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Note

Automated precolumn derivatization of amino acids, small peptides, brain amines and drugs with primary amino groups for reversed-phase high-performance liquid chromatography using naphthalenedialdehyde as the fluorogenic label

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One of the most extensively used methods for the assay of primary amines is based on the precolumn derivatization with *o*-phthaldialdehyde (OPA) in the presence of a thiol [such as 2-mercaptoethanol (2-ME)] as the nucleophile at basic pH in aqueous solution [1]. Previously, our laboratory has reported automated fluorimetric procedures for the precolumn derivatization, which were applied to amines using OPA and 2-ME as reagents [2] and to carboxylic acid [3,4]. The OPA–2-ME method suffers from instability of the fluorescent isoindole derivatives and it is only useful for detecting primary aliphatic amines and amino acids. Most oligopeptides and proteins do not give sufficient fluorescent products because their formation seems to be dependent on a C-terminal lysine [5]. The search for the formation of more stable fluorescent adducts has led to an improved derivatization method. The extension of the single benzene nucleus to a naphthalene-2,3-dicarboxaldehyde (NDA) and the use of cyanide (CN) as a nucleophile provide intensely fluorescent N-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives of amino acids, peptides and other compounds possessing primary amino groups [6–8]. Another advantage is that NDA derivatives are also suitable for chemiluminescence detection [9]. The derivatization reaction, carried out in aqueous solution at basic pH, to yield N-substituted CBIs is complete within 15 min.

The aim of this study was to develop an automated precolumn derivatization procedure to demonstrate the versatility of NDA as a fluorogenic label for the high-performance liquid chromatographic (HPLC) assay of compounds with primary amino groups. The method can be applied to a wide variety of biologically or medically interesting compounds with minimum sample pretreatment. Additional biomedical applications of the method may include the assay in brain

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dialysate, cerebrospinal fluid, plasma or urine of drugs such as amphetamine [a stimulant of the central nervous system (CNS)], fluvoxamine (an antidepressant), baclofen (for the treatment of spasticity), tyramine (a depression marker) [10,11] and spermine and spermidine (tumour markers).

EXPERIMENTAL

Chemicals, reagents and solutions

Monobasic and dibasic sodium and potassium phosphate, sodium cyanide, sodium tetraborate decahydrate, methanol, tetrahydrofuran, acetonitrile, taurine and β-alanine were purchased from E. Merck (Darmstadt, F.R.G.), L-aspartic acid, L-asparagine, L-alanine, L-valine, L-leucine, L-dihydroxyphenylalanine (L-DOPA) and *trans*-2-phenylcyclopropylamine hydrochloride (tranylcypromine) from Sigma (St. Louis, MO, U.S.A.), L-glutamic acid, L-glutamine, 4-aminobutyric acid (GABA), L-2-aminobutyric acid and glycylglycine from Fluka (Buchs, Switzerland), DL-homoserine and phenylethylamine from Janssen Chimica (Beerse, Belgium), DL-normetanephrine hydrochloride and tyramine hydrochlo-

