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ASSESSMENT OF THE CURRENT STATUS OF REACTION LIQUID CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

The introduction of chemical reactions in liquid chromatography as an aid to improving the detection properties and selectivities for certain groups of compounds has become well known in recent years, and is demonstrated by the rapidly increasing number of publications and the appearance of reviews and books[1–3] in this area. Pre- and post-chromatographic derivatization techniques can be distinguished, and are treated separately in this paper.

2. PRE-CHROMATOGRAPHIC TECHNIQUES

The major reasons for separating compounds as derivatives in liquid chromatography are to improve the detection properties and to improve the selectivity. The latter can be attained by using the selectivity of the reagent in a clean-up process.

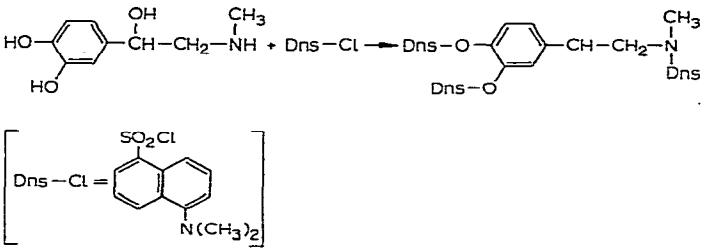
Pre-chromatographic techniques offer several advantages over post-chromatographic methods. One is that the reaction is independent of the mobile phase. The kinetics of the reaction are also not very critical provided that we have a reproducible and relatively simple procedure. The possibility of working with a large excess of reagent and having no difficulty in eliminating such an excess is attractive.

On the negative side we have the high risk of artefact formation, which often necessitates an extensive study of the reaction conditions and of the resulting derivatives.

A typical example which illustrates the problems and limitations of these techniques is the 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatization of

adrenaline to form a fluorescent derivative suitable for blood analysis. The possibilities of the fluorescence labelling of amines and phenolic compounds with Dns-Cl and related reagents have recently been discussed[3], and the applications of this technique to biogenic amines, particularly catecholamines, have been studied by Schwedt and Bussemans[4].

The derivatization of the adrenaline proceeds according to the following reaction:



This reaction has been shown by Nachtmann *et al.*[5] to proceed quantitatively by the use of titration procedures.

As can be seen in Fig. 1 (curve A), the reaction proceeds to complete substitution after 10 min, 3 moles of Dns-Cl reacting with 1 mole adrenaline. Some other alkaloids that were also investigated showed ratios of 2:1 and 1:1, respectively. These results were confirmed by nuclear magnetic resonance (NMR) studies on the isolated derivatives. Studies of the fluorescence properties of these derivatives showed that the tri-Dns-adrenaline, in spite of being the most substituted derivative, had the lowest fluorescence yield (Fig. 2) and is unstable in that the fluorescence increases rapidly (*ca.* 50% in 25 min) upon irradiation with UV light. The other derivatives, in contrast,

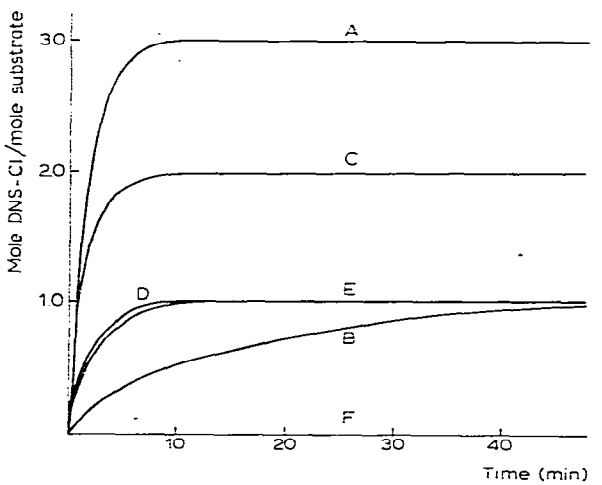
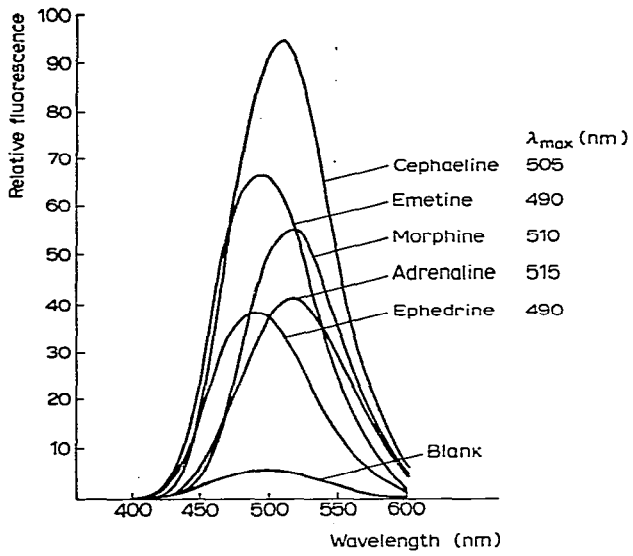


