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Chemical derivatization as a strategy to enhance detectability of agents used in cancer management*

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Abstract: The bioanalysis of drugs used in the management of cancer is often complicated by the lack of selectivity and sensitivity. Chemical derivatization of these drugs prior to their chromatographic analysis represents a viable strategy to improve chromatographic resolution and to enhance detectability. This review provides examples of how this approach can meet these objectives. Derivatization of racemic cyclophosphamide with a chiral acylating agent, following hydroxyalkylation to introduce a reactive centre into the molecule, provides the basis for its stereospecific analysis. The analysis of dianhydrogalactitol is described, in which diethyldithiocarbamate is used as a nucleophilic derivatizing agent that improves chromatographic behaviour and analytical sensitivity. The final example that is described is the design and preparation of improved fluorogenic reagents (*o*-phthalaldehyde analogues) for the derivatization of peptides and application of these reagents to the trace analysis of leu-enkephalin in plasma.

Keywords: *Pre-column derivatization; stereoselectivity; HPLC; cyclophosphamide; dianhydrogalactitol;* o*-phthalaldehyde analogues; peptide (leu-enkephalin) analysis.*

Introduction

Drugs used in the management of cancer, unlike members of most other therapeutic classes, are highly toxic and are usually administered at doses approaching those that produce life-threatening toxicities. The incidence and intensity of both chemotherapeutic and toxic effects are usually dose-dependent. Thus, there is particular value in being able to monitor plasma levels of these drugs and their biologically active degradation products.

Monitoring anti-cancer agents and other drugs used in cancer therapy is complicated by their limited stability in biological fluids. In many cases such drugs undergo facile degradation to form both biologically active and inactive products or require metabolic activation to produce the ultimate therapeutic entity. The degradation products are often structurally similar to the parent drug and to components normally present in biological

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matrices. Thus, analytical methods must be capable of providing a high degree of selectivity. The inherent chemical reactivity and chemotherapeutic potency of such agents generally result in low concentrations of drug in plasma, thus creating an increased demand for analytical sensitivity.

Overall, high-performance liquid chromatography (HPLC) has emerged as the technique of choice for the separation of many of these compounds because of its speed, capability of handling thermally unstable and non-volatile materials and compatibility with biological samples. The major disadvantage of HPLC is the lack of sensitive, universal detectors. In many instances, the particular analytical problems associated with anti-cancer agents have necessitated the development and incorporation of special approaches to facilitate their analysis in biological fluids. This review focuses on the use of chemical derivatization as a strategy to enhance analyte detectability and chromatographic resolution [1].

Examples of pre-column derivatization (i.e. chemical modification of the analyte prior to chromatographic separation) will be used to illustrate its value as part of an analytical protocol in: (a) enhancing the stability of the analyte; (b) improving the separation of the analyte from the sample matrix; (c) refining chromatographic separations (by improving band shape and/or increasing resolution of adjacent bands); and (d) enhancing detectability by increasing the response to the detector or by introducing an additional element of specificity into the determination due to the limited reaction possibilities of the reagent.

Cyclophosphamide

Cyclophosphamide (CP),**1**, is an alkylating agent that is widely used clinically in the management of a wide variety of haemolytic and solid neoplasms. CP is a chiral molecule by virtue of an asymmetric phosphorus atom [2]; however, it is the racemate that is used clinically. Therapeutic monitoring of blood and urinary levels of this drug generally has failed to consider differences in disposition of its enantiomers. However, chiral recognition may be important in understanding its chemotherapeutic behaviour, since CP is a pro-drug that is converted to active alkylating species by hepatic cytochrome P-450 dependent enzymes [3], i.e. "chiral catalysts". The activating reaction sequence would thus be expected to be influenced by the chirality of the substrate, CP. Differences have, in fact, been noted in the biological activity of organophosphorus molecules that are chiral at phosphorus [4, 5], including CP [6–8]. The metabolism of a deuterated racemate of CP was studied in mice, rats and rabbits [9, 10]. The amount of CP enantiomers was quantitated mass spectrometrically by stable isotope dilution. Stereoselective metabolism of CP was demonstrated in all three species, although the extent and direction of this stereoselectivity was different in each species. In man, CP recovered from patient urine was laevorotatory [6, 7], further supporting the stereoselectivity of CP activation.

Analytical methods that have been developed to monitor CP blood levels are not stereoselective. Both GLC [11–17] and HPLC [18, 19] methods have been described for monitoring CP in formulations and biological fluids. These methods involve separation of either the underivatized molecule [15–19] or its trifluoroacetylated derivative, **1a** [11–14].

