laboratories (Table I).

Regeneration of the polymeric reagents

Since both polymeric reagents swell in ACN it was often sufficient to wash the

TABLE I

QUANTITATIVE DATA FOR POLYMERIC REAGENTS

Benzophenone elemental analysis				
Polystyrene–DVB	91.88% C,	8.02% H		
Polymeric phenol	77.00% C,	5.93% H,	2.67% N,	10.30% O
Tagged phenol	71.82% C,	5.12% H,	4.46% N,	14.56% O
Benzophenone elemental analysis results[a]				
1.91 ± 0.02 mequiv. phenol/g of polymer				
0.64 ± 0.02 mequiv. label/g of polymer				
Benzophenone hydrolysis results[b]				
0.62 ± 0.02 mequiv. label/g of polymer				
Benzotriazole hydrolysis results[c]				
0.31 ± 0.01 mequiv. label/g of polymer				

[a] Based on increase in nitrogen, less than 0.014 mequiv. Cl^- found, indicating near quantitative conversion of intermediate to the polymeric phenol.

[b] Hydrolysis was performed on polymer acylated with benzoyl chloride due to instability of the 3,5-dinitrobenzoate anion to the hydrolysis conditions.

[c] Hydrolysis was performed on polymer tagged with *o*-acetylsalicyloyl chloride.

reagent with several aliquots of ACN, allowing each aliquot to sit for 60 s to allow for impurities to diffuse out of the pores of the polymer. Occasionally, an overnight Soxhlet extraction was performed using ACN to thoroughly clean the polymer. The polymer was then dried and acylated with DNBCl to yield the final tagged reagent.

Solid-phase on-line derivatization

Only the polymeric benzophenone–DNB reagent was used on-line, due to the instability of the polymeric benzotriazole–DNB reagent to water. The on-line reactor was assembled from stainless-steel hardware (27 mm × 2.2 mm I.D., 0.2-μm frits). Similar-dimension columns are commercially available for facile in-house packing of guard columns from Upchurch Scientific (Oak Harbor, WA, U.S.A.). The reactor was attached to an injector through the injection-loop ports (ports 1 and 4, Rheodyne Model 7010), and this configuration was used as a switching valve [5]. The analyte was injected and allowed to pass through the reactor for real-time, on-line derivatization, or was switched to a bypass mode to trap the analyte to attain longer reaction times (Fig. 2). The reactor was attached to the switching valve using 15 cm of small bore (0.009 in. I.D.) stainless-steel tubing. It could then be lowered into a thermostated hot water reservoir to increase the temperature of the reaction.

Reversed-phase derivatization. The derivatization of nucleophiles in aqueous organic solvents on-line, was complicated by the reactivity of the polymer. A dual-pump apparatus was configured such that only anhydrous organic solvent passed through the reactor. A mixing tee was placed after the reactor and before the analytical column, where the strength of the final eluent was adjusted through proportioning of the pumps (Fig. 2). The derivatization solvent was ACN or tetrahydrofuran (THF) and the aqueous solvent was generally ACN–water (10:90) or THF–water (10:90). A mixture of three amines (morpholine, *n*-propylamine and *n*-butylamine; 20 ppm each), was derivatized on-line using the dual-pump system. The derivatization of amines was tested in two different anhydrous organic eluents, THF–ACN (1:1) and ACN (Fig. 3).

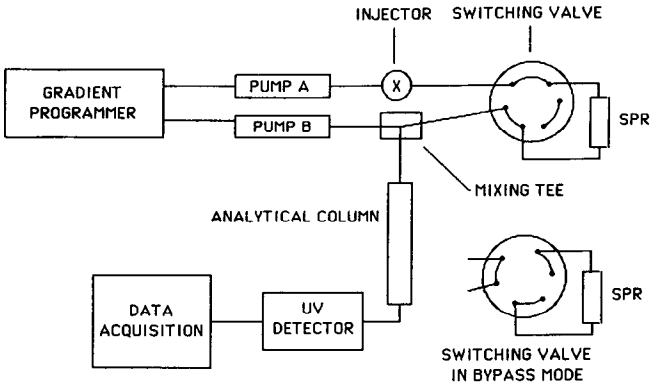

Fig. 2. Diagram of dual-mobile-phase HPLC system. Pump A contains anhydrous ACN, pump B contains ACN–water (10:90). Column, LiChrosorb 5 μm C_{18} cartridge, 125 × 4.0 mm I.D.

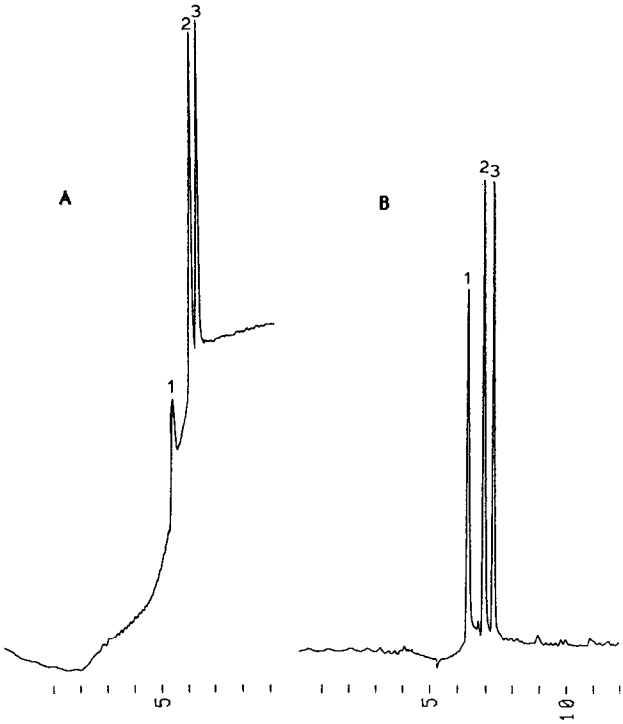

Fig. 3. On-line derivatization of (1) morpholine, (2) propylamine and (3) butylamine using two mobile phases. (A) THF–ACN (1:1); (B) ACN. Both systems are split mobile-phase gradients in which only pure organic flows through the on-line reactor. Conditions: injection volume, 20 μl; column, LiChrosorb 5 μm C_{18} cartridge, 125 × 4.0 mm I.D.; pump A: 100% organic; pump B: 10% organic. Gradient No. 8, 5–85% A over 5 min. Retention time in min.

Normal-phase derivatization. The derivatization of amines and amine analogues in normal-phase was expected to be more facile than in reversed-phase due to the lack of water in the mobile phase. However, since the reactivity of the polymer was enhanced at elevated temperatures (50–80°C), the reactor had to be protected from alcohols as well. Reactions were accomplished using a single programmable pump (Waters, Model 590) with flow-programmed step gradient. The analyte flowed through the reactor at 0.1 ml/min and was then stepped up to higher flow-rates at the rate of 1 ml increments every ten seconds, to a final flow-rate of 3.1 ml/min. The reactor column was placed in a hot water bath to equilibrate 2 min before injection of the analyte, and was removed from the bath after 4 min to prolong its lifetime. Analysis of the enantiomers of amphetamine was accomplished in <20 min from injection to derivatization, separation and detection of the on-line formed derivatives (Fig. 4).

Solid-phase off-line derivatizations

An amount of the polymeric reagent was added to a Pasteur pipet which had been scored and broken *ca.* 5 cm above the tapered section. The tapered end was plugged with a small amount of tissue paper which kept the polymer bed intact, and filtered any particulates from the final solution containing the derivatives. A volume of analyte (30–100 μl) was added to a fixed mass of the polymer (30–150 mg) such that the

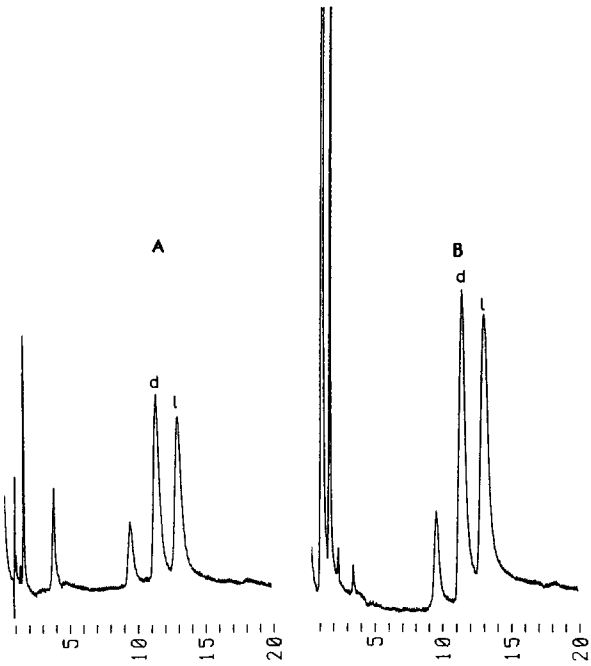

Fig. 4. On-line derivatization and separation of the enantiomers of amphetamine. (A) Injection of 75 ppm of the standard amphetamine–3,5-dinitrobenzamide; (B) injection of 100 ppm amphetamine free base in ACN–DCM (1:1) with 0.05% TEA added. Real-time derivatization at 72°C in a mobile phase of hexane–DCM–THF (70:27:3). Derivatization occurred at 0.1 ml/min for 40 s. The flow-rate was increased in increments of 1 ml every 10 s to a final flow-rate of 3.1 ml/min. Conditions: injection volume, 10 μl; column, Supelco 5 μm, LC-(*R*)-Naphthyl Urea 250 × 4.6 mm I.D. Retention time in min.

and the reaction was quenched with 200 μl of ACN–water (80:20), which was adjusted to a concentration of 2 m*M* HNO_3. Quantitation of percent conversion was performed as before. The off-line derivatization of three amines was performed (Fig. 5).