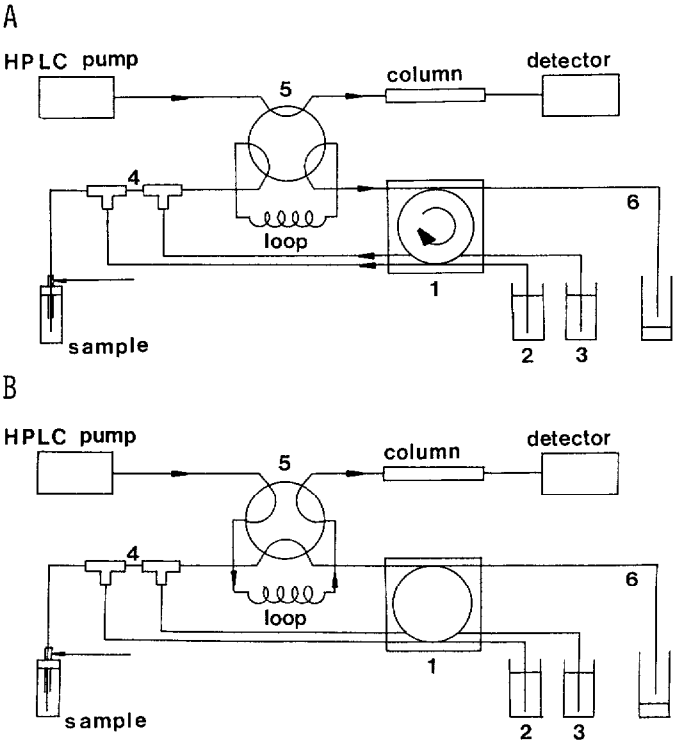

Fig 1 Schematic diagram of the precolumn derivatization device The principal components are described in detail in the text (A) Load position; (B) inject position

ride from Calbiochem (La Jolla, CA, U.S.A.), glycyl-DL-aspartic acid and glycylglycyl-L-aspartyl-L-alanine from Bachem (Bubendorf, Switzerland), DL-norvaline from Serva (Heidelberg, F.R.G.), glycine (AnalaR) from BDH (Poole, U.K.), L-serine from Nutritional Biochemicals (Cleveland, OH, U.S.A.) and dexamphetamine sulphate from the Department of Pharmacy, University Hospital Groningen (Groningen, The Netherlands). Baclofen was a gift from Miss E. W. Wuis (Department of Clinical Pharmacy, Sint Radboud Hospital, University of Nijmegen, Nijmegen, The Netherlands). NDA was obtained from TCI-Tokyo Kasei Kogyo (Tokyo, Japan) All compounds were of analytical-reagent grade.

Solutions and buffers were prepared in water doubly distilled in quartz apparatus. Stock solutions of the amino acids (1 mg/ml) and amino acid mixtures in water were stored at −25°C and diluted before use. All the other amine compounds were freshly prepared. NDA was prepared daily by dissolving the required amount in methanol. Stock solutions of sodium cyanide (5 mg/ml) were prepared in borate buffer (pH 9.3) and were diluted before use with the same buffer. The sample/CN reagent/NDA reagent mixing ratio (dependent only on the internal diameters of the three pump tubes) was adjusted to 3:1:1. Sodium phosphate buffer (pH 7.0, 0.05 *M*) and potassium phosphate buffer (pH 6.8, 0.05 *M*) were prepared by mixing solutions of monobasic and dibasic sodium and potassium phosphate, respectively. Sodium borate buffer (pH 9.3, 0.05 *M*) was made from sodium tetraborate decahydrate.

Precolumn derivatization procedure

A Spark (Emmen, The Netherlands) PROMIS autosampler is equipped with a modified precolumn derivatization unit which enables two reagents to be delivered instead of one (Fig. 1). A peristaltic pump (1) transports (flow-rate 75 μl/min, tube I.D. 0.11 mm) the solutions of cyanide (2) and NDA (3) into the mixing tees (4) when the injection valve (5) (Model 7010, Rheodyne, Cotati, CA, U.S.A.), equipped with a 50-μl sample loop which serves as a reaction coil, is in the load/react position (Fig 1A). At the same time, a third waste tube (6) with a higher flow-rate of 375 μl/min (due to a larger inner diameter of 1.29 mm) withdraws solution from the mixing tees. The difference between the flow-rates of the removal and reagents is compensated for by sample in-flow (225 μl/min). When the sample loop is filled (programmable pump time) and the derivatization is complete (programmable reaction time), the valve switches into the inject position (Fig. 1B) and the sample is injected onto the column.