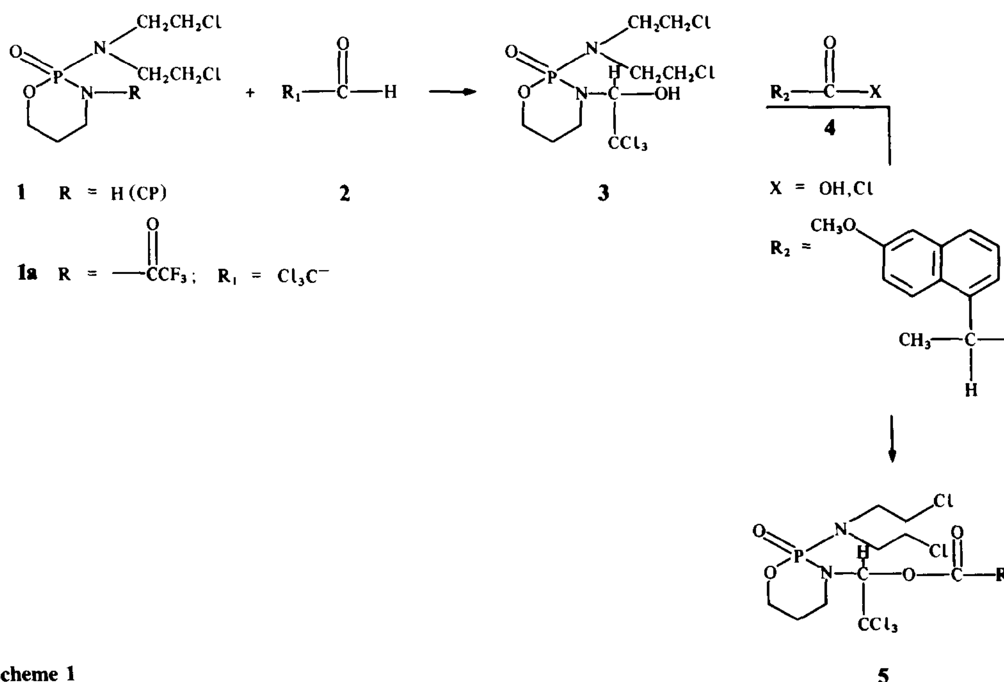
Several approaches were investigated in this laboratory to develop an HPLC assay for CP that was stereoselective. Chiral columns such as the Pirkle covalent phenylglycine

column [20] and the Supercoil (R)-urea column [21] failed to resolve the CP enantiomers. Although the addition of β-cyclodextrin to the mobile phase of a reversed-phase system reduced the retention volume of CP, it failed to resolve the CP enantiomers. Thus, although CP appears to form an inclusion complex with β-cyclodextrin, the differences in the stability of the complex with each enantiomer were insufficient to effect their resolution.

An alternative strategy involved attempting to derivatize CP with a chiral reagent that would convert the enantiomers to diastereomers. The success of this approach required that (a) the reaction could be performed with trace quantities of CP; (b) the integrity of the chiral centres was maintained during the derivatization sequence; (c) the diastereomeric derivatives could be resolved by HPLC; and (d) a "reporter group" could be introduced into the molecule that would allow detection of CP at physiologically relevant levels.

Attempts to acylate CP at the phosphoramide nitrogen directly with chiral carboxylic acids or their acid chlorides were unsuccessful, due to the poor nucleophilicity of the CP amide nitrogen. These disappointing, yet not unexpected, findings let to an investigation of a two-step derivatization sequence involving initial *N*-hydroxyalkylation through a Mannich-type reaction with an aldehyde, **2**, followed by esterification of the resultant carbinolamine, **3**, with a chiral chromophoric acylating agent, **4**, to form an ester, **5** [22]. Selection of the aldehyde reagent was limited to those that are strongly electrophilic, due to the poor reactivity of the phosphoramide nitrogen. Accordingly, chloral was chosen as the derivatizing species.

Oxyalkylation of CP with a chlorinated aldehyde introduces a functional group (HO) of significantly greater reactivity than is present in the parent compound, and provides a carbon spacer that may place the second reactive centre (hydroxyl group) in an



Scheme 1

environment where steric effects of a subsequent derivatization step may be reduced. In this way, selection of the chiral reagent is less constrained by reactivity considerations, so that the reagent and conditions for the second step of the derivatization (**4**→**5**) can be selected to maximize product yield and optimize the chromatographic and detection properties of the product.