The same experiment was performed using a base catalyst. An equimolar amount of TEA was added to the 200-ppm solution of *n*-butylamine–diethylamine in pure ACN. The identical study was performed using a 4 m*M* HNO_3 quenching solution (Fig. 6).

An analogous experiment was performed using the polymeric benzotriazole–DNB reagent. Since nearly quantitative conversion of the amine, in the presence of a base catalyst, to the 3,5-dinitrobenzamide (DNBa) occurred at room temperature (RT) in $\leqslant$60 s with both polymeric reagents, no temperature optimizations were performed.

Concentration of base catalyst vs. amine concentration

The amount of TEA used as a base catalyst was varied from 0.5 to 2 equiv. *vs.* *n*-butylamine concentration. Derivatization conditions were identical to those used for the optimization of time.

pH study vs. percent derivatization

For partially or wholly aqueous solutions, the pH of the solution was an important factor, since ionization of the analyte rendered the amine functionality non-nucleophilic. Eight buffers were prepared by adjusting the pH of a 50-ml aliquot of 0.4 *M* H_3BO_3 to the pH of interest using 0.5 *M* KOH and diluting to a 100 ml volumetric. Thus, 0.2 *M* H_3BO_3 buffers with pH values between 7.0 and 10.0 in 0.5 pH increments were prepared. A 400-ppm solution of *n*-butylamine was prepared and 25-ml aliquots of the amine solution were mixed with 25-ml aliquots of the appropriate buffer solution to prepare the final analyte solution. The final composition was 200 ppm *n*-butylamine in ACN–0.1 *M* borate buffer (50:50) at different pH values. Derivatization conditions and quantitation of percent conversion were identical to those used for the optimization of time. No analogous pH study was performed with the polymeric benzotriazole–DNB reagent.

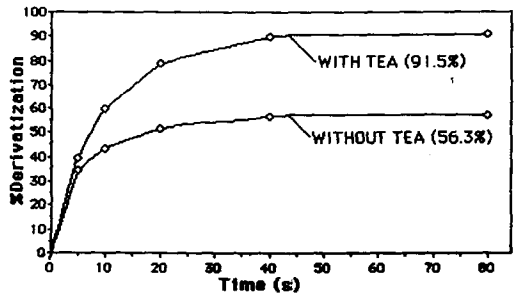

Fig. 6. Percentage of derivatization *vs.* time of butylamine with polymeric benzophenone–DNB in ACN with and without equimolar TEA present. Conditions: 30 μl of 200 ppm in ACN × 70 mg polymeric benzophenone–DNB; 2 min at RT, elute with 500 μl ACN, dilute with 500 μl water; injection volume, 10 μl; column, LDC-Milton Roy 3 μm Spherisorb ODS-II, 100 × 4.0 mm I.D., ACN–water (50:50) at 1.5 ml/min.

polymer was wetted without the analyte reaching the tissue plug. The solid-phase reaction was allowed to proceed for an amount of time determined by the kinetics of the substrate, and was washed with 500 μl ACN. The eluent was then diluted with water to match the mobile phase. For normal-phase work, the derivatives were eluted from the reactor using 1 ml of the mobile phase. For normal-sensitivity work (ppm and above), 20 μl of the final solution was injected into the HPLC. After each derivatization, the polymer was removed from the cartridge, the tissue paper replaced, and the reactor filled with fresh polymer. The used polymer was placed aside to be regenerated.

Optimization of off-line derivatization conditions

Time and temperature. Typical primary and secondary amine substrates were used to characterize the kinetics of the solid-phase reagents with amines. Thus, a 200-ppm solution of *n*-butylamine in ACN and a 200-ppm solution of diethylamine in ACN were used as test substrates to determine the dependence of percent derivatization as a function of time. Each analyte was derivatized separately, off-line for different intervals, and the product obtained was compared to the external standard that had been synthesized and characterized. The percent conversion of the amine to the amide was quantitated. Reaction time intervals were 5, 10, 20, 40 and 80 s. A 30 μl volume of the amine solution was injected onto 70 mg of the polymeric reagent,

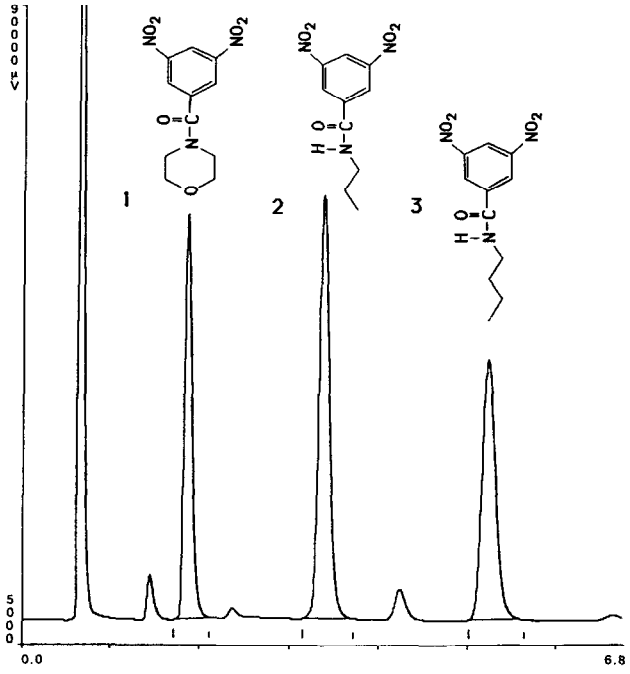

Fig. 5. Analysis of amines using off-line derivatization. Derivatives in order of elution: 1 = morpholine–DNBa, 2 = *n*-propyl–DNBa, 3 = *n*-butyl–DNBa. Conditions: 20 ppm each in ACN, 30 μl × 70 mg polymeric benzophenone–DNB; 1 min at RT, elute to 1 ml ACN, inject 10 μl. Column, LiChrosorb 5 μm C_{18} cartridge, 125 × 4.0 mm I.D., ACN–water (50:50) at 1.5 ml/min. Retention time in min.

Percent derivatization vs. amine concentration

The working range of amine concentrations over which the percent derivatization remained constant was determined. Solutions of *n*-butylamine were prepared in the concentration range of 500 ppb[a] to 20 000 ppm. No base catalyst was used. Derivatizations and quantitation using the polymeric benzophenone–DNB were performed as before.

Derivatization protocol for amines, amino alcohols, amino acids and amino acid methyl esters using both polymeric reagents

Amines. Amines as the free base were weighed or injected into a volumetric flask containing ACN and an equimolar amount of TEA. The TEA had to be specially treated to remove 1° and 2° amine impurities, which otherwise complicated the chromatograms [27]. Amines as the hydrochloride or sulphate were prepared in ACN–0.05 *M* NaOH (80:20). For derivatization of these solutions followed by normal-phase chromatography, the water was trapped using 500 mg of anhydrous Na_2SO_4, which was placed below the polymer bed in the off-line reactors. Thus, the derivative and solution were made anhydrous as they eluted from the reactor. The comparison of percent derivatization *vs.* time for both polymers is given in Fig. 7. Quantitation was performed using peak areas (Table II).

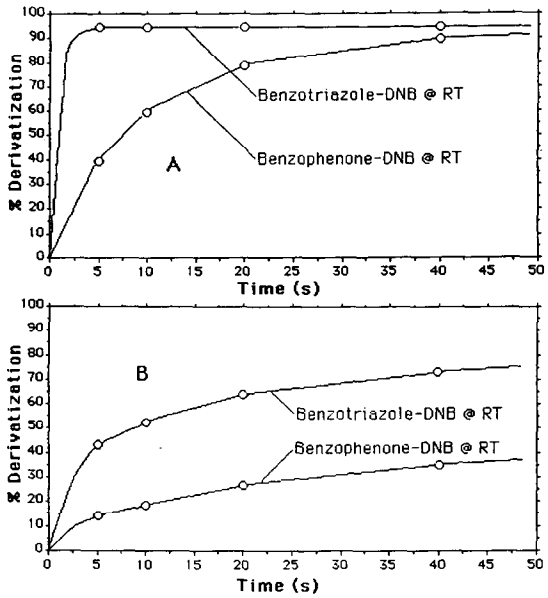

Fig. 7. Time dependence of derivatization of *n*-butylamine and diethylamine with the two polymeric reagents. (A) Kinetic curve for *n*-butylamine, (B) kinetic curve for diethylamine. Conditions: 200 ppm *n*-butylamine–diethylamine in ACN with equimolar TEA. 30 μl × 70 mg polymeric benzophenone–DNB, 50 μl × 30 mg polymeric benzotriazole–DNB; 2 min at RT, elute with 500 μl ACN, dilute with 500 μl water. Chromatography, injection volume, 20 μl; LiChrosorb 5 μm C_{18}, 125 × 4.0 mm I.D., ACN–water (50:50) at 1.5 ml/min.

[a] Throughout the article, the American billion (10^9) and trillion (10^{12}) is meant.

