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PRE-COLUMN DERIVATISATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

In the past few years the use of derivatisation procedures in high-performance liquid chromatography (HPLC) has expanded rapidly. Frei and Santi[1] have defined two sorts of derivatisation HPLC, namely pre- and post-column. Post-column derivatisation, which consists of mixing the column effluent with a suitable chromogenic agent before detection, is a logical development from amino acid analysis techniques relying on the use of ninhydrin as a chromogenic agent.

Frei and Santi[1] include ion-pair separations as examples of pre-column derivatisation. We intend to consider pre-column derivatisation only in the more commonly accepted sense of the actual conversion of one chemical entity to another for the purpose of HPLC separation and detection. It is felt that this is more in line with the current usage of the phrase and is already well established in derivatisation for gas–liquid chromatography.

The major purpose of derivatisation in HPLC is to overcome the lack of universally sensitive detectors. Refractive index (RI) detectors are renowned for their insensitivity and there are no reports of the use of derivatives designed to increase the response of such detectors. Ultraviolet (UV) spectrophotometers are the most common detectors coupled to HPLC systems and here the main function of derivatisation is to increase the UV adsorbing properties of the compounds under investigation. It is also convenient that so doing normally results in UV maxima above 254 nm, thus increasing the choice of suitable eluents.

The use of fixed-wavelength detectors operating at 254 nm is a disadvantage in this work as most derivatives have UV maxima above this wavelength and, although monitoring at 254 nm may be adequate, some degree of sensitivity must be sacrificed. The greater availability of commercial chromatographs equipped with variable-wavelength detectors may well stimulate more interest in the use of derivatising agents.

The use of spectrofluorimetric detectors in conjunction with HPLC is increasing, and fluorescent derivatives may give an appreciable improvement in sensitivity over UV detection although there is some indication that correct selection of the UV monitoring wavelength rather than the arbitrary selection of 254 nm may rival spectrofluorimetric detection in some cases.

Specificity may be an advantage where a complex mixture is to be analysed, only some of the components of which react with a specific derivatisation agent. It may be even possible to adopt a functional group analysis approach with a suitable range of reagents.

In addition to their function in increasing the sensitivity of detection, derivatising agents may be used to alter the chromatographic character of compounds. This may be a gross effect for example to allow separation on underivatised silica instead of ion-exchange columns, or, in a minor way, to increase the separation of previously unresolved compounds.

Chemically the ideal derivatisation agent should be rapid and quantitative in reaction, there should be no side products and excess derivatising agent should be easily removed. Although the ideal is rarely met, suitable procedures may be adopted including the use of internal standards, sealed reaction vials, appropriate HPLC conditions which separate product from reactant, etc.

The derivatisation procedures are discussed on the basis of the functional group of the parent compound which is involved in the derivatisation reaction.

2. HYDROXYL DERIVATISATION

Although most reports deal with derivatives designed to increase the levels of detection of hydroxylated compounds there are a number of accounts of derivatisation for the prime purpose of improving the chromatographic characteristics of compounds. The methylation of hydroxyanthrene-9-ones does not enhance their detection at 254 nm but does allow their efficient separation on Micropak-CN or -NH_2 columns[2]. The free hydroxyanthrene-9-ones produce badly tailing peaks under similar operating conditions. In a similar manner, the separation of the triacetyl derivatives of adrenaline and noradrenaline can be seen as an attempt to improve their chromatographic properties (and presumably stability) rather than detectability[3].

Rees *et al.*[4] compared the HPLC characteristics of sterols and their acetates and, in an attempt to separate cholesterol, sitosterol and campesterol, found that separation of the acetates on reverse-phase columns was better than separation of the free sterols. They also reported that reverse-phase separation was more promising than the use of argentised columns. Detection was by differential refractometry. The use of steryl benzoates allowed more sensitive monitoring by UV at 254 nm, but campesterol and stigmasterol benzoates could not be resolved. Ikekawa and Koizumi[5] reported the separation of a number of vitamin D_3 metabolites, most of which were resolvable under ordinary conditions. However the 24-R and 24-S epimers of both

24-hydroxy and 24,25-dihydroxy vitamin D_3 had identical retention times. Conversion to their trimethylsilyl derivatives allowed separation of the epimers on Zorbax-Sil columns. Ikekawa and Koizumi also report the separation of the C-24 epimers of a number of hydroxylated cholesterol benzoates and related compounds.