The reaction of CP with chloral, **2**, formed an alkyloxy product, **3**, as previously described by Bodor *et al.* [22]. The reaction media required the presence of 1% dimethylformamide to avoid oxidative degradation of the reagent [23] and **3**. Reaction products were extracted into dichloromethane and the extract was subjected to solid phase extraction on a silica gel column. After elution of **3** with dichloromethane: methanol, and evaporation of the eluent, the residue was acylated with the acid chloride

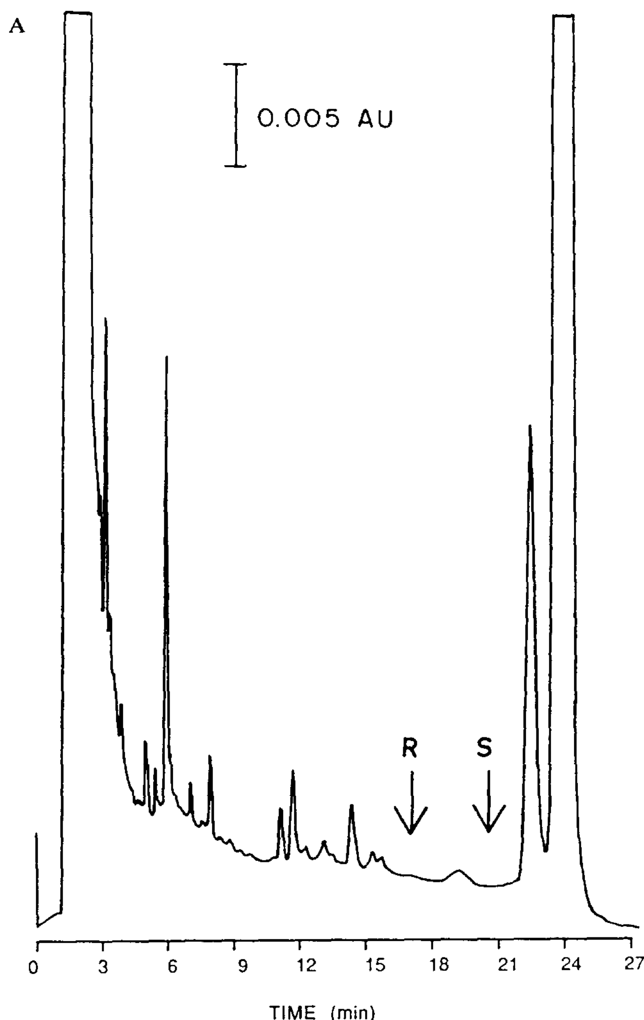


Figure 1
Chromatograms of the diastereomeric products following extraction and derivatization of (A) blank plasma, and (B) plasma containing racemic CP (2.0 μg ml^{-1}). Chromatograms were obtained on an ODS Hypersil column (150 ×= 4.6 mm i.d.; 3 μm particles) eluted with a mobile phase of acetonitrile: potassium phosphate buffer, 0.01 *M*, pH 6.65 (60:40) at a flow rate of 1.0 ml min^{-1} and detected at a wavelength of 232 nm (range = 0.05 a.u.f.s.).

of (+)-6-methoxy-α-methyl-1-naphthaleneacetic acid [24], Naproxen, **4**. Naproxen has the advantages over other chiral acylating agents of being a stable solid at room temperature and containing a strong chromophore. The diastereomers (**5**) formed in this derivatization sequence were successfully resolved on a reversed-phase HPLC column (Fig. 1), providing a convenient and sensitive (detection limit ca. 1 μg ml^{-1} of CP in plasma) means of monitoring CP levels in plasma with quantitative assessment of stereochemical disposition of the drug.