TABLE II

ENANTIOMERIC PURITY OF AMINES[a,e]

Sample	*d* (%)	*l* (%)
(±)-1-amino-1-phenylpropane[b] (lot KM63206KM)	50.45 ± 0.05	49.55 ± 0.05
(*R*)-(+)-1-amino-1-phenylethane[c] (lot AP03909TM)	98.86 ± 0.04	1.14 ± 0.04
(*S*)-(−)-1-amino-1-phenylethane[c] (lot 00901JP)	1.95 ± 0.16	98.05 ± 0.16
(*R*)-(+)-1-amino-1-(1-naphthyl)ethane[d] (lot 03424HM)	99.11 ± 0.05	0.89 ± 0.05
(*S*)-(−)-1-amino-1-(1-naphthyl)ethane[d] (lot 88F0342)	0.78 ± 0.07	99.22 ± 0.07

[a] 40 μl amine solution × 80 mg benzophenone–DNB, 2 min at RT, elute to 1 ml with mobile phase, LC-(*S*)-Naphthyl Urea column, 250 × 4.6 mm I.D., 20 μl × 0.016 a.u.f.s. at 254 nm.

[b] Hexane–DCM–methanol (90:7:3) at 1 ml/min.

[c] Hexane–DCM–methanol (80:15:5) at 2 ml/min.

[d] Hexane–DCM–methanol (70:25:5) at 2 ml/min.

[e] All amines purchased through Aldrich.

Amino alcohols. The amino alcohols were dissolved in ACN containing equimolar TEA. For reversed-phase work, the derivatives were eluted from the off-line reactor with 500 μl of ACN and diluted with water prior to injection (Fig. 8). For normal-phase chromatography, the derivatives were eluted from the off-line reactor using 1 ml of the mobile phase.

Amino acids. The amino acids were dissolved in ACN–0.05 *M* NaOH (80:20). This allowed the amine functionality to remain as the free base and provided good wetting and swelling properties for the polymer. Derivatization was as above for the amines, and separation was performed on a C_{18} column with 0.1% trifluoroacetic acid (TFA) added for pH suppression (Fig. 9).

Amino acid methyl esters. The methyl esters of the amino acids studied were either synthesized using BF_3–methanol or methanol–$HCl_{(g)}$, or were purchased as the hydrochloride salt and were neutralized in 0.1 *M* NaOH. The solution was made partially organic (*ca.* 12%), and the analyte was extracted using a solid-phase technique. The preparation of a 200-ppm solution of the methyl ester of phenylalanine was as follows: 24.1 mg of the hydrochloride was weighed directly into a 5-ml Reacti-vial and 2 ml of 0.1 *M* NaOH was added. A 0.250 ml volume of ACN was added

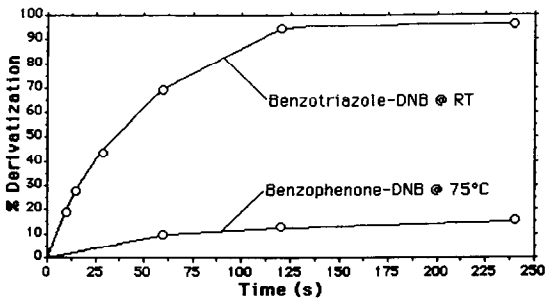

Fig. 8. Time dependence of derivatization of ethanolamine with both polymers. Same conditions as Fig. 7, except separation was performed on a Spherisorb 3 μm CN 100 × 4.6 mm I.D., ACN–water (50:50) at 1.5 ml/min.

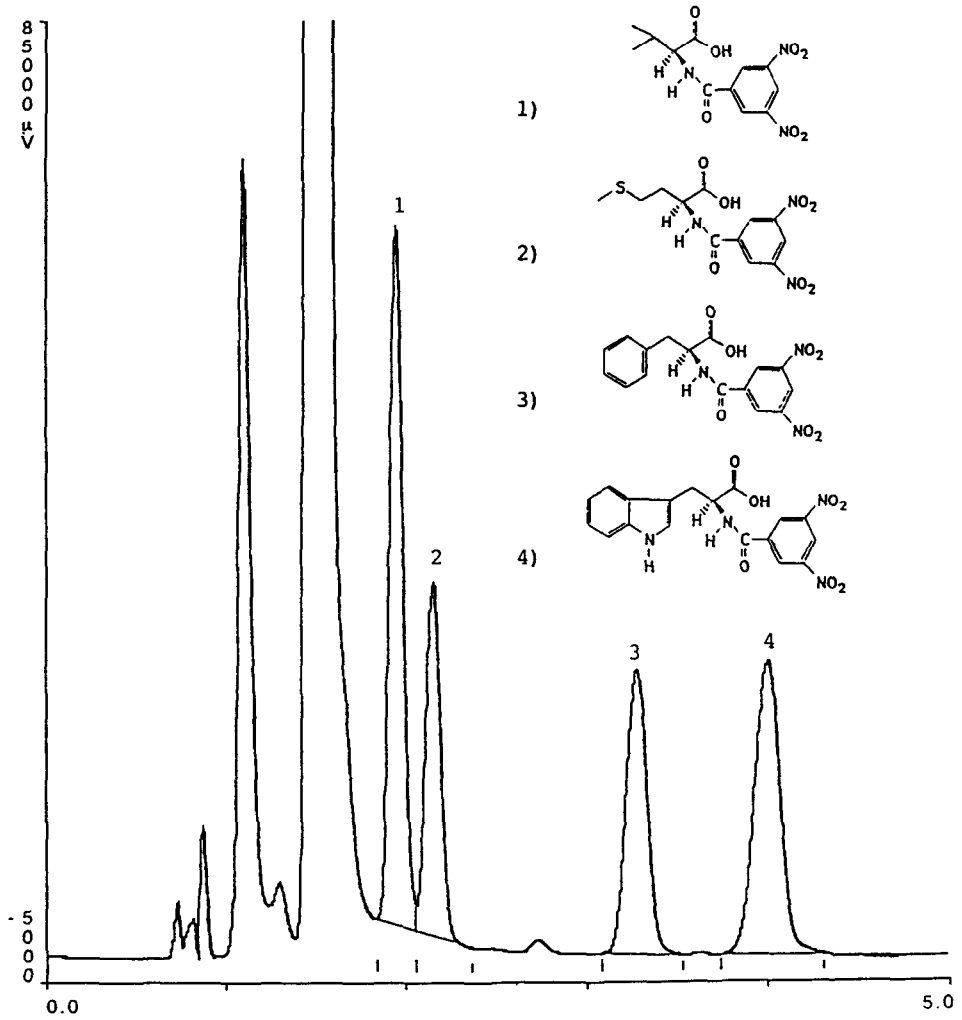

Fig. 9. Derivatization of (1) valine; (2) methionine; (3) phenylalanine; and (4) tryptophan dissolved in ACN–0.05 *M* NaOH (80:20). Conditions: 50 μl (200 ppm each) × 30 mg polymeric benzotriazole–DNB; 2 min at RT, elute to 500 μl ACN, dilute with 500 μl water; injection volume, 20 μl; column, Spherisorb 3 μm CN 100 × 4.6 mm I.D., ACN–water (20:80) (0.1% TFA). Retention time in min.

to aid in the dissolution of the free base. The solution was taken up in a 5-ml gastight syringe and passed over an RP-C_{18} Sep-Pak cartridge which had been conditioned with 3 ml of ACN and 1 ml of distilled water. The eluate was discarded and the cartridge washed with 1 ml of distilled water. The amino acid methyl ester was eluted into a 100-ml volumetric flask with two 5-ml aliquots of pure ACN. The solution was made equimolar with TEA and the volumetric flask was brought to the mark. Derivatization of this solution was identical to that of a free amine in ACN (Figs. 10 and 11). Quantitation was performed using peak areas (Tables III and IV).