Fitzpatric and Siggia[6] described the separation, on Corasil C_{18} and Permaphase ODS, of seven steroids as their benzoyl esters. They also describe the use of *p*-nitrobenzoates as derivatives giving detection levels of 1 ng. Attempts to produce steryl 3,5-dinitrobenzoates were unsuccessful although Carey and Persinger[7] used 3,5-dinitrobenzoate derivatives for the determination of diethylene glycols in various polyethylene glycols. Higgins[7a] reports the separation of the benzoate esters of sapogenins isolated from *Agave* species.

Benzoylation was also used by Lehrfeld[8] to produce perbenzoxylated carbohydrates. Gradient elution with ether in hexane on Corasil II allowed the separation of glucose, rhamnose, mannose, maltose and galactose. It was important, with these compounds, to control the reaction carefully as isomerisation could occur if the sample was allowed to stand in pyridine before the derivatising agent, benzoyl chloride, was added. Thus α-D-glycopyranose could produce a mixture of α- and β-D-glucopyranose pentabenzoates.

Anisoyl chloride has been used as a derivatisation agent for two pharmaceutical products. Hexachlorophene forms a di-*p*-methoxybenzoate derivative detectable at less than 40-ng levels[9] (Fig. 1) and pentaerythritol forms a tetra-*p*-methoxybenzoate derivative capable of detection at 12 ng[10]. Both derivatisation products were separated on silica and detected at 254 nm.

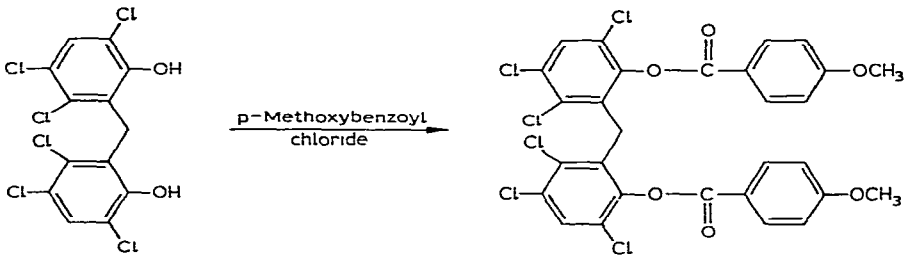


Fig. 1. Hexachlorophene derivatisation.

The fact that polyhydroxy compounds will form polysubstituted derivatives was found particularly useful by Nachtmann *et al.*[11] in their examination of cardiac glycosides by HPLC. The cardiac glycosides from *Digitalis* species all possess an unsaturated lactone moiety which gives an adsorption maximum around 230 nm. Reaction with *p*-nitrobenzyl chloride allows the formation of polynitrobenzoates of the sugars attached to the 3-position of the aglycone. As there are 1–4 sugar units so attached the analysis is obviously complicated but it is greatly helped by the fact that in the reported separation the mono-sugar derivatives elute first progressing in sequence to the tetrasaccharide derivatives. Conventionally the later peaks eluted from a column are broader and less easy to determine but in this case these peaks are precisely the ones due to the compounds with the greatest number of chromophoric groups and hence the greatest detector response factor (Fig. 2).

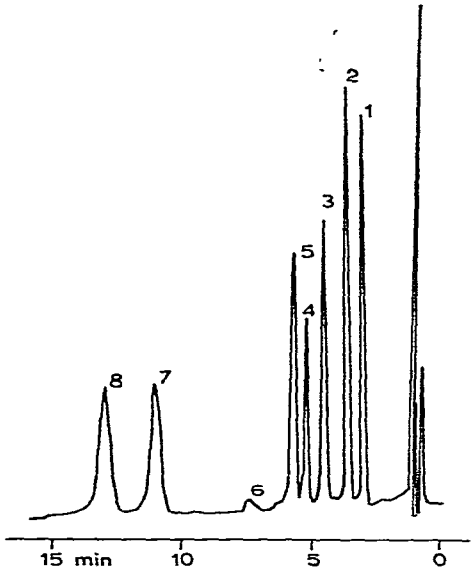


Fig. 2. HPLC of some *p*-nitrobenzoate derivatives of cardiac glycosides[11]. 1 = Digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = acetyldigoxin; 5 = digoxin; 6 = unknown; 7 = lanatoside C; 8 = desacetyl lanatoside C. Solvent system: *n*-hexane–methylene chloride–acetonitrile (10:3:3). Column: SI 60, 5 μm. UV detection at 254 nm.