Fig. 1. Corrected molar ratios obtained by continuous titration monitoring of the dansylation reaction. (A) Adrenaline; (B) ephedrine; (C) cephaeline; (D) emetine; (E) morphine; (F) codeine with no reaction occurring[5].



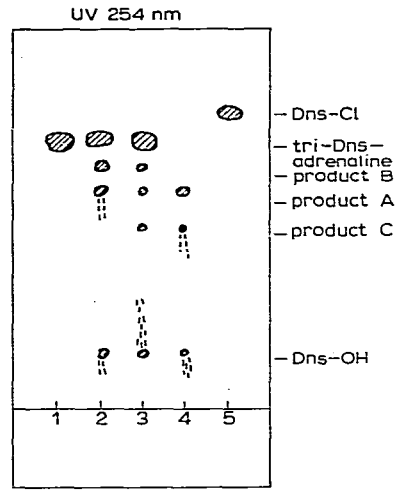
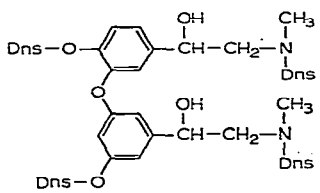


Fig. 2. Fluorescence emission spectra of the Dns derivatives recorded with a Zeiss PMQ 2 instrument equipped with a ZFM4 fluorescence attachment. Excitation at 365 nm[5].

Fig. 3. Thin-layer chromatogram of tri-Dns-adrenaline and its photodegradation products[6]. (1) Tri-Dns-adrenaline (pure); (2) tri-Dns-adrenaline irradiated in benzene solution; (3) tri-Dns-adrenaline irradiated on the plate; (4) product A irradiated in benzene solution; (5) Dns-Cl. Chromatography carried out on Merck SI-60 F_{254} silica gel plates. Mobile phase: benzene–toluene (3:1).

showed a decrease of a few percent in this same irradiation time. This led us to believe that adrenaline is one of the borderline cases for a meaningful application to pre-column derivatization and prompted us to study this system further.

Irradiation of purified tri-Dns-adrenaline (structure confirmed by infrared spectroscopy and NMR) produced several photochemical degradation products, as can be seen in Fig. 3. Tri-Dns-adrenaline and Dns-OH, split off the derivative in the degradation process, were positively identified on the basis of R_F-values. Product A (Fig. 3) was isolated and the structure determined by NMR. Product A seems to correspond to an asymmetrical dimer with the following structure:



There is good evidence that product B corresponds to the symmetrical dimer. Upon irradiation of products A and B, further degradation occurs (Fig. 3), resulting in a rapid increase in fluorescence followed by a decrease at a lower rate.

The following explanation may serve to explain these phenomena. The tri-substituted adrenaline is strained owing to steric hindrance, as can be clearly demon-

strated on a model; this would also explain the relatively low fluorescence yield (Fig. 2). Upon degradation, one Dns moeity splits off in the neighbouring hydroxyl positions of the adrenaline molecule and the dimers are formed. This results in a less strained molecule with a higher fluorescence yield. The fluorescence is further enhanced when the dimers split into the di-substituted monomers (probably product C in Fig. 3). Prolonged irradiation results in a further degradation to the mono-Dns derivative and hence a net decrease in fluorescence.

Practice has shown that with sufficient care it is still possible to use the tri-Dns derivative of adrenaline for analytical purposes. A method has been developed for the determination of Dns-adrenaline and Dns-noradrenaline in blood plasma by high-performance liquid chromatography (HPLC)[6]. On the other hand, this example also draws attention to the general risk of artefact formation in pre-chromatographic derivatization techniques and the need to carry out studies of this nature for critical systems before the development of an analytical technique is perfected.

In some instances UV labelling may be preferred to fluorescence labelling, in spite of losing the inherent sensitivity of fluorescence techniques. The situation can often be considerably simpler and more predictable for UV derivatization and the additivity of extinction values, depending only on the degree of substitution, can be useful in quantitative work, as has been shown in recent studies with sugars and cardiac glycosides[7–9]. For the above system, which involves *p*-nitrobenzoylation of non-aromatic hydroxyl groups, not only a better UV detectability but also a better chromatographic selectivity was obtained, which permitted the analysis of complex mixtures such as that shown in Fig. 4 for an extract from digitalis plants.