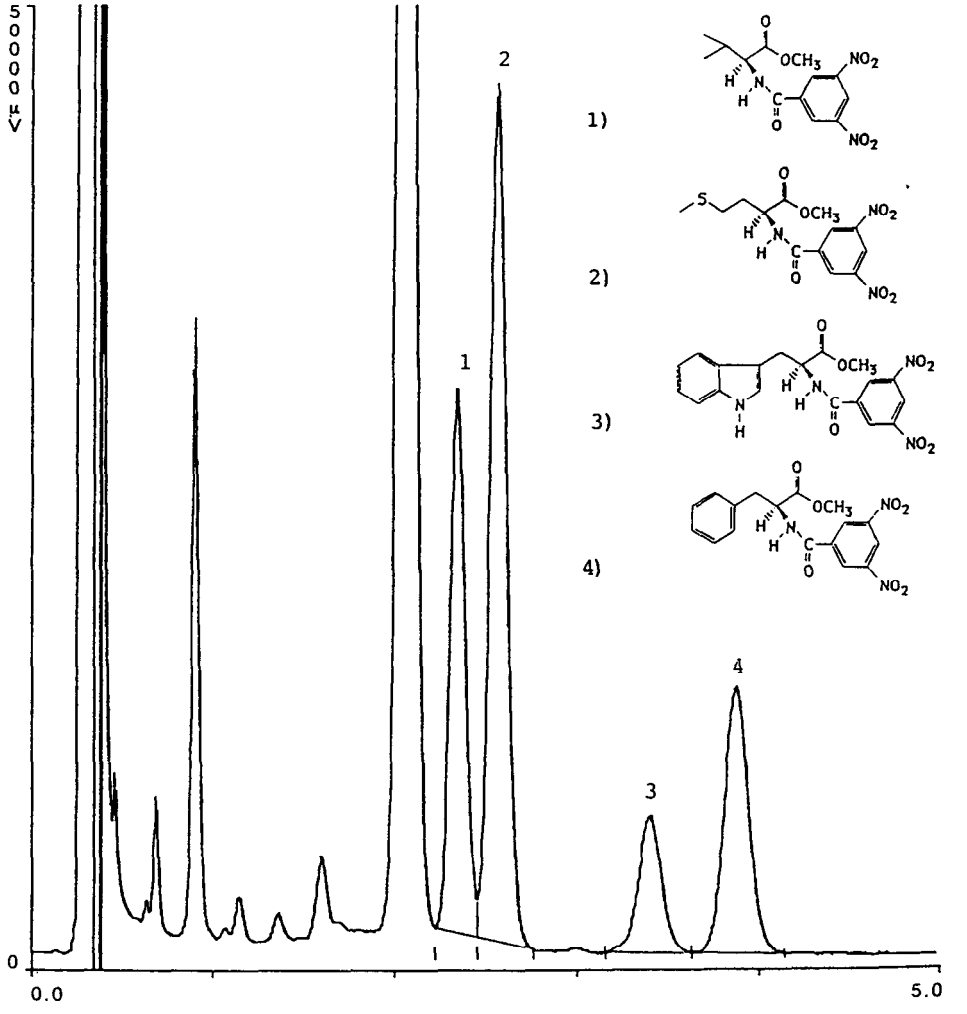

Fig. 10. Derivatization of the methyl esters of (1) valine; (2) methionine; (3) tryptophan; and (4) phenylalanine. Conditions: 100 μl (100 ppm each) × 60 mg polymeric benzotriazole-DNB; 2 min at RT, elute to 500 μl ACN, dilute with 500 μl water; injection volume, 20 μl; column, LiChrosorb 5 μm C_{18}, 125 × 4.0 mm I.D., ACN–water (45:55) at 2.5 ml/min. Retention time in min.

Single-blind chiral analysis of amphetamine

The enantiomeric ratio of amphetamine isomers was determined in five samples, spiked with varying ratios of *d*- and *l*-amphetamine. The samples were prepared from the pure enantiomers of amphetamine sulphate, which was dissolved in ACN–0.25 *M* KOH (50:50). Unknown ratios of *d*- and *l*-amphetamine were prepared by adding different amounts of the two stock solutions to a third vial. The authors had no previous knowledge of the enantiomeric compositions.

Determination of the enantiomeric composition was via derivatization and chromatographic analysis of the samples. Five samples were analyzed by injecting

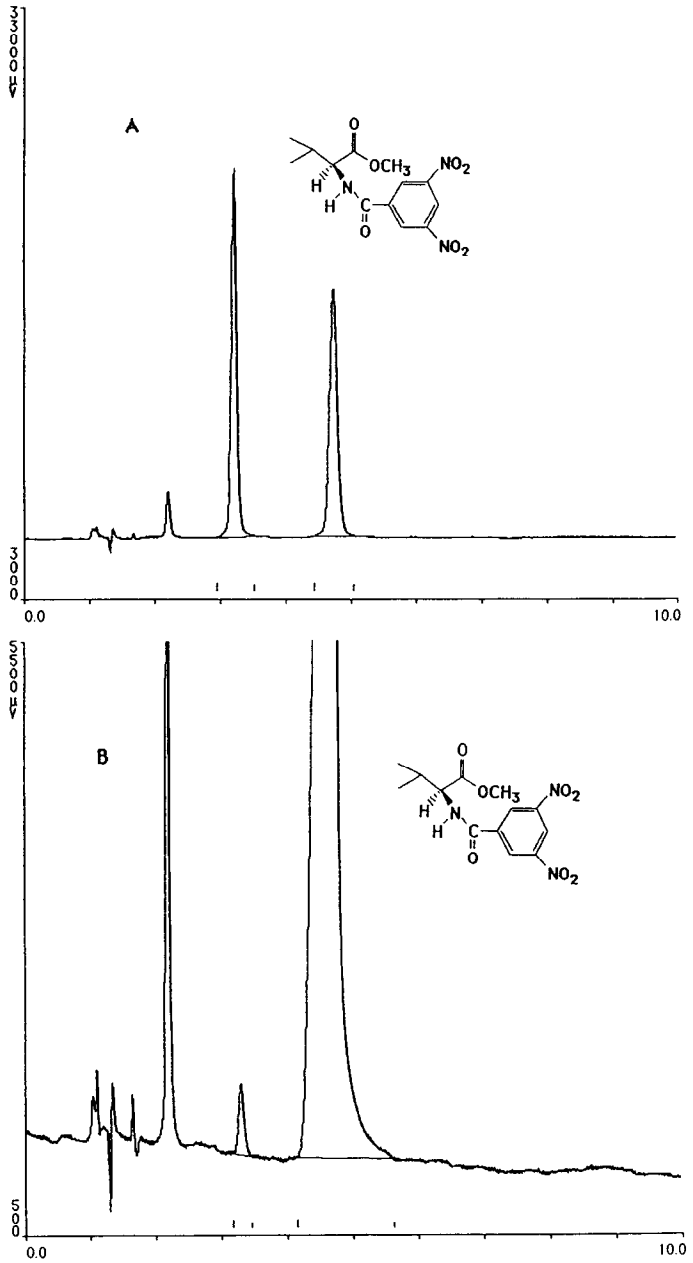

Fig. 11. Chiral separation of *d*- and *l*-valine methyl esters as the off-line formed 3,5-dinitrobenzamide. (A) 1 ppth *d,l*-Valine showing 50:50 composition by area; (B) 2 ppth *l*-valine showing 0.18 ± 0.02% enantiomeric contamination of the *d*-isomer. Conditions: 20 μl × 30 mg polymeric benzophenone–DNB, 3 min at RT, elute to 1 ml with mobile phase, injection volume 20 μl. Chromatography, 5 μm LC-(*S*)-Naphthyl Urea 250 × 4.6 mm I.D., hexane–DCM–methanol (90:5:5) at 3.0 ml/min. Retention time in min.

TABLE III

CHROMATOGRAPHIC FIGURES OF MERIT FOR THE AMINO ACID DERIVATIVES[a]

Compound as 3,5-DNBa (methyl ester)	Column efficiency $(N)^f$	k' $[(t_r - t_0)/t_0]$	α (k'_2/k'_1)
Phenylalanine[b]			
d-form	7780	2.12	1.38
l-form	7610	2.92	
Alanine[c]			
d-form	7820	0.95	1.66
l-form	7580	1.75	
Valine[d]			
d-form	7590	2.21	1.68
l-form	7510	3.72	
Tryptophan[e]			
d-form	8540	1.49	1.74
l-form	7630	2.59	

[a] 40 μl × 80 mg benzophenone–DNB, 3 min at RT, elute to 1 ml with mobile phase. Supelco LC-(*S*)-Naphthyl Urea column, 250 × 4.6 mm I.D., 20 μl × 0.004 a.u.f.s.
[b] Hexane–DCM–methanol (75:23:2) at 2 ml/min.
[c] Hexane–DCM–methanol (70:25:5) at 2 ml/min.
[d] Hexane–DCM–methanol (90:5:5) at 3 ml/min.
[e] Hexane–DCM–methanol–ACN (70:22:5:3) at 2 ml/min.
[f] N = number of plates.

70 μl of the amine solution onto 150 mg of the polymeric benzophenone–DNB reagent, allowing the reaction to occur at RT for 60 s and eluting the derivative to 0.7 ml with 100% THF. Four derivatizations were performed with 2–3 injections per derivatization. Determination of enantiomeric composition was performed using peak areas (Table V).

TABLE IV

ENANTIOMERIC PURITY OF AMINO ACIDS[a]

Amino acid	*d*/*l*	*d*	*l*
Phenylalanine[b]	(49.95/50.05) ± 0.03	99.15 ± 0.02	99.62 ± 0.02
Alanine[c]	(50.01/49.99) ± 0.04	98.90 ± 0.05	97.90 ± 0.05
Valine[d]	(50.21/49.79) ± 0.10	–[f]	99.82 ± 0.02
Tryptophan[e]	(49.99/50.01) ± 0.01	100.00 ± 0.00	100.00 ± 0.00

[a] 40 μl sample × 80 mg polymeric benzophenone–DNB, 1 min at RT, elute to 1 ml with mobile phase. Supelco LC-(*S*)-Naphthyl Urea column, 250 × 4.6 mm I.D., 20 μl × 0.004 a.u.f.s. at 254 nm.
[b] Hexane–DCM–methanol (75:23:2) at 2 ml/min.
[c] Hexane–DCM–methanol (70:25:5) at 2 ml/min.
[d] Hexane–DCM–methanol (90:5:5) at 3 ml/min.
[e] Hexane–DCM–methanol–ACN (70:22:5:3) at 2 ml/min.
[f] *d*–Enantiomer not analyzed.