A number of fluorescent derivatives have also been investigated, in all cases in an attempt to further increase limits of detection in biological situations. For example the HPLC of catecholamines as their acetates has already been described[3] but much greater sensitivity can be obtained by forming the dansyl (1-dimethylamino-naphthalene-5-sulphonyl) derivatives of adrenaline, noradrenaline and dopamine which can be detected at levels below 50 pg after separation on Lichrosorb SI[12].

Cassidy *et al.*[13] prepared the dansyl derivatives of a number of hydroxy-biphenyls (of importance as fungicides used in the preservation of citrus fruits during storage). Separation on silica was good and sensitive detection was possible with UV monitoring at 254 nm. They reported that the use of a fluorescence detector increased sensitivity 8-fold over UV detection at 254 nm, but were hampered by design problems in the spectrofluorimeter flow cell. The method was also used for the detection of phenolic compounds in urine.

Frei *et al.*[14] report the use of dansyl derivatives of alkaloids including morphine and ephedrine and apply this derivatisation procedure to the analysis of a cough syrup containing ephedrine, cephaeline and emetine. The method is only applicable to those alkaloids containing free hydroxyl groups. Alkaloids such as narcotine and codeine did not form dansyl derivatives but were still easily detectable by UV monitoring as opposed to spectrofluorimetry. Similarly fluorogenic labelling of organophosphorus pesticides is reported, the pesticide being hydrolysed to the free phenol before derivatisation with dansyl chloride[14a].

More complex molecules have also been subjected to derivatisation HPLC. McCluer and Evans[15] describe the use of benzyl chloride to derivatise cerebrosides which were analysed on Zipax columns with UV monitoring at 254 nm. This paper

indicates that earlier work[16] which assumed only O-acylation was incorrect in that cerebrosides containing non-hydroxy fatty acids undergo amide acylation in addition to O-acylation. This N-acylation is avoidable by using benzoic anhydride as the derivatising agent[15].

3. CARBONYL DERIVATISATION

Carey and Persinger[7] report the analysis of a number of simple aldehydes and ketones as their 2,4-dinitrophenylhydrazine (2,4-DNP) derivatives using Corasil II and UV monitoring at 254 nm. Papa and Turner[17] use Permaphase ETH or 1% tris(2-cyanoethoxy)propane stationary phase on Zipax for the separation of the 2,4-DNP derivatives of thirteen carbonyl compounds including pyruvic acid, pyruvaldehyde and salicaldehyde. They report detectable limits of 5 ng and apply the method to the analysis of carbonyl constituents of car exhaust gases. Steroidal ketones have been studied as their 2,4-DNP derivatives by two groups of workers. Henry *et al.*[18] showed that a great increase in detection limits could be obtained by converting steroidal ketones to their 2,4-DNP derivatives although those steroidal ketones possessing an α,β-unsaturated carbonyl moiety could be detected at 254 nm. They quote 1 μg detection for androsterone and 1 ng for its 2,4-DNP derivative. Fitzpatrick *et al.*[19] separated the 2,4-DNP derivatives of four epimeric forms of androsterone and dehydroepiandrosterone by reverse-phase HPLC on Corasil C_{18} or using 1.5% oxydipropionitrile (ODPN) on Zipax. This procedure was successfully used for the analysis of steroids in urine.

A preliminary report by Frei and Lawrence[20] indicates the possibility of fluorogenic labelling for ketones and aldehydes using dansyl hydrazine.