Dianhydrogalactitol

The HPLC analysis of the anti-neoplastic agent dianhydrogalactitol (DAG,**6**) includes a pre-separation derivatization step that incorporates many of the features described above. The hydrophilicity of the drug prevents its efficient extraction into water-

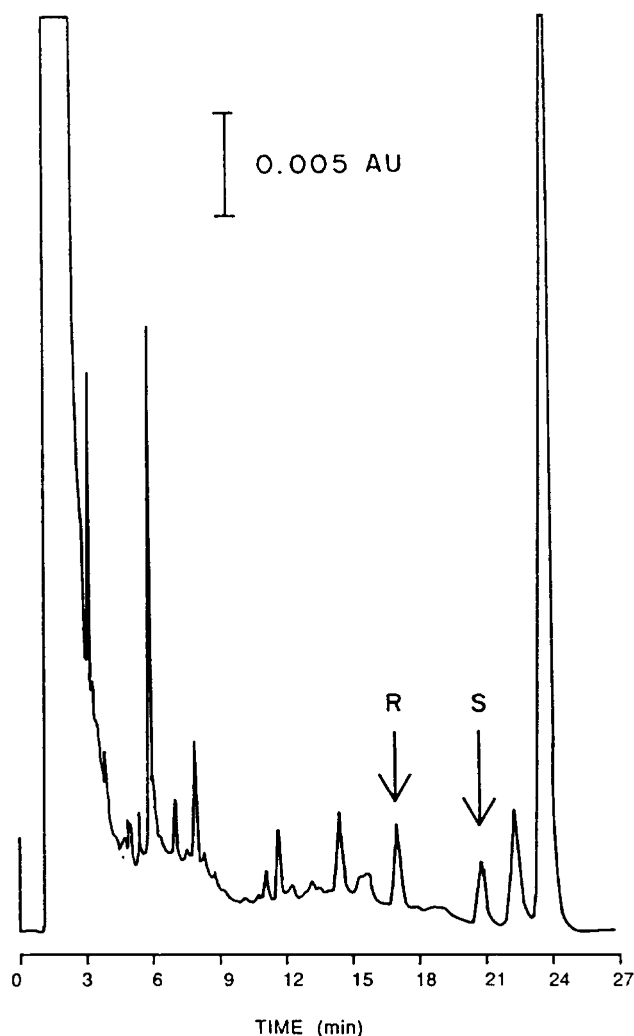
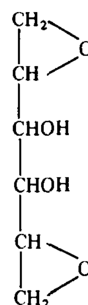


Figure 1 (continued)



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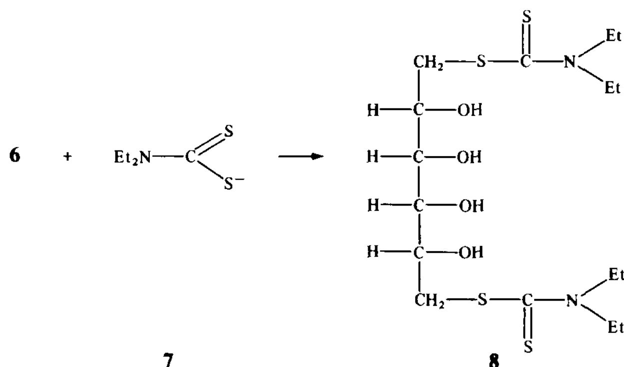
immiscible solvents, even from salt-saturated solutions. Furthermore, DAG is unstable, binding irreversibly to red blood cells (in sample containers) through attack by endogenous nucleophiles at the epoxide, and also undergoing intramolecular rearrangement to the thermodynamically more stable 2,3-epoxy isomer. The lack of chromophoric groups in **6** results in detection limits (≥ 10 μg ml^{-1} of plasma) that are above that necessary for clinical monitoring of the drug. These difficulties were overcome by derivatizing DAG with diethyldithiocarbamate, **7** [25]. Reaction was carried out directly in the blood sample (at room temperature) and conversion to the *bis*-dithiocarbamate, **8**, was quantitative and complete in <5 min. Elimination of the epoxides by conversion of **6** to **8** stabilized the analyte from subsequent nucleophilic attack. The derivative was more hydrophobic than the parent drug and could be extracted quantitatively into chloroform. The derivative separates efficiently on a normal-phase (CN) column and strongly absorbs UV light (absorptivity at 254 nm = 2.8×10^4 log cm^{-1}), thus providing a route to its clinical monitoring in blood.

Although this example illustrates the usefulness of pre-column derivatization, the approach has been primarily used to extend the utility of sensitive HPLC detectors to compounds that are relatively insensitive to them, by chemically transforming the molecule of interest to a more readily detectable species. Unlike the situation encountered in GLC, derivatization is rarely employed in HPLC to improve chromatographic separation, because of the flexibility in retention characteristics available by mobile phase manipulation and stationary phase selection.