TABLE V

SINGLE-BLIND SPIKED AMPHETAMINE ANALYSIS[a]

Sample	Spiked composition (%)	Determined (%)	R.S.D. (%)	RE[b] (%)
5-119-1				
d-form	65.0	64.7 ± 0.2	0.3	−0.6
l-form	35.0	35.3 ± 0.2	0.6	+0.9
5-119-2				
d-form	75.0	73.4 ± 0.5	0.7	−2.1
l-form	25.0	26.6 ± 0.5	1.9	+6.4
5-119-3				
d-form	50.0	50.5 ± 0.3	0.6	+1.0
l-form	50.0	49.5 ± 0.3	0.6	−1.0
6-13-2				
d-form	65.0	64.6 ± 0.4	0.6	−0.6
l-form	35.0	35.4 ± 0.4	1.1	+1.1
6-13-3				
d-form	50.0	50.1 ± 0.2	0.4	+0.2
l-form	50.0	49.1 ± 0.2	0.4	−0.2

[a] 70 μl amine solution × 150 mg polymeric benzophenone–DNB, 1 min at RT, elute to 1 ml with mobile phase hexane–ethanol (97:3) at 3 ml/min. Supelco LC-(*R*)-Naphthyl Urea column, 250 × 4.6 mm I.D., 5 μm; 10 μl injection × 0.016 a.u.f.s. at 254 nm.

[b] Relative error (% RE) = [(found − spiked)/spiked] · 100%.

Single-blind achiral analysis of amphetamine in urine using a mixed-bed reactor

A solution of amphetamine sulphate was spiked into a urine sample (0.1 *M* NaOH) to a representative concentration of levels found when the substance is abused [28]. The presence of amphetamine in the sample was shown by derivatization with the polymeric benzotriazole–DNB reagent. The derivative retention time matched that of the standard amphetamine-DNBa which had been synthesized earlier. The presence of amphetamine was confirmed by the use of a second labelling reagent, *p*-nitrobenzoyl (pNB). The polymer was tagged with this reagent and used as above to prove the identity of the amphetamine derivative peak, again compared to the retention time of the standard amphetamine-pNBa. To quantitate the amount of amphetamine present in the spiked samples both polymers were simultaneously used. A 1:1 mixture of the two polymeric reagents (pNB–DNB) was prepared and the sample again derivatized. This time, two derivative peaks were formed, indicating reaction of the amphetamine with both polymeric reagents (Fig. 12). A calibration curve of amphetamine from 5 to 50 ppm was prepared in a urine matrix which had been adjusted to contain 60% ACN. Three single-blind spiked samples were prepared in the same manner at concentrations within the standard curve. The standard curve and samples were derivatized using 100 μl sample *vs.* 60 mg of the 1:1 labelled polymeric benzotriazoles. Both derivatives were used to quantitate the concentration of amphetamine present (Table VI).

Single-blind analysis of amino acid enantiomeric composition

To further validate the method with a different class of analyte, an experiment was performed with a known amount of phenylalanine of unknown enantiomeric

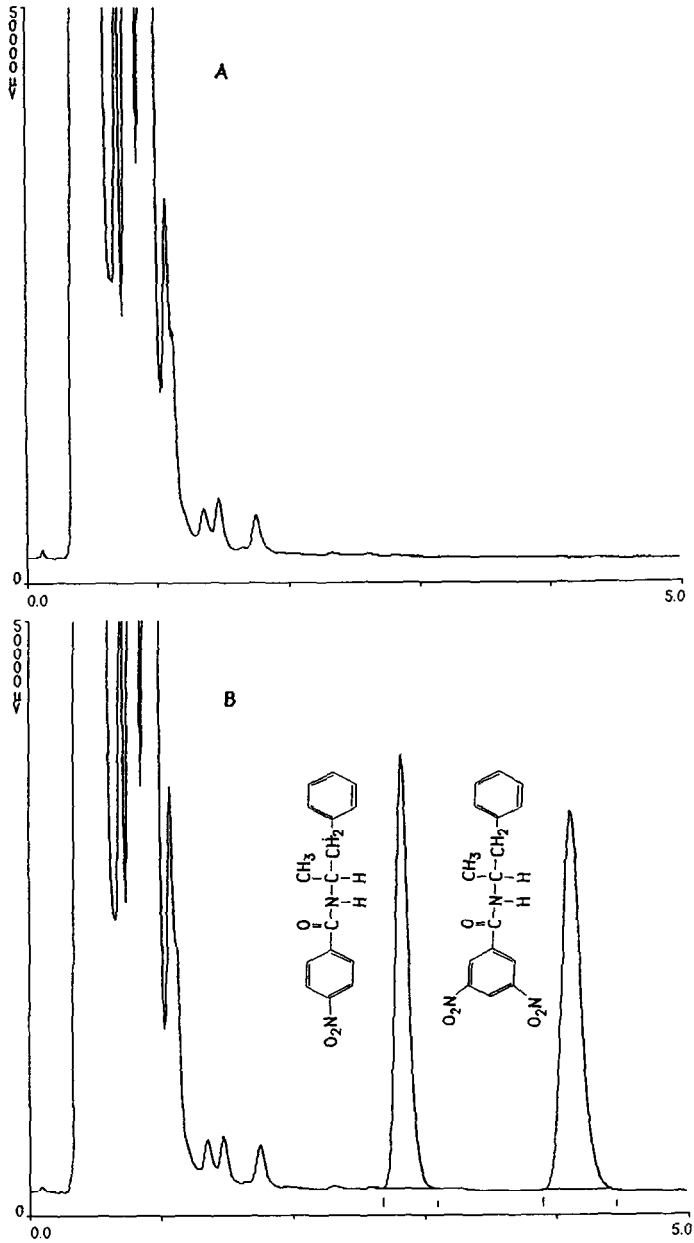

Fig. 12. Mixed-bed derivatization of amphetamine in urine. (A) Urine blank derivatized with polymeric benzotriazole–(pNB/DNB) (1:1); (B) spiked sample (35 ppm) derivatized with polymeric benzotriazole–(pNB/DNB) (1:1). Conditions: derivatization, 100 μl sample × 60 mg polymeric benzotriazole; 2 min at RT, elute with 500 μl ACN, dilute with 500 μl 0.05 *M* NaOH, injection volume 20 μl. Chromatography, LiChrosorb 5 μm C_{18}, 125 × 4.0 mm I.D., ACN–water (50:50) adjusted to 0.05% (v/v) NH_4OH at 2.0 ml/min. Retention time in min.

TABLE VI

SINGLE-BLIND SPIKED AMPHETAMINE ANALYSIS USING POLYMERIC MIXED BED REACTOR OFF-LINE[a]

Sample	Spiked concentration (ppm)	Determined (ppm)			
		pNP–amphetamine	RE (%)	DNP–amphetamine	RE (%)
8-84-1	32.3	33.0 ± 0.4	+2.2	34.0 ± 0.5	+5.2
8-84-2	28.2	28.7 ± 0.3	+1.6	29.8 ± 0.8	+5.6
8-84-3	40.4	40.9 ± 0.1	+1.3	42.8 ± 0.5	+5.9

[a] 100 μl sample × 60 mg (pNP/DNB)–benzotriazole (1:1), 2 min at RT, elute with 0.5 ml ACN, dilute with 0.5 ml 0.05 *M* NaOH, ACN–water (50:50) adjusted to 0.05% (v/v) NH_4OH at 2 ml/min, 125 × 4.0 mm I.D. LiChrosorb 5 μm C_{18}, 20 μl injection × 0.016 a.u.f.s. at 254 nm.

composition. The experiment simulated a real-world analysis to determine enantiomeric purity of a lot of amino acid. It was necessary to prepare the methyl ester of the amino acid for chiral recognition. Thus, each lot was alkylated using 2 ml BF_3–methanol (50:50, w/w) and 20 ml of anhydrous methanol at 70°C for 30 min. The methanol was evaporated under reduced pressure and the residue dissolved in 5 ml of 5 *M* KOH. The KOH was extracted with 5 × 1 ml aliquots of dichloromethane (DCM), and the DCM extracts were combined and dried over Na_2SO_4 directly into a 10-ml volumetric flask. The Na_2SO_4 was washed with a 5-ml aliquot of ACN, and the volumetric flask was brought to the mark with DCM. A 40-μl sample of this solution was injected onto 80 mg of the polymeric benzophenone–DNB reagent, and after 3 min at RT the derivative was washed from the reactor cartridge with 1 ml THF–hexane (15:85). A 20-μl sample was injected. Separations were performed on a 5-μm LC-(*S*)-Naphthyl Urea 250 × 4.6 mm I.D. column using hexane–DCM–methanol (75:23:2) at 1.5 ml/min. The ratio of enantiomers was determined via area counts of the two enantiomers (Table VII).

TABLE VII

SINGLE-BLIND SPIKED PHENYLALANINE ANALYSIS[a]

Sample	Spiked comp. (%)	Determined (%)	R.S.D. (%)	RE (%)
6-73-2				
d-form	33.6	33.3 ± 0.3	0.9	−0.9
l-form	66.4	66.7 ± 0.3	0.4	+0.5
6-74-1				
d-form	11.2	11.1 ± 0.4	3.6	−0.9
l-form	88.8	88.9 ± 0.4	0.4	+0.1
6-74-2				
d-form	11.2	11.2 ± 0.2	1.6	–
l-form	88.8	88.8 ± 0.2	0.2	–

[a] 40 μl sample × 80 mg polymeric benzophenone–DNB, 3 min at RT, to 1 ml of THF–hexane (15:85), hexane–DCM–methanol (75:23:2) at 3 ml/min. Supelco LC-(*S*)-Naphthyl Urea column, 250 × 4.6 mm I.D., 20 μl injection × 0.004 a.u.f.s. at 254 nm.