4. CARBOXYLIC ACID DERIVATISATION

Commonly, carboxylic acids are converted to their methyl esters before analysis by GLC. A similar derivatisation has been used for HPLC by both Pei *et al.*[21] and Scholfield[22]. In both cases separation was by reverse-phase HPLC using an RI detector. Fan *et al.*[23] analysed 11- and 12-hydroxylauric acid, produced by microsomal metabolism of sodium laurate, by reverse-phase HPLC of their methyl esters. Refractometry was again used as the detection method. Mikes *et al.*[24] also investigated the separation of fatty acid methyl esters using Corasil II with a stationary phase of silver nitrate–ethylene glycol. In all these cases detection levels are poor although such derivatives are certainly useful for preparative work[25].

Where improved detectability is required, esterification with aromatic derivatisation agents is an obvious approach and numerous methods have been designed for producing suitable derivatives for HPLC analysis. Phenacyl esters, prepared by reaction with 2-bromoacetophenone and triethylamine as base, have been investigated by Borch[26] using μBondapak C_{18} with UV detection at 254 nm. Cooper and Anders[27] chose 2-naphthacyl bromide to produce the naphthacyl esters of fatty acids which were also separated on reverse-phase material (Corasil C_{18}) with UV monitoring. At 254 nm they report the detection of 4–90 ng depending on the chain length of the fatty acid. Cooper and Anders[28] have also reviewed general aspects of the HPLC of fatty acids and other lipids.

Benzyl esters, prepared using 1-benzyl-3-*p*-tolyltriazine as derivatising agent, have been separated on Corasil II with detection at 254 nm by Politzer *et al.*[29]. They report the detection of 1–2 μg benzyl stearate and suggest that nitrobenzoates would give enhanced detection but would result in less efficient separation. This parallels our findings on benzoate and *p*-nitrobenzoate derivatives of phytosterols[30]. Politzer *et al.* also point out that as the UV characteristics of the derivatives are entirely due to the derivatising agent there is no need to determine individual response factors for each acid. This will, of course, hold true for any simple fatty acid derivative detected in the UV mode.

Nitrobenzoates have been reported in an application note where fatty acids were derivatised using 1-*p*-nitrobenzyl-3-tolytriazine giving detectability of 1–10 ng depending on molecular weight[31]. Knapp and Krueger[32] prefer the use of O-*p*-nitrobenzyl-N,N′-diisopropylurea to produce the same *p*-nitrobenzyl esters on the basis of convenience (the triazine reaction liberates nitrogen, thus precluding the use of sealed reaction vials) and safety (a number of triazines have been reported as carcinogenic). Separation of their fatty acid derivatives was carried out on Micropak 5 μm silica with UV detection at 254 nm allowing determination in the picomole range for stearic acid.

p-Nitrophenacyl esters of ten series F prostaglandins, eight series E prostaglandins and a number of related compounds were produced by reaction with *p*-nitrophenacyl bromide in the presence of N,N-diisopropylethylamine as base[33]. The reaction occurred quantitatively in under 15 min. Compounds were separated on Zorbax-Sil and, with UV detection at 254 nm, it was possible to detect certain of these derivatives at the 1-ng level. The same procedure has recently been reported for the quality control of the 15- epimer of prostaglandin $F_{2\alpha}$ in pure $F_{2\alpha}$[34]. Dunham and Anders[35] determined prostaglandin B_2 directly by UV detection at 280 nm and prostaglandins E and A, after conversion by treatment with base, to B_2.

Durst *et al.*[36] and Grushka *et al.*[37] report the use of *crown* ethers as catalysts for producing phenacyl derivatives to enhance the UV detection of fatty acids. Both reports use a Corasil II column with a C9 chemically bonded phase and UV detection at 254 nm. Durst *et al.*[36] report the preparation and detection of the *p*-bromophenacyl derivatives of simple fatty acids, and Grushka *et al.*[37] discuss the use of α-bromoacetophenone, α,*p*-dibromoacetophenone and *p*-nitrobenzyl chloride with *crown* catalysts to produce derivatives of some important dicarboxylic acids and report their detection at nanogram levels and below. Fatty acid derivatisation agents are summarised in Table I.