Pre-separation derivatization can involve oxidation, reduction, displacement or addition reactions. The last two categories include attack by either nucleophilic or electrophilic species, depending on the reactivity of the substrate. Most of the reactions described to date employ electrophilic reagents that interact with nucleophilic sites on target molecules. In clinical analysis this approach has the distinct disadvantage that extensive clean-up is often necessary prior to the derivatization step because of the wide variety of endogenous nucleophiles present in biological fluids (at high concentrations relative to the compound of interest) which also react with the derivatizing agent reducing the specificity of the method, potentially causing consumption of large amounts of reagent, and making separations more difficult. Also, such reagents (e.g. acylating agents) are often susceptible to degradation by water (the most abundant nucleophile in biological fluid). Nucelophilic reagents such as diethyldithiocarbamate are much more specific because of the absence of electrophilic functionalities in endogenous compounds and are often stable in water. It is these properties that allow the derivatization of DAG with diethyldithiocarbamate to be carried out directly in blood. Such derivatization could not be accomplished as readily with electrophilic agents. Unfortunately, a relatively small percentage of pharmaceuticals is amenable to derivatization with nucleophilic reagents.

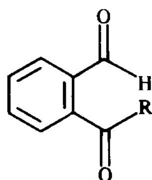
Peptide (leu-enkephalin)

Peptides are an emerging chemical class of drugs. Their application in the management of cancer patients is only beginning to be considered, as chemotherapeutic agents, as hormonal supplements and immunomodulators, and as potential analgesics, e.g. endorphins and enkephalins. Of course, this chemical class also has broad potential value both as diagnostic probes and as therapeutic agents in a variety of other disease states. The analysis of peptides from biological matrices is complicated by their chemical reactivity, i.e. propensity for undergoing enzyme-mediated and spontaneous hydrolysis. Furthermore, the detection limits that can be achieved in these analyses are limited by the physical properties of the amino acid monomers from which the peptides are derived.

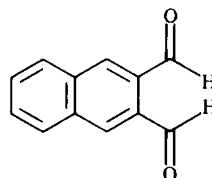
Chemical derivatization of peptides is an obvious strategy that could be employed to enhance their detectability. Unfortunately the success in applying this approach to bioanalysis of peptides has been limited. Because of the inherent sensitivity potential of fluorimetry, conversion of peptides to fluorescent derivatives has been a commonly considered strategy. Of fluorogenic reagents capable of reacting with primary amines, *o*-phthalaldehyde (OPA),**9**, has been favoured due to its ease of handling, low cost, spectral emission properties and high reactivity [26]. Major disadvantages encountered with OPA include the chemical lability (toward auto-oxidation) [27, 28], poor quantum yield of fluorescence (Φ) [29] ($\sim$0.1) of the isoindole derivative and the restricted reactivity of OPA [29, 30], i.e. inability to form fluorescent products with peptides, except those containing a lysine residue (application has otherwise been restricted to amino acids).

Recently, two analogues [29, 31] of OPA were designed and synthesized, **10** and **11**, that form fluorescent isoindoles upon reaction with the primary amine portion of amino acids. The resulting isoindoles are resistant to chemical degradation.

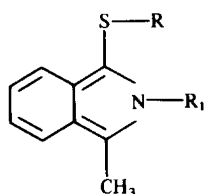
The methylated analogue of OPA, **10** (OAB) was designed to block C-1 of the isoindole (formed on reaction with amine), which is the initiation site for auto-oxidation [27, 28]. OAB reacts [31] with amino acids in the presence of thiol in alkaline media, at a



R = H **9**, OPA
R = CH_3 **10**, OAB



11, NDA



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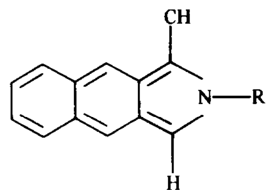