The many other possibilities that exist for pre-chromatographic derivatization for fluorescence and UV labelling can be found in detail in two books[2,3].

For molecules that possess no suitable active group for derivatization but have a reasonable basic or acidic activity, there remains the possibility of ion-pair chromatography, the idea again being to choose an ion partner with a good chromophore or fluorophore. Much of the original work in this area was done by Schill and co-workers[3,10], who also treated the theoretical aspects of ion-pair chromatography. Santi *et al.*[11] have demonstrated the application of the technique to tropane alkaloids forming picrate ion pairs. The use of these techniques in routine pharmaceutical analysis in dissolution rate and content uniformity testing has been demonstrated by the same group[12]. An application of this principle to the analysis of active principles in a tablet formulation for the treatment of migraine is shown in Fig. 5.

The problem was to determine small amounts of ergotamine and hyoscyamine in the presence of up to a 1000-fold excess of caffeine and butalbital. By forming picrate ion pairs for the two basic components ergotamine and hyoscyamine, and detection at the UV maximum of the picrate ion (254 nm), it is possible to discriminate partially between the other two components (see Fig. 5). Complete supression of the absorbance of caffeine and butalbital is possible at the second absorption maximum of the picrate ion (345 nm) (see Fig. 6), and a sensitive and reproducible quantitation of the two alkaloids is therefore feasible. The search for other ion partners with still better detection properties is continuing[13].

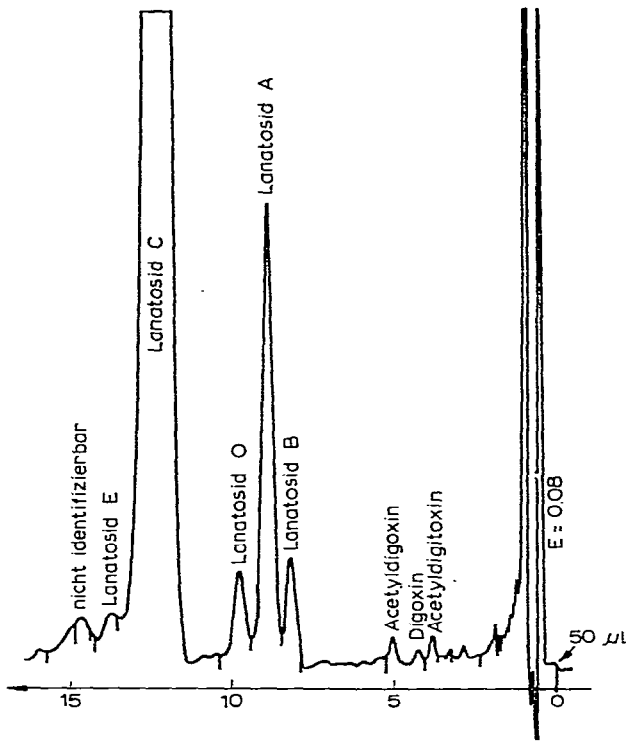


Fig. 4. HPLC of 4-nitrobenzoyl derivatives of some digitalis glycosides in a plant extract. Chromatography: *n*-hexane–methylene chloride–acetonitrile (10:3:3). Column, SI-60 (5 μm), 15 cm × 3 mm I.D.; flow-rate, 1.5 ml/min; Δp, 120 atm. Apparatus: Hewlett-Packard UFC 1000 with DuPont 842 UV detector (254 nm). Rheodyne 7105 injection system; 20-μl injection volume in chloroform.

3. POST-CHROMATOGRAPHIC TECHNIQUES

The developments of post-column reactors in liquid chromatography in recent years can be traced to the lack of detectors with sufficient sensitivity and selectivity for certain analytical problems. The advantages and disadvantages have been elaborated on earlier[1,2], but certainly the major advantages of the reaction detector are that artefact formation is not critical and that the reaction does not have to go to completion or give well defined derivatives, provided that it is reproducible.

The most serious disadvantage, on the other hand, is the interdependence between the mobile phase and the reaction medium, and this has probably been one of the major reasons why the development of post-column techniques in HPLC has not been very widespread until now.

Obviously the kinetics of a reaction are also very important and determines to a large extent the feasibility of a reaction detector and which type should be used. There are three different principles for the construction of such reactors: (1) tubular or capillary reactors, (2) bed reactors and (3) air-segmented streams such as are used in automated analysers.