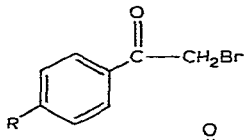
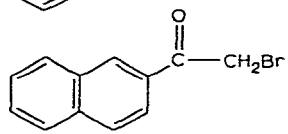
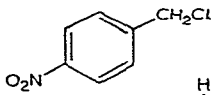
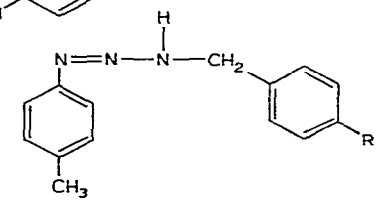
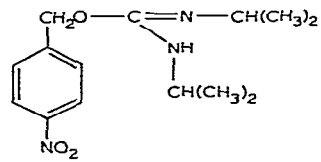
Scott *et al.*[38] resolve some optically active isoprenoidal acids by the production of diastereoisomeric derivatives of isoprenoidal acid enantiomers by reaction with R- or S-α-methyl-*p*-nitrobenzylamine. Separation was on 10-μm Partisil with UV detection.

5. AMINE DERIVATISATION

Most derivatisation HPLC studies of simple amines have been concerned with aliphatic amines and the production of derivatives that are easily detected by UV or spectrophotometric detectors. Aromatic amines are normally adequately examined without derivatisation. Röder and Merzhäuser[39] do report the use of acetate deriva-

TABLE I

CARBOXYLIC ACID DERIVATISATION AGENTS

Reagent	*R*	*Reference*
	H	26, 36, 37
	Br	37
	NO_2	33, 34
		27
		37
	H	29
	NO_2	31
		32

tives of some biogenically important amines to give better chromatographic properties but they required a wavelength of 225 nm for detection. *p*-Nitrobenzoyl chloride is used as a derivatising agent for ketamine (a parenteral anaesthetic) (Fig. 3) and two of its derivatives[40]. These compounds are poor chromophores. The formation of the *p*-nitrobenzamide derivatives greatly enhances detectability at 254 nm after separation on μBondapak C_{18}.

Dopamine, noradrenaline and related compounds have been converted to fluorescent derivatives both by the reaction with dansyl chloride[12] and by the use of

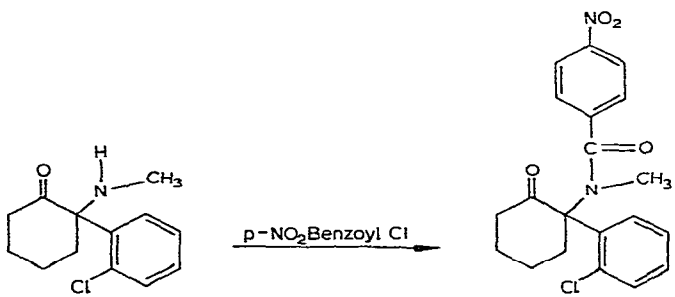


Fig. 3. Ketamine derivatisation.

fluorescamine. Fluorescamine has been used for some time as a post-derivatisation agent as a substitute for ninhydrin and has attracted considerable attention as a pre-column derivatising agent. Imai[41] reports the detection of 10-nmoles/ml catecholamines by fluorescence detection of the fluorescamine derivatives. Schwedt[42] reports lower detectable limits for dopamine and noradrenaline but demonstrates that noradrenaline derivatisation produces a mixture of two compounds.

Yoshioku and Tamura[43] report the use of chloroacetaldehyde as a means of producing fluorescent derivatives of adenine, adenosine and some nucleotides (Fig. 4). Separation on Hitachi gel No. 3010 with $\lambda_{ex} = 233.7$ and $\lambda_{em} > 410$ nm allowed the detection of 1 pmole of derivative.

Fig. 4. Adenine derivatisation with chloroacetaldehyde.

The optical purity of amines has been investigated using HPLC for the separation of diastereoisomeric derivatives produced by reaction with (S)-O-methylmandeloyl chloride[44]. The derivatives of R- and S-α-methylbenzylamine were adequately resolved on Merkosorb Si60.