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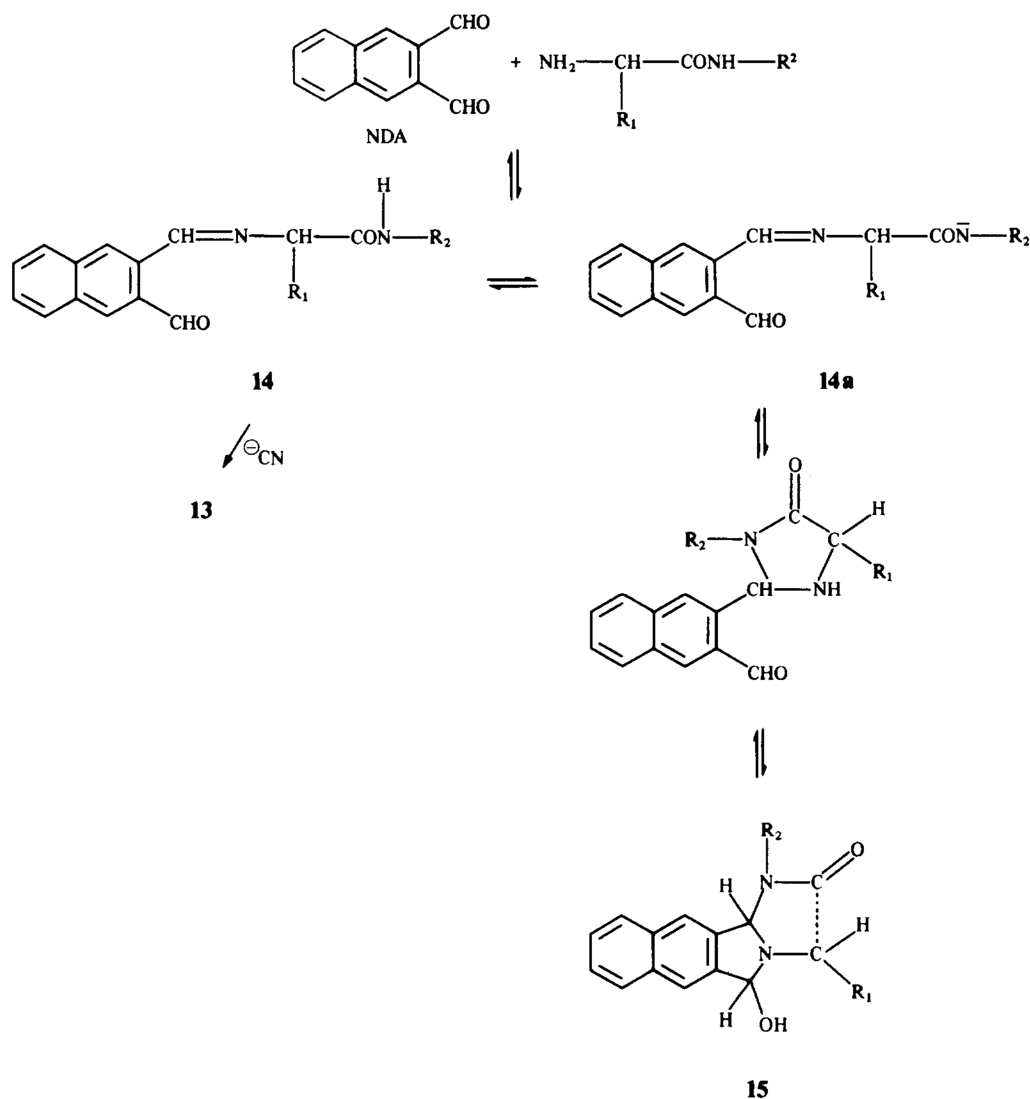
rate similar to that observed with OPA, but the corresponding isoindole derivative, **12**, is resistant to auto-oxidation ($t_{1/2}$ 1 h; a 125% improvement in stability of that observed with the corresponding OPA derivatives). OAB fails, however, to form fluorescent products with peptides. The sensitivity achievable for amino acid analysis using OAB is similar to that achievable with the parent, OPA. To improve sensitivity, the naphthalene analogue of OPA, **11** (NDA), was prepared [29]. Although NDA was poorly reactive toward amino acids when thiols were used as co-reactant, cyanide proved to be a more acceptable nucleophile. In the presence of cyanide, NDA reacts with amino acids at a rate comparable to that observed with OPA and OAB (under similar reaction conditions) to form the corresponding benz-isoindole, **13**. The reaction product was even more resistant to degradation than that formed with OAB ($t_{1/2} > 24$ h) and the quantum yield was considerably improved over that observed with **12** ($\Phi = 0.71$). Although this reagent provides a considerable improvement in sensitivity over OPA and OAB for amino acid analysis, it failed to form fluorescent products with peptides under the conditions used for amino acid derivatization [29, 30]. However, it was noted that NDA was consumed on exposure to peptides in the presence of cyanide under the conditions used for amino acid derivatization [32]. A major reaction product was isolated following exposure of NDA to tri-alanine (employed as a model substrate) under these alkaline conditions, and identified as the imidazol-3-one, **15**. This product probably forms according to Scheme 1, through a Schiff's base intermediate **14**. At alkaline pH, the amide nitrogen is ionized, forming a nucleophilic centre, **14a** which can participate in an intramolecular electrophilic attack at the Schiff's base carbon to form **15**. If this mechanism is correct, then the suppression of ionization of the amide by lowering the pH should minimize the contribution from this pathway and increase the likelihood for reaction of the Schiff's base intermediate, **14**, with cyanide to form the desired fluorescent isoindole analogue, **13**.