Comparison of chiral resolution of urea vs. amide derivatives of d,l-norephedrine

The 3,5-dinitrobenzamide and 3,5-dinitrophenylurea derivatives of *d,l*-norephedrine were synthesized via the solution-phase reactions of *d,l*-norephedrine with

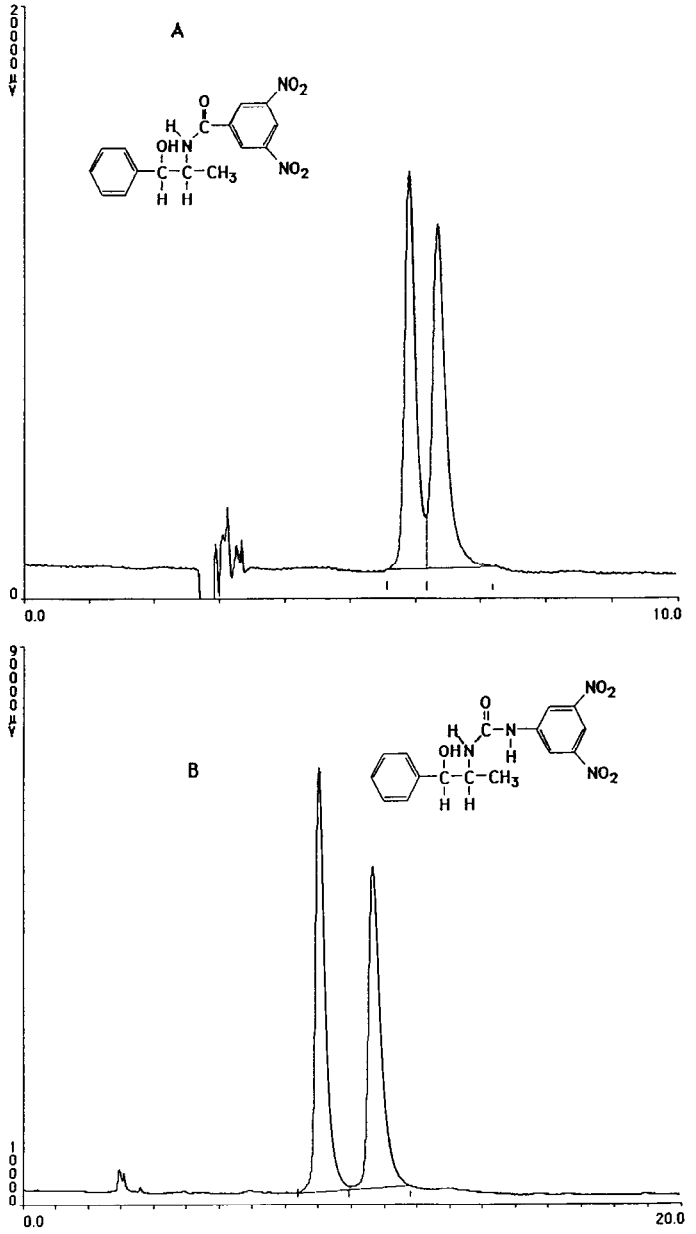

Fig. 13. Comparison of chiral resolution of the standards of the (A) 3,5-dinitrobenzamide and the (B) 3,5-dinitrophenylurea of *d,l*-norephedrine. Conditions: 40 ppm each in mobile phase, injection volume 10 μl. Chromatography, 5-μm LC-(*S*)-Naphthyl Urea 250 × 4.6 mm I.D., methyl-*tert.*-butyl ether (MtBE)–ACN–methanol (92:7:1) at 1.0 ml/min. Retention time in min.

3,5-dinitrophenylacylazide (DNPAA) and DNBCl, respectively. The dinitrophenylurea was formed by decomposition of the DNPAA at 80°C in toluene to form dinitrophenylisocyanate *in situ* [29]. The *d,l*-norephedrine was added to this solution and allowed to react for 30 min. The urea was isolated by rotary evaporation and recrystallized from methanol–water. The derivatives were purified via recrystallization and a 40-ppm solution of each was prepared in the mobile phase. The chiral resolution of each under identical separation conditions was determined (Fig. 13).

RESULTS AND DISCUSSION

We describe here a solid-phase approach for the derivatization of nucleophiles for improved separation and detection in HPLC with UV detection. Two polymeric reagents have been prepared by modifying commercially available polymeric supports. The first polymeric reagent contained an *o*-nitrobenzophenone activated ester linkage which had been proven useful for on- and off-line derivatizations of amines and related nucleophiles. The second polymeric reagent contained a benzotriazole ester, which was >2 orders of magnitude more reactive towards nucleophiles [25]. This was due to the polymeric matrix which differed from the first reagent in structure as well as mesh size, surface area and possibly pore size. This reagent allowed for enhanced derivatization of less nucleophilic species, such as amino acids and amino alcohols. It was, however, sensitive to moisture and could not be used on-line for HPLC derivatizations [6]. A comparison of the reactivity of the two polymers is made in this paper, but we have not compared specific rate constants. We are currently synthesizing new batches of both polymers from the same polystyrene–divinylbenzene matrix and a true comparison of rate constants is forthcoming [30].

Synthesis/characterization of derivatives

For true calculations of the percent conversion of analytes, the expected derivatives from the solid-phase reactions with amine substrates were synthesized on a preparative scale. This allowed knowledge of the chromatographic behavior of the derivatives, as well as the efficiency of conversion of analyte to derivative. The derivatives of amines, amino acids and amino alcohols were synthesized via solution phase techniques and then characterized for physical and spectral purity. Derivatives which contained a secondary amide, obtained from a primary amine analogue, had a maximum at 207 nm. Those containing a tertiary amide, had a maximum at 233 nm. For HPLC determinations, the UV wavelength used was 254 nm, which provided good sensitivity for both 2° and 3° amides.

Characterization of the polymeric reagents

Hydrolysis. The polymers were characterized via a hydrolysis procedure, which quantitatively hydrolyzed the ester groups, as well as by elemental analysis. The hydrolysis procedure afforded the absolute amount of analytical label covalently attached to the polymeric benzophenol or the polymeric hydroxybenzotriazole. The benzoic acid released from the polymers under high pH conditions, was quantitated via HPLC using external standard calibration. The hydrolysis procedure for the benzophenone was performed with a benzoyl, not 3,5-dinitrobenzoyl, moiety attached to the polymer. A significant amount of the 3,5-dinitrobenzoate anion underwent

decarboxylation under conditions of the experiment. Unsubstituted benzoyl chloride (BCl) was used to tag the polymer under the assumption that the two different acid chlorides would add to the polymer to the same extent. The BCl-tagged polymer was hydrolyzed and the benzoic acid generated, then quantitated. The recovery of benzoic acid from the hydrolysis procedure was constant at 96.1 ± 0.4%, from 30 min to 2 h. This suggested that none of the benzoic acid released from the polymer was lost. The amount of benzoic acid found was a true representation of the amount originally covalently attached to the polymer (Table I).

The polymeric hydroxybenzotriazole was tagged with *o*-acetylsalicyloyl chloride, washed and dried prior to the loading determination. The hydrolysis procedure was performed previously and included a liquid–liquid extraction procedure [6]. The amount obtained for the polymeric hydroxybenzotriazole was roughly one half of the loading of the polymeric benzophenone (Table I).

Elemental analysis. The elemental analysis of the polymeric benzophenone was performed on the virgin polystyrene, the intermediate polymeric phenol and the final reagent which had been acylated with DNBCl. Though the reactivity of the polymers acylated with BCl and DNBCl would be expected to be different due to the inductive effects of the nitro groups in the 3,5-DNB label, the two acid chlorides added to the polymer in nearly equivalent amounts as indicated by the agreement between hydrolysis and elemental analysis data (Table I). The quantitative conversion of the polymeric *p*-chloro intermediate to the benzophenol was indicated by the lack of chlorine. From the difference in nitrogen content in the starting polystyrene and the polymeric benzophenol intermediate, the absolute loading possible, if all polymeric benzophenol sites were converted to the active ester, was calculated to be 1.9 mmol/g. The difference in nitrogen content between the polymeric phenol and the final analytical reagent gave the amount of label which was covalently attached to the solid-phase reagent. The elemental analysis data indicated 30% of the phenol sites were esterified. The reason for the unlabelled sites may be a combination of incomplete acylation of accessible phenol sites, hydrolysis of the activated ester during the washing procedure, and phenol sites within the pores of the polymer which were sterically inaccessible to the DNBCl. The loading obtained, however, still created a local molar excess of labeling reagent of >350-fold for ppm concentrations of analyte.

The elemental analysis of the polymeric hydroxybenzotriazole reagent was performed using only the starting polystyrene and the final reagent tagged with an *o*-acetylsalicyloyl group [6]. The percentage of each element was calculated based on the hydrolysis data of 0.31 mequiv. *o*-acetylsalicyloyl/g polymer. The calculated percentages matched closely the actual percentages of each element present, which suggested agreement between hydrolysis data and elemental analysis data for the polymeric hydroxybenzotriazole (Table I).

Regeneration of polymeric reagents

Regeneration of the polymeric reagents was accomplished by washing with ACN, and retagging with DNBCl. After >50 regenerations, the polymeric benzophenone–DNB still acylated butylamine to over 90% completion in <60 s. After two years, the polymeric hydroxybenzotriazole was washed with ACN and tagged. The reagent showed very high reactivity for amines, amino alcohols and amino acids. This corroborated our theory that the polymeric intermediates were totally regenerable and only the final tagging reaction need be repeated.