Diamines and polyamines have received special attention in that they are biochemically important and difficult to analyse by the majority of chromatographic procedures. Satisfactory analyses have been reported of compounds such as spermidine, spermine and putrescine as their *p*-toluenesulphonyl derivatives with UV monitoring[45], as their dansyl derivatives with both UV monitoring[46] and spectrofluorimetry[47], and as their fluorescamine derivatives by spectrofluorimetry[48].

Nitrogenous compounds related to amines have also been subjected to derivatisation HPLC by reaction involving the nitrogen atom. Frei and Lawrence mention the possibility of producing the dansyl derivatives of N-methyl carbamates[20] and Frei *et al.*[49] use this technique for the examination of carbamate insecticides, detecting between 2 and 10 ng of most of the insecticides studied. Dansyl chloride is also used as a derivatising agent for barbiturates allowing, after separation on 30-μm pellicular silica C_{18}, the detection of 2-ng quantities by fluorimetry with $\lambda_{ex} = 360$ and $\lambda_{em} = 520$ nm (ref. 50).

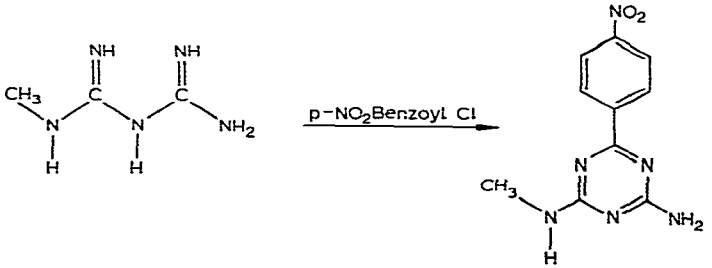


Fig. 5. Triazine formation from metformin.

Ross[51] reports the use of *p*-nitrobenzoyl chloride for the derivatisation of metforim (a hypoglycaemic biguanide). In this case the derivative is formed by acylation followed by cyclisation to yield a substituted triazine (Fig. 5) which is detected in urine by UV monitoring at 280 nm after separation on Corasil C phenyl. Evans *et al.*[52] report a method for estimating the amount of ethylenimine in polyethylenimine by the use of Folin's reagent (1,2-naphthaquinone-4-sulponate) which reacts to produce 4-(1-aziridinyl)-1,2-naphthaquinone which is separated on 10-μm Micropak with detection at 254 nm.

6. AMINO ACID AND PEPTIDE DERIVATISATION

Amino acid autoanalysers using post-column derivatisation have been in use for a number of years and transfer from low-pressure LC to HPLC using ion-exchange columns has resulted in a decrease of overall analysis times. Post-column derivatisation is still applicable to HPLC analysis but a number of reports have appeared concerning pre-column derivatisation.

There are several reports on the separation of amino acids as their phenylthiohydantoin derivatives. The popularity of this derivative is due to its production during the Edman degradative method for sequential analysis of peptides and proteins. The Edman method cleaves the N-terminal residue as its anilinothiazolinone derivative which forms the phenylthiohydantoin derivative on treatment with aqueous acid. Separation conditions vary to include silica[53–57], chemically bonded reversed-phase silica[58,59], Micropak CN[59a] and polystyrene gel[60] as column packings. All reports describe the use of UV for monitoring the eluent. Muramoto *et al.*[61] report the separation of Fluorescein–thiohydantoin derivatives of amino acids on Jascorex SV-02-500 (octadecylsilyl groups bonded to glass).

The use of dansyl amino acid derivatives as products of the Edman procedure is described by Engelhardt *et al.*[62] and Yamabe *et al.*[63]. This derivatisation procedure allows the use of fluorimetric detection as does the use of 5-dibutylaminonaphthalene-1-sulphonyl chloride which is suggested by Seiler *et al.*[64] as a replacement for dansyl chloride in the fluorescence labelling of amines, amino acids and peptides. Although essentially the same derivative it does allow the detection of methylamine which is not possible with dansyl chloride as methylamine is liberated during the derivatisation procedure.