Equipment and chromatography

Two LKB (Bromma, Sweden) Model 2150 HPLC pumps, controlled by an LKB Model 2152 solvent programmer were used for gradient elution at a flow-rate of 0.5 ml/min. The amino acid derivatives were eluted with a gradient of the following buffers: (A) sodium phosphate buffer (pH 7.0, 0.05 *M*) and (B) methanol–tetrahydrofuran–water (50:20:30, v/v) (Fig 2A). The peptides, biogenic

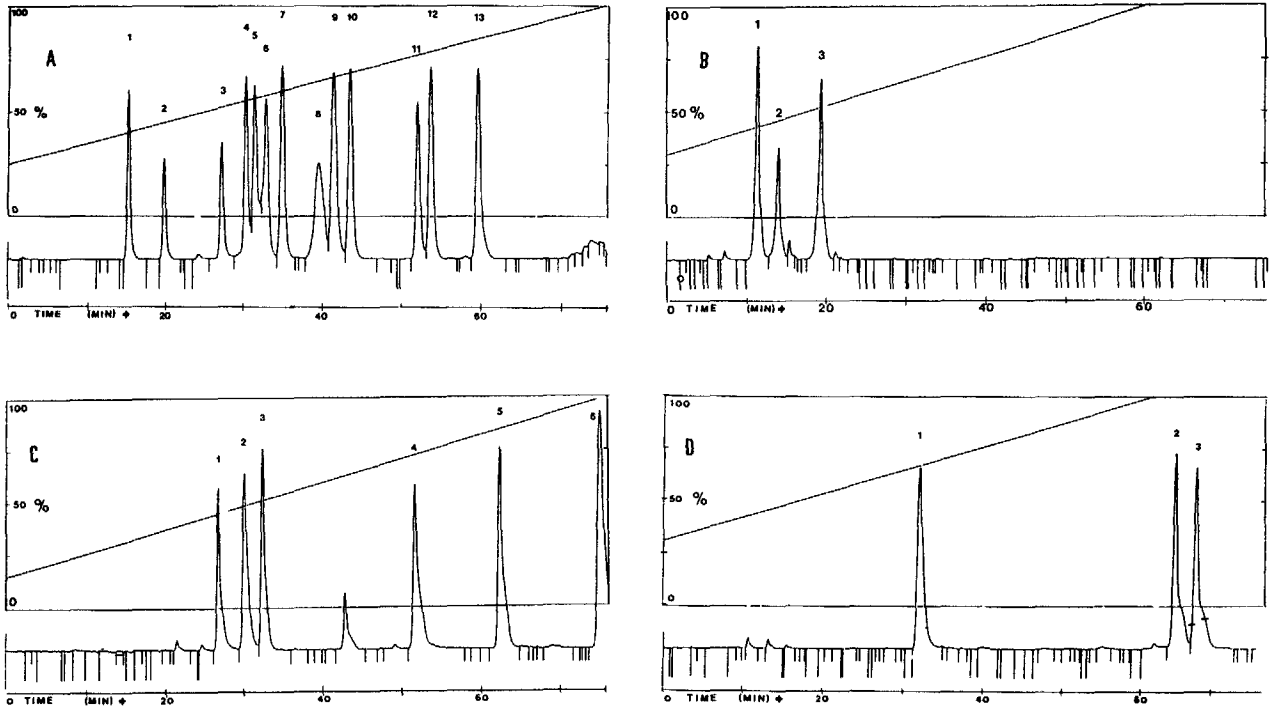

Fig 2 Chromatograms of a standard mixture of (A) amino acids, (B) peptides, (C) brain amines and (D) drugs Ordinate solvent gradient (% B) Amounts injected, 100 pmol Peaks. (A) 1 = aspartic acid; 2 = glutamic acid, 3 = asparagine, 4 = glutamine; 5 = serine, 6 = homoserine, 7 = glycine, 8 = taurine, 9 = alanine, 10 = β-alanine, 11 = norvaline, 12 = valine, 13 = leucine, (B) 1 = glycyl-DL-aspartic acid; 2 = glycylglycyl-L-aspartyl-L-alanine, 3 = glycylglycine, (C) 1 = L-dihydroxyphenylalanine, 2 = γ-aminobutyric acid, 3 = α-aminobutyric acid; 4 = normetanephrine, 5 = tyramine, 6 = phenylethylamine, (D) 1 = baclofen; 2 = amphetamine; 3 = tranylcypromine

amines and drugs were eluted with mobile phases composed of solvent A, tetrahydrofuran–potassium phosphate buffer (pH 6.8, 0.05 *M*) (5:95, v/v), and solvent B, acetonitrile–methanol–potassium phosphate buffer (pH 6.8, 0,05 *M*) (55:10:35, v/v) (Fig 2B–D). Prior to use, the buffers were degassed and passed through a 0 2-μm membrane filter; during analysis, helium was passed continuously through both buffer solutions