On-line derivatization

Only the polymeric benzophenone–DNB was used on-line for real-time or delayed conversion of the amine analogues to the corresponding amides. The solvent used for on-line derivatizations was the mobile phase or, at times, a single component of the mobile phase. The solvents used for optimal percent derivatizations had to be chosen with the separation scheme in mind. Again, ACN-, THF- and DCM-containing mobile phases offered the highest percent of conversions. Not unexpectedly, the solvents which yielded the highest percent of conversions, also led to increased reactor bleed and reduced lifetime. The average useful analytical lifetime of the reactor was one day's work; the reactor had to be dismantled and repacked at the beginning of each day for efficient, reproducible results.

For on-line derivatizations in reversed-phase solvents, a dual-mobile-phase system was configured in which only anhydrous organic solvent flowed through the reactor (Fig. 2) [5]. This was necessary due to the reduced lifetime of the polymeric benzophenone–DNB in aqueous solutions. The analytes were injected in pure ACN and were passed through the reactor at low flow-rates and elevated temperatures. The derivatives formed on-line were preconcentrated on the head of the analytical column by forming an initially weak isocratic mobile phase. When the derivatives had left the reactor column, the gradient was increased to remove the derivatives from the analytical column. In this manner, all band broadening contributions prior to the analytical column were negated. While in theory very large injection volumes could be used, only 20-μl injections were attempted. The on-line derivatization of three amines, morpholine, *n*-propylamine and *n*-butylamine, was performed using two different solvent systems. The first was a 1:1 mixture of ACN and THF. The anhydrous oganic contained a 1:1 mixture of these solvents, and the aqueous pump contained 10% of the 1:1 organic mixture. This was done because of the difference in reactivity in THF compared to ACN. The derivatives were more easily formed in solvents which were partially or wholly ACN (Fig. 3).

For normal-phase on-line derivatizations, a single pump was configured due to the inability of the system to effectively precipitate the derivatives at the head of the chiral column. The polymeric benzophenone–DNB was more stable towards normal-phase solvents due to their non-polar, anhydrous nature. It was found that the polymeric benzophenone–DNB effectively acylated alcohols which were used as polar modifiers, and these had to be substituted with other non-nucleophilic polar modifiers. The amount of time the reaction was allowed to continue could be chosen through a choice of flow-rate in real-time derivatizations, or the amount of time the analyte was allowed to reside within the reactor under bypass conditions (Fig. 2). The real-time derivatizations gave less baseline disturbance and more symmetric peak shapes. The baseline disturbance was understandable, since a small amount of analytical label was constantly bleeding through the system due to the instability of the reagent. To switch the reactor on- and off-line, caused a break in this constant bleed. The better symmetry of the peaks was due to reduced diffusion within the reactor under steady flow conditions. When the analyte was trapped, the heat from the water bath caused thermal convection inside the reactor which lead to diffusional band broadening. Interestingly, less reactive polymeric reagents yielded more symmetric derivative peaks in the bypass mode than for real-time conversions [31]. With less reactive polymers, diffusional band broadening was not as important as reaction band broadening. While

less reactive, these polymeric reagents proved useful for the on-line conversion of many pharmaceutically important amines [32]. Another factor in the symmetry of the derivative peaks was the kinetics of reaction. Slow reaction kinetics with a moving analyte will lead to what is termed, "reaction band broadening" which means that the analyte plug experiences derivatization throughout the length of the polymeric reactor, instead of instantaneous derivatization. This would appear most noticeably for real-time derivatizations at higher flow-rates or, as mentioned, with less reactive polymers [33]. This was noticed for the injected amphetamine *vs.* the injected standard, amphetamine–DNBa (Fig. 5). The standard had some asymmetry due to void volume and eddy diffusion band broadening, but the derivative peak tailed more so, indicating reaction band broadening had also occurred.

For on-line derivatizations, the temperature of reaction was raised to enhance the rates. Since kinetics are based on activation energy, concentration of reactants and temperature, higher temperatures yielded higher overall conversions. The increase in temperature had a two-fold effect; it raised the energy of the reactants closer to the activation energy of the tetrahedral intermediate, and it decreased the viscosity of the solution, increasing mass transfer to and from the active ester sites. For on-line studies, derivatization temperatures of 50–70°C were used, depending on the mobile phase. The optimal temperature was chosen by allowing the injected analyte to flow through the reactor at low flow-rates and determining the increase in derivatization with increases in temperature.

Optimization of off-line derivatization conditions

Solvent. The solvent must be a polar, aprotic, non-nucleophilic solvent of low viscosity to properly stabilize the tetrahedral adduct formed upon nucleophilic addition to the carbonyl carbon. The solvent must be compatible with the separation scheme and the detection mode. The solvent must also swell the polymer, to allow for better mass transfer of analyte into the pores of the polymer. The optimum solvent for derivatization of primary and secondary amines was determined to be ACN [6].

Temperature and time. The proper amount of time was determined for amines by plotting the percentage of analyte converted *vs.* time. The carbonyl carbon of the ester linkage is highly activated towards nucleophilic attack, due to the *ortho*-nitro group and the *para*-carbonyl group in the polymeric phenol, which act to delocalize the negative charge on the leaving group. However, activation of the carbonyl center now was due largely to the inductive effect of the two nitro groups in the analytical label. This was determined by a comparison of the reactivity of the same polymeric benzophenol which had been tagged with other reagents such as 9-fluorenylchloroformate and *o*-acetylsalicyloyl chloride. These polymeric reagents were less activated due to the structure of the labelling moiety, and yielded lower percent of derivatizations. Under ambient temperature conditions, the polymeric benzophenone–DNB reacted with a 200-ppm concentration of *n*-butylamine to more than 90% completion within 60 s. This was convenient, and thus, no attempts at optimizing reaction temperature were performed.

The same optimization of time was performed with indentical substrate and solvent conditions. The polymeric hydroxybenzotriazole–DNB reagent was more reactive than the polymeric benzophenone–DNB. The rate of acylation of a primary amine, *n*-butylamine, could not be measured at RT. A 10-s derivatization yielded 95%

conversion, while a 5-s derivatization yielded 96%. The reaction was assumed to occur instantaneously. The reaction of diethylamine was slower due to steric hindrance of the nucleophilic center, conversion was 48% in 5 s.

Effect of base catalyst on percent conversions

The kinetic plot of percent conversion *vs.* time asymptoted to a constant value after reaching 50% conversion (Fig. 6). At this point, the hydrogen ion generated during the final stage of the addition–elimination reaction was protonating unreacted amines. Addition of TEA scavenged this proton, allowing the reaction to proceed to completion [34]. TEA also deprotonated any residual phenol sites, thus removing a source of hydrogen bonding and possible loss of analyte due to ion-pair formation. This was first noticed when *n*-butylamine and diethylamine were derivatized simultaneously. *n*-Butylamine proceeded to a higher percent conversion, while the percent conversion of diethylamine was reduced. Addition of TEA allowed for the derivatization of three amines simultaneously, without any interaction between the analytes (Fig. 5). Concentrations of the base catalyst in greater than an equimolar amount gave no higher percent derivatizations, but were deleterious due to derivatization of impurities in the TEA. For aqueous derivatizations, the pH was adjusted with hydroxide, which kept the analyte as the free amine. The only drawback was that the analytes and hydroxide were now competing for the same active ester sites. The high molar excess of the derivatizing reagent assured that at pH 11 or lower, this was not a major factor.

pH dependence of derivatization

The pH dependence was determined using partially aqueous solutions. Borate buffer was adjusted to appropriate pH values and mixed with an equal amount of ACN. The derivatization maximum, 70%, occurred at pH 9.5. At pH 7 the analyte was partially protonated and non-nucleophilic, while at higher pH values there may have been competition of the amine with hydroxide for the active ester sites, and/or alkaline hydrolysis of the derivatized analyte.

Linearity of derivatization with substrate concentration

Theoretically, the reproducibility of the percentage of derivatization should remain constant, regardless of the concentration of amine. This was true when the polymeric ester was in large molar excess. For 30 μl of 200 ppm *n*-butylamine, the immobilized reagent (70 mg) was in 366-fold excess. This was important for standard addition experiments, where additional analyte added must produce a linear response. The linear range of *n*-butylamine was from 10 parts per thousand (ppth) to the detection limit, which for amines derivatized precolumn, off-line, was 1 ppm. Above 10 ppth, the percent derivatization began to drop. The polymer became the limiting reagent at this concentration.

Derivatization characteristics

Detection limits. The high limit of detection was due to the dilution of the analyte as it was extracted from the polymeric matrix, off-line. The injection volume was 20 μl; a larger injection volume would have allowed lower concentrations to be detected. A larger amount of analyte injected onto a larger mass of polymer would have

accomplished the same feat. Solid-phase extraction of the analyte using ion-exchange sorbents or ion-pair formation using hexanesulphonic acid and hydrophobic (C_{18}) solid-phase extraction could also be used to lower detection limits [35]. Evaporation of the solvent after elution from the solid-phase derivatization cartridge was considered, but this usually leads to a loss in precision. A detection limit of approximately 1 ppm was deemed appropriate for most applications.

Optimization of analyte/reagent ratios

Ideally, the polymer should remain in at least 40-fold molar excess if derivatization of the analyte is dependent only on concentration of the analyte. When performing off-line reactions, the polymer bed should not be completely wetted with the sample. If this occurred, some of the analyte would be absorbed by the tissue plug and reproducibility of the method would suffer. The ratio of injected volume to mass of polymer used was different for the two polymers and was a function of surface area. The ratio of volume to mass in μl/mg for the two polymers was 1:2 for the polymeric benzophenone–DNB and 1.7:1 for the polymeric benzotriazole–DNB. The higher surface area of the polymeric benzotriazole reagent allowed for a smaller mass to adsorb a larger volume of injected analyte.