The use of fluorescamine as a derivatisation agent for polyamines has already been mentioned[48] and it has also been used for post-column derivatisation of amino acids and peptide hormone hydrolysates. Its use as a pre-column derivatisation agent for amino acids has been investigated by McHugh *et al.*[65] who found that HPLC of the derivatives gave two peaks. This was shown to be due to an equilibrium reaction involving lactone formation between the free carboxylic acid group of the amino acid derivative and a proximal hydroxyl group (Fig. 6). It was found that this reagent was suitable for derivatisation of peptides where the free carboxylic acid is far enough away from the nitrogen which is derivatised. In this case lactonisation cannot occur.

Optical activity of amino acids has been investigated by Furukawa *et al.*[66] using alanine, isoleucine and phenylalanine as models. The production of diastereoisomers by using an optically active derivatising agent was used in a similar fashion to the method of Helmchen and Strubert[44] except that, in this case, a double derivati-

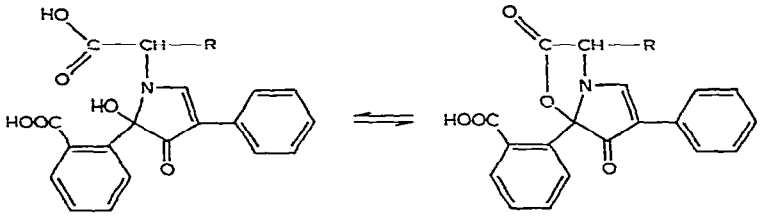


Fig. 6. Lactonisation of fluorescamine amino acid derivatives.

sation was undertaken. The amino acids were first reacted with d-10-camphorsulphonyl chloride to introduce the second asymmetric carbon centre and then derivatised with *p*-nitrobenzyl chloride to allow UV detection. Separation was carried out on Micropak Si5 with detection at 254 nm.

More specialist application of derivatisation is found in the reaction of 4-biphenylcarbonyl chloride to produce the N-acyl derivatives of L-α-distearoyl, L-α-dipalmitoyl and L-α-dioleoyl phosphatidylethanolamines[67]. These were separated by HPLC on Micropak Si 10 with UV monitoring at 280 nm. Naturally occurring mixtures from microsomal fractions and myelin from rat brain and liver were also examined. Serine-containing phosphoglycerides could also be analysed by this procedure.

7. MISCELLANEOUS

Certain pre-column treatments which are specific to particular compounds rather than methods of general applicability have been reported.

Benomyl, a systemic fungicide, has been the subject of two derivatisation procedures. Kirkland *et al.*[68] described the controlled acid hydrolysis of benomyl to afford methyl benzimidazol-2-yl carbamate which is separated on Zipax SCX with UV detection at 254 nm allowing the determination of 0.05 ppm from soil and plant tissues. Maeda and Tsuji[69] described the more vigorous hydrolysis of carbaryl to afford 2-aminobenzimidazole. Separation on Hitachi gel no. 3010 (a porous polystyrene polymer) allows detection of 25 ng of derivative at 277 nm. An alternative approach is described based on the fact that, in base, 2-aminobenzimidazole fluoresces. Thus detection is possible by mixing the column effluent with base and monitoring at $\lambda_{ex} = 285$ and $\lambda_{em} = 315$ nm.

Diphenylhydantoin, on oxidation with alkaline permanganate, affords benzophenone which is chromatographed on Merkosorb Si 60 with naphthalene as an internal standard[70]. The method serves to detect phenylhydantoin at plasma levels of 1.0 mg/l. The non-barbiturate sedative, ethchlorovynol, is an α-hydroxyvinyl chloride which, on treatment with acid, gives an α,β-unsaturated aldehyde. Needham and Kochhar[71] utilise this reaction, which is followed by formation of the semicarbazide, to detect 0.05 μg/ml by UV monitoring at 280 nm after separation on μ-Bondapak C_{18} (Fig. 7).

Isocyanates (found as air pollutants) have been estimated by passing air more through a solution of 4-nitro-N-propylbenzylamine[72]. The isocyanates are converted into urea derivatives which are chromatographed on Pellosil. By UV monitoring, isocyanotoluene was detected at a level of 0.2 ppb* in 20 l of air.

* The American billion (10^9) is meant here.