The CBI derivatives were separated on a Chrompack (Middelburg, The Netherlands) ODS-2 Chromspher C_{18} column (200 mm × 3.0 mm I.D.; particle size 5 μm) and detected with a Kontron SFM 23 spectrofluorimeter (VH Instruments, Maarsen, The Netherlands) equipped with a high-pressure xenon lamp (150 W). The chromatograms were recorded with a CI-10 integrator (LDC-Milton Roy, Shannon, Ireland).

RESULTS

Reaction conditions and analysis

The derivatization reaction is carried out in aqueous solution at pH 9.3 in 15 min. Optimum reaction conditions have already been described [6–8]. The peak area *versus* the concentration for aspartic acid, glycylglycine, tyramine and amphetamine was linear over the range 10 fmol–100 pmol injected. The correlation coefficients were better than 0.974. The standard deviation of the determination of aspartic acid samples ($n = 8$, 100 pmol injected) was 0.113. The detection limits, based on a signal-to-noise ratio of 2, were 10 fmol for aspartic acid, 25 fmol for glycylglycine, 50 fmol for tyramine and 50 fmol for amphetamine (50 μl of sample injected). These detection limits are similar to or lower than those reported in the literature [8,12].

Fractions of rat brain dialysate were collected by means of perfusion with artificial cerebrospinal fluid (CSF) of U-shaped cellulose dialysis fibers which were bilaterally implanted in the rat striatum, as described in detail elsewhere [13].

The urine sample for the determination of tyramine (see *Applications*) were heated at 100°C for 30 min at pH 0.9 to hydrolyse tyramine-O-sulphate. The same procedure was followed for the determination of tyramine in cheddar cheese (200 mg of crumbled cheese stirred for 60 min in 100 ml of water at pH 0.9, filtered and then hydrolysed). Human plasma was diluted as much as 200-fold before injection, still allowing the detection of amphetamine in the clinically relevant range. Rat brain dialysate and human urine (tyramine determination) could be analysed without extraction, purification or dilution.

Fluorescence was produced with a xenon source at an excitation wavelength of 420 nm. The waker tungsten–halogen source results in lower sensitivity. Excitation at 420 nm is not optimum for fluorescence detection, hence shifting the excitation wavelength to the ultraviolet region (246 nm) using a deuterium lamp may improve the detection limits further [7,8]. Extremely low detection limits can be obtained with laser-induced fluorescence. Detection limits for NDA deriv-

atives utilizing the UV lines of an argon ion laser were in the range 200–500 amol [12]. Currently, chemiluminescence is also used and the light source is not necessary because the fluorescence is generated chemically. NDA derivatives could be detected at levels as low as 200 amol [9].

Applications

As an example of a clinical application of the procedure, we chose the tyramine conjugation test, which has been used to predict the treatment response to tricyclic antidepressant medication. After an oral dose of tyramine hydrochlo-