Reproducibility of the method

For derivatization off-line of analyte concentrations at $10\times$ the detection limit, the reproducibility of derivatization was good. The percent relative standard deviation (R.S.D.) of peak areas obtained was in most cases less than 5%. This, in conjunction with a 2% R.S.D. for injection from a fixed-loop injector, gave good reproducibility for injection volumes of $\geqslant 10$ μl. Thus, reproducibility of the entire method was $\leqslant 7\%$ R.S.D.

Achiral analyses

Amines. The achiral analysis of amines was shown for three amines derivatized off-line (Fig. 5). Aliphatic amines are not easily analyzed by HPLC in the absence of derivatization due to their poor molar absorptivity, ionization at neutral pH and poor behavior on silica-based supports. The facile derivatization of these analytes using solid-phase reagents solved these problems, and the final derivatives could be easily detected to low ppm levels. Comparison of the reaction of *n*-butylamine and diethylamine with the two polymers indicated their difference in relative reactivity (Fig. 7).

Amino alcohols. Amino alcohols had even less desirable qualities for separation and detection than amines. This was due to the increased intramolecular hydrogen bonding, high water solubility and lower vapor pressure. Thus, even GC analyses are not effective in the absence of derivatization. The polymeric benzophenone–DNB reagent reacted slowly with these compounds, due to intramolecular hydrogen bonding which slowed mass transfer to the polymeric surface. For a homologous series of amino alcohols this meant that the nucleophilicity of the amine group increased as the carbon chain separating the alcohol oxygen and the amine increased. This was due to inductive effects of the electronegative oxygen, which removed electron density from the nitrogen atom. However, viscosity increased with chain length, due to intramolecular hydrogen bonding, which was possible when the chain length was >3.

The polymeric benzophenone–DNB accomplished a 15% derivatization of ethanolamine at 75°C for 10 min, while the polymeric benzotriazole–DNB accomplished a 96% conversion in under 2 min at RT (Fig. 8).

Amino acids. Amino acids were slow to undergo derivatization with either reagent due to their zwitterionic nature at neutral pH. For most amino acids, at pH 10 the carboxyl group was ionized, leaving the amine group unprotonated and nucleophilic. However, the hydration sphere around the carboxyl group slowed mass transfer to the polymer, and electron induction from the carboxyl group lowered the nucleophilicity of the amine. Methionine and phenylalanine were dissolved in ACN–0.05 *M* NaOH (80:20) and reacted with both polymeric reagents. The polymeric benzophenone–DNB reagent derivatized both amino acids 2% in 2 min at RT while the polymeric benzotriazole–DNB reagent derivatized both amino acids *ca.* 25%. The separation of valine, methionine, phenylalanine and tryptophan, derivatized using the polymeric benzotriazole–DNB reagent off-line, showed a hydrolysis peak due to reaction of the hydroxide ions (Fig. 9). The hydrolysis product, 3,5-dinitrobenzoate anion, was retained due to pH suppression of the mobile phase. Use of a gradient would have yielded better resolution and smaller peak volumes; isocratic conditions, however, were sufficient.

Amino acid methyl esters. The derivatization of amino acid methyl esters yielded higher percent derivatizations, since the amine group was more accessible. The methyl ester hydrochlorides were neutralized, solid-phase extracted from solution, and eluted into ACN. Using the polymeric benzotriazole–DNB for 2 min at RT, the percent derivatization of phenylalanine and methionine was 75%. A better separation from early-eluting compounds was obtained, since the methyl esters were more hydrophobic. In addition, the hydrolysis peak was not as severe since there was no pH suppression (Fig. 10). The hydrolysis peak was removed in subsequent analyses by using a mobile phase with ACN–water (45:55) adjusted to 0.05% (v/v) NH_4OH. Again, a gradient system would be necessary for analysis of all amino acids, and these amino acid methyl esters were chosen to show efficacy of the methodology.

Chiral analyses

The derivatization of chiral analytes with an achiral π-acid label, allowed for direct chiral recognition on Pirkle brush-type stationary phases [16–18]. All chiral work was performed in normal phases, because solvation of the chiral analytes with water obscured the 1:1 (analyte–stationary phase), 3-point molecular recognition. The use of a π-acid-labelled analyte and a π-base stationary phase, offers higher resolution of enantiomers than the opposite logic. In addition, π-acid derivatizing reagents are more powerful acylating reagents, and allow for facile conversions of weak nucleophiles. Thus, the use of a π-acid immobilized reagent seemed justified.

The off-line derivatization of chiral amines was accomplished using the polymeric benzophenone–DNB to exemplify the usefulness of the analytical methodology. α Values for some substrates were as high as 1.8 and this recognition allowed for sensitive measurements of enantiomeric excess. Although all chiral amines analyzed were less than 100% optically pure, most contained less than 1% of the other isomer (Table II). The 3,5-dinitrobenzamides of secondary amines have no proton on the amide nitrogen and it appeared that this hydrogen bonding with the stationary phase was important for chiral recognition. None of the secondary amines were baseline

resolved as the 3,5-dinitrobenzamides. Generally, these compounds were reacted with 3,5-dinitrophenylisocyanate in solution to form a dinitrophenyl urea [29]. The separation of *d,l*-norephedrine as the 3,5-dinitrobenzamide *vs.* the 3,5-dinitrophenyl urea derivative gave an indication of the effect of the urea's enhanced recognition (Fig. 13).

Derivatives of the amino alcohols did not separate well, due to the achiral interactions of the alcohol group with the stationary phase. α Values ranged from 1.0 to 1.2. The compounds studied did not contain an aromatic center, which enhances the recognition through π–π interactions and increased steric bulk of the ligand. The distance of the chiral center from the site of acylation also had a profound effect on the recognition. As the chiral site moved further from the π-acid label, recognition became less due to a poorer fit of the necessary sites of interaction with the stationary phase (*i.e.*, dipole interaction, hydrogen bonding and steric fit).

Chiral recognition of amino acid methyl esters was enhanced due to dipole interaction of the carbonyl group adjacent to the chiral carbon. Only tryptophan methyl ester was found to be 100% optically pure (Tables III and IV). The detection limit of enantiomeric excess was assumed to be $\leqslant 0.1\%$ based on the detection limit of *d*-valine methyl ester, which showed 0.18% optical impurity. This allowed for approximately 0.06% to be detected at a signal-to-noise ratio of 2 (Fig. 11). The separation values for many of the amino acid methyl esters were comparable to values obtained with similar chiral stationary phases [36,37].

Single-blind spiked chiral analysis of amphetamine

Artificial spiked samples in ACN–0.1 *M* NaOH (50:50) of *d,l*-amphetamine sulphate were prepared at different enantiomeric compositions. Analysis of the samples involved injection of the analyte solution onto the polymeric benzophenone–DNB off-line, and elution of the derivatives after a set amount of time. Injection of the solution containing the derivatives yielded the enantiomeric ratio of the amphetamine enantiomers from peak areas (Table V).

Single-blind achiral analysis of amphetamine in urine using a mixed-bed reactor

The analysis of amphetamine spiked into urine was accomplished using a 1:1 molar mix of polymeric benzotriazole–(pNB/DNB) off-line. Quantitation was performed via comparison to an external-standard calibration plot which was obtained from known amounts of amphetamine, spiked into the same urine matrix. The pNB derivative of amphetamine was more accurate than the DNB derivative, possibly due to matrix coelution. This possibility was negated since there was no peak present in the blank at that retention time. Also, the standard curve should have accounted for that problem, since it was prepared in the same matrix. We are uncertain as to the determinant error. Numbers for each derivative were within an acceptable percent relative error of the true amount (Table VI and Fig. 12).

Single-blind analysis of enantiomeric composition of phenylalanine

This experiment was designed to show the validity of the approach to a real-world lot of amino acid which could be used for pharmaceutical preparations or peptide synthesis. It also proved a lack of racemization inherent in the methodology. The phenylalanine was alkylated, extracted and derivatized off-line using the

polymeric benzophenone–DNB reagent. Chiral analysis was performed using peak areas of the enantiomers. Excellent agreement was seen between the spiked ratios and those determined using off-line solid-phase derivatization followed by chiral chromatography (Table VII).

CONCLUSIONS

We described two polymeric reagents useful for the conversion of nucleophilic substrates to more detectable species for HPLC–UV detection. These derivatives were amenable to reversed- or normal-phase chromatography, and the polymers generated derivatives which were amenable to Pirkle-type enantioselective separations. In previous work, the derivatives had been engineered to contain a chiral center, in which case derivatives of enantiomeric analytes were separated as diastereomers on achiral stationary phases [7]. This approach was shown to be a viable alternative to solution-phase derivatizations for indirect enantiomeric recognition. However, the preferred approach to analysis of chiral analytes is direct enantiomeric recognition on a chiral stationary phase, and the use of a polymeric reagent which imparted an achiral tag to the analyte has now allowed for this. Sensitive, facile determinations of enantiomeric excess for chiral analytes has been shown possible using these solid-phase reagents. The use of a polymer containing more than one labelling moiety yielded additional information about an analyte in a complex matrix. Future work will include the detection of catecholamine metabolites in serum and/or urine to further exemplify the utility of the methodology.

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