Although no reaction was detected at pH 8–9 when the reactivity of NDA toward alanine oligomers in the presence of cyanide was monitored fluorimetrically as a function of pH [32], acidification to pH 7 resulted in fluorescence (Figs 2 and 3). The fluorescent



product was isolated and shown to be the corresponding benz-isoindole, **13**. The corresponding amino acid monomer failed to react at pH 7, but showed the expected reaction at pH $\geqslant 9$. Thus, NDA is capable of forming fluorescent derivatives of peptides, but reaction conditions have to be modified to avoid predominance of the side reaction (which is not capable of taking place when amino acids are derivatized) in which the key Shiff's base intermediate, **14**, is consumed.

The reaction was applied to the analysis of leu-enkephalin from plasma [32]. After liquid–solid extraction of deproteinized plasma enriched with leu-enkephalin (with leu-enkephalinamide added as internal standard) on octasilyl silica columns, the eluent was allowed to react with NDA/CN. As shown in Fig. 4, maximum fluorescence was obtained, as predicted, when reaction was carried out at pH 7; under these conditions maximum yield was obtained in 3 min. The reaction mixture was applied to an octyldecyl

Figure 2
pH-Rate formation profiles of the cyanobenzisoindole derivatives of trialanine (A), dialanine (B) and alanine (C). For each rate profile derivatization was carried with NDA (2×10^{-6}M), sodium cyanide (1×10^{-3}M) and the amine (1×10^{-3}M). Product formation was monitored spectrofluorometrically (λ_{ex} = 420 nm; λ_{em} = 470 nm), following HPLC separation on an Hypersil ODS 5 μm (250 × 4.6 mm i.d.) column eluted with 35% acetonitrile in potassium phosphate buffer (0.05 M, pH 6.8).

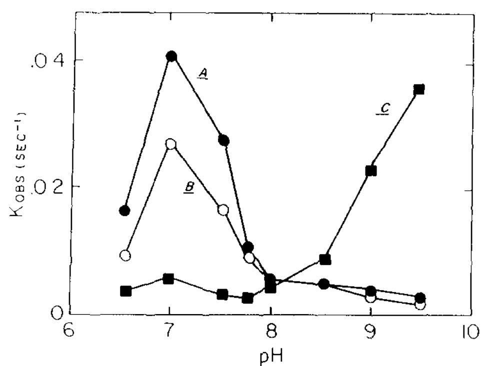
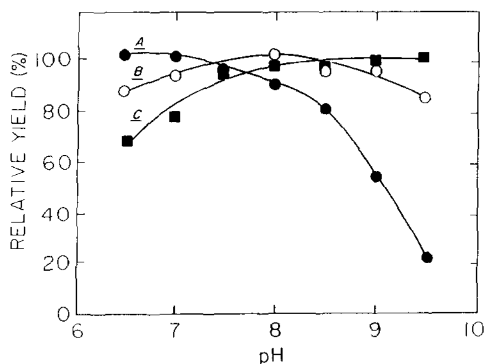


Figure 3
pH-Chemical yield plots of the CBI-derivatives of trialanine (A), dialanine (B) and alanine (C). Studies were carried out using the conditions described in Fig. 2.