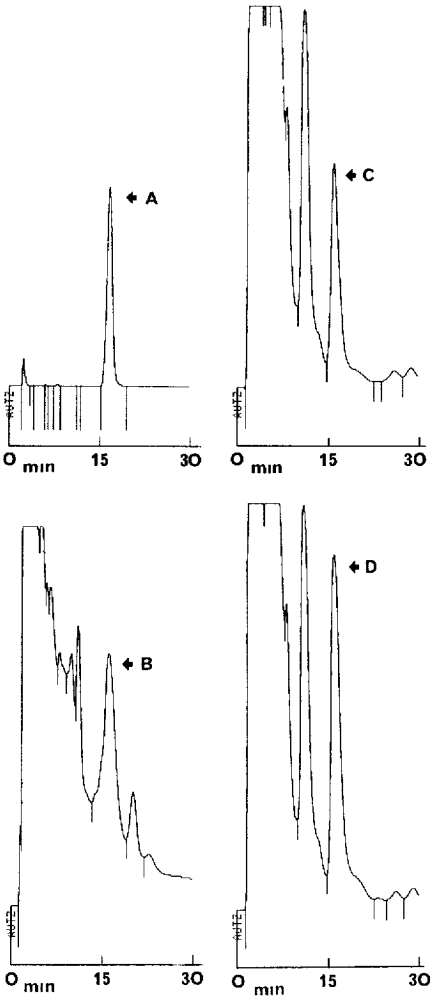

Fig 3 Chromatograms of tyramine in (C) "blank" human urine and (D) urine obtained form a subject after eating cheddar cheese, (B) in cheddar cheese and (A) in a reference solution (100 pmol injected). Same mobile phases as used for peptides, biogenic amines and drugs Isocratic separation (25% A, 75% B)

ride, a subgroup of endogenously depressed patients excreted subnormal amounts of tyramine-O-sulphate in the urine. Hence this tyramine conjugation deficit test has been regarded as a depression marker of these patients [10,11]. We determined tyramine in human urine before and after the subjects had eaten 100 g of cheddar cheese and in the cheese itself (Fig. 3). We found 180 μg of tyramine in 1 g of cheese, 0.45 μg/ml in "blank" urine and almost double that concentration in urine after eating the cheese. We also determined the polyamines spermine and spermidine, whose urinary excretion has been used in the diagnosis of tumours, and the CNS-stimulant amphetamine in human plasma and brain dialysate of the rat (chromatograms not shown).

DISCUSSION

The determination of primary amines is of significance in the areas of food and clinical pharmacology, among many others, such as the determination of dissolved free amino acids in sea water. Further, the assay of drugs with primary amino groups (*e.g.*, amphetamine, baclofen, fluvoxamine and their metabolites) in biological fluids is often important for optimizing clinical treatment regimes and, of course, is necessary to obtain pharmacokinetic parameters. Several peptides, *e g.*, peripheral peptide hormones or peptides with a CNS action, are of interest in clinical and experimental conditions. In some instances, the HPLC assay of physiological concentrations of peptides in biological fluids still suffers from difficulties. Low endogenous levels of peptides are often inaccessible with conventional (non-laser) chromatographic fluorescence detection (except for small peptides fragments), owing to a decrease in the fluorescence efficiency on extending the peptide chain length [7]. Moreover, it is necessary to control pH and the cyanide concentration for the efficient derivatization of peptides with NDA–CN [14].

Numerous fluorogenic reagents for liquid chromatographic analysis and more recently a wide variety of precolumn derivatization methods have been developed for the determination of amino acids, peptides and other compounds with amino groups. The literature clearly indicates that the NDA–CN derivatization reagents give high fluorescence quantum efficiencies of the CBI-fluorophores and yield adducts of superior stability, especially compared with other labels commonly applied for the determination of primary amines [6–8]. Another advantage is that the NDA–CN combination is transparent to the detector in the absence of analytes, so it is not necessary to remove the excess of reagent after derivatization. The versatility of the reagent combination is also reflected by the fluorimetric assay of cyanide based on the same reaction with NDA and the amino acid taurine at basic pH [15].

The precolumn derivatization method described here lends itself to clinical pharmacokinetic studies. Precolumn derivatization is used to change the physical nature of the analytes and to improve the sensitivity or selectivity of analytical

detection. Precolumn HPLC derivatization procedures are, in general, very suitable for biomedical investigation provided that they can be automated Automation of the procedure considerably improves the sample throughput, as large numbers of samples usually have to be analysed [16]. We developed a fully automated precolumn derivatization procedure for compounds containing primary amino groups To our knowledge, this is the first time that the automation of the precolumn derivatization of primary amines with NDA has been adressed. The procedure is simple and requires no or little sample pretreatment.

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