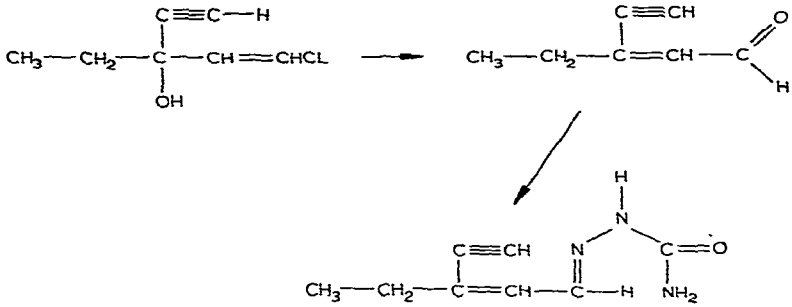


Fig. 7. Ethchlorovynol derivatisation.

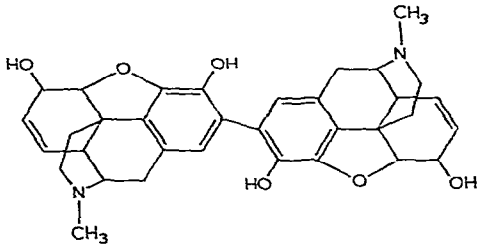


Fig. 8. Pseudomorphine.

A unique derivatisation procedure has been adopted by Jane and Taylor[73] for the sensitive fluorescence detection of morphine in urine. Morphine, on oxidation with ferricyanide, affords a fluorescent dimer termed "pseudomorphine" (Fig. 8). Dihydromorphine is added to the sample before dimerisation, as an internal standard, producing three compounds *in toto*, "pseudomorphine", dihydromorphine dimer and morphine–dihydromorphine dimer. These are easily separated on 7-μm Partisil and the morphine content calculated from relative peak heights. With fluorescence detection at $\lambda_{ex} = 320$ and $\lambda_{em} = 436$ nm it was possible to determine 4 ng of morphine accurately.

Hayashi *et al.*[74] use naphthalene-2,3-diamine sulphate, in the presence of an antioxidant, to derivatise phenylpyruvic acid. The product, 3-benzyl-2-hydroxybenzoquinoxaline (Fig. 9), is separated on Permaphase ETH, and UV detection at 254 nm allows the assay of phenylpyruvic acid at microgram levels. Chlorothioxanthren-9-one is used as an internal standard.

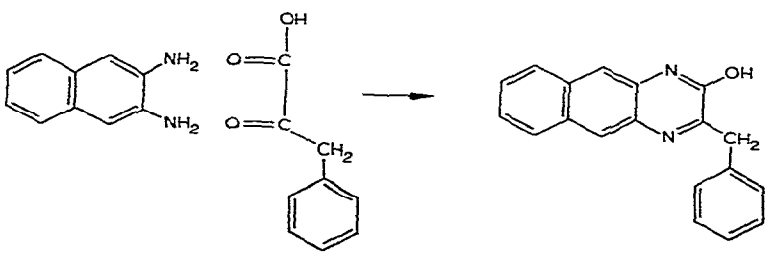


Fig. 9. Phenylpyruvic acid derivatisation.

8. CONCLUSION

It would appear that the increasing use of pre-column derivatisation in HPLC is closely following the use of HPLC *per se*. Most of the applications at this time are designed to circumvent problems with detection rather than difficulties in separation. It is therefore predictable that interest in derivatisation HPLC will increase as variable-wavelength UV detectors and spectrofluorimeters become standard additions to commercial equipment.

As yet, little use has been made of the selectivity of derivatisation to simplify the analysis of complex mixtures. It is likely that such an approach will be of value in disciplines such as chemotaxonomy and phytochemistry, particularly as the methodology developed for analysis should be directly convertible to preparative scale. The advantages of selectivity will become more evident when satisfactory universal detection systems are available.

9. SUMMARY

Pre-column derivatisation in high-performance liquid chromatography is mainly used to increase the detectability of compounds requiring analysis. This normally involves reaction with chromogenic or fluorogenic reagents, and thus the use of the more commonly available HPLC detectors. Derivatisation is more rarely used to alter chromatographic properties of compounds or to allow the resolution of optical isomers. Applications of pre-column derivatisation are discussed by reference to the functional group of the substrate involved in the derivatisation reaction.

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