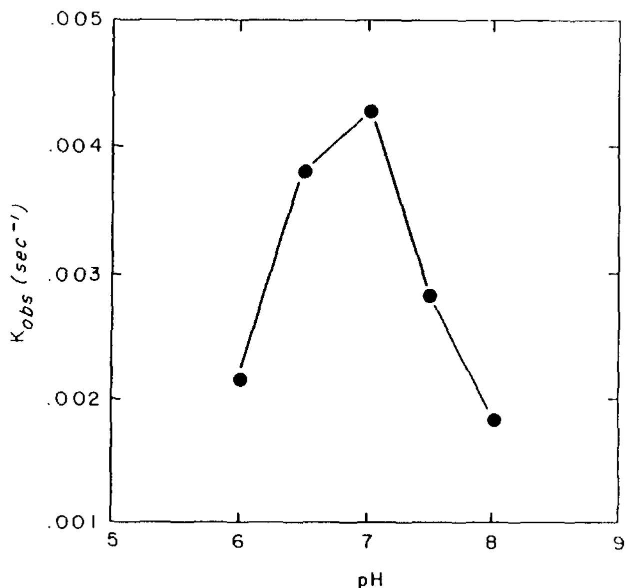


Figure 4
pH-Rate profile for CBI-derivative formation arising from the reaction of leu-enkephalin (5×10^{-6}M) with NDA (1×10^{-4}M) and sodium cyanide (2×10^{-4}M) in phosphate buffer (0.25 M) at 25.0 ± 0.1°C determined by monitoring reaction mixtures spectrofluorometrically. (λ_{ex} = 420 nm; λ_{em} = 470 nm).

silica column which was eluted with acetonitrile:THF:phosphate buffer (pH 3.0) (45:4:51). A sample chromatogram of a processed plasma sample containing 320 pmol ml^{-1} of leu-enkephalin in plasma is shown in Fig. 5 and demonstrates the application of this derivatization sequence as an approach for the trace determination of peptides from biological matrices.

Conclusion

These examples illustrate some of the advantages to be gained by introducing a chemical derivatization step into an analytical sequence. Substantial improvements in detectability can be achieved by selection of appropriate reagents that can "selectively" introduce reporter groups that permit detection at levels considerably lower than the detectability of the underivatized form (e.g. use of OPA analogues to derivatize peptides). Alternatively, derivatization can be used to alter the physical/chemical properties of an analyte, thereby offering improved chromatographic selectivity. In this

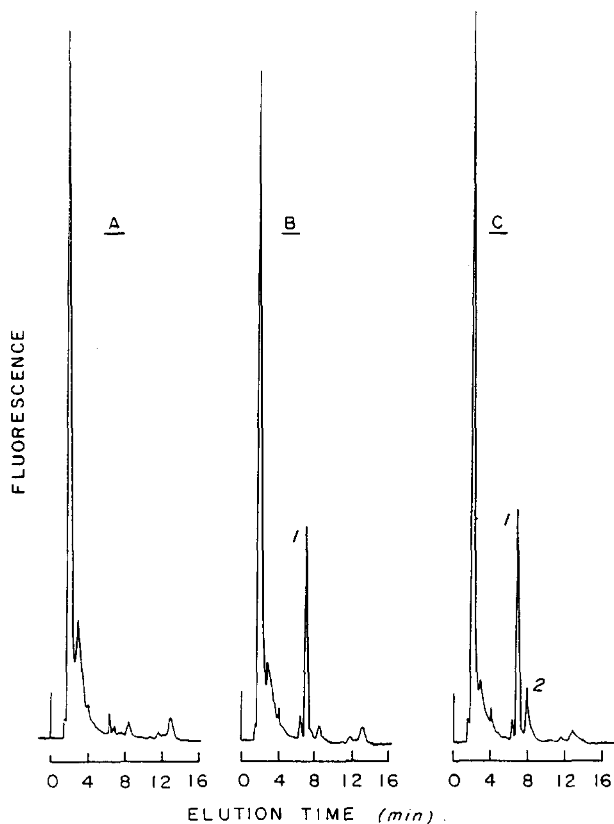


Figure 5
Chromatograms of blank (A) and plasma (B, C) samples spiked with leu-enkephalinamide (peak 1, 1.25 nmol ml^{-1}) and leu-enkephalin (peak 2, 0.32 nmol ml^{-1}); processing includes derivatization with NDA/cyanide. Chromatograms were obtained on a Shandon Hypersil ODS 5 μm guard column (50 × 4.6 mm i.d.) directly attached to a Shandon Hypersil ODS 3 μm analytical column (150 × 4.6 mm i.d.). Columns were eluted using a mobile phase of 45% acetonitrile/4% tetrahydrofuran/51% phosphate buffer (0.05 M, pH 3.0). Flow rate 1 ml min^{-1}. Column effluent was monitored spectrofluorimetrically; λ_{ex} = 420 nm; λ_{em} = 470 nm.

review, derivatization provided increased hydrophobicity of dianhydrogalactitol permitting its extraction from plasma and subsequent separation by conventional HPLC techniques. Derivatization with a chiral reagent offered a means for resolving enantiomeric pairs on an achiral column by conversion of the racemate to a diasteromeric mixture. In considering the use of pre-chromatographic derivatization, however, caution must be exercised to avoid losses that can compromise the precision and accuracy of the analysis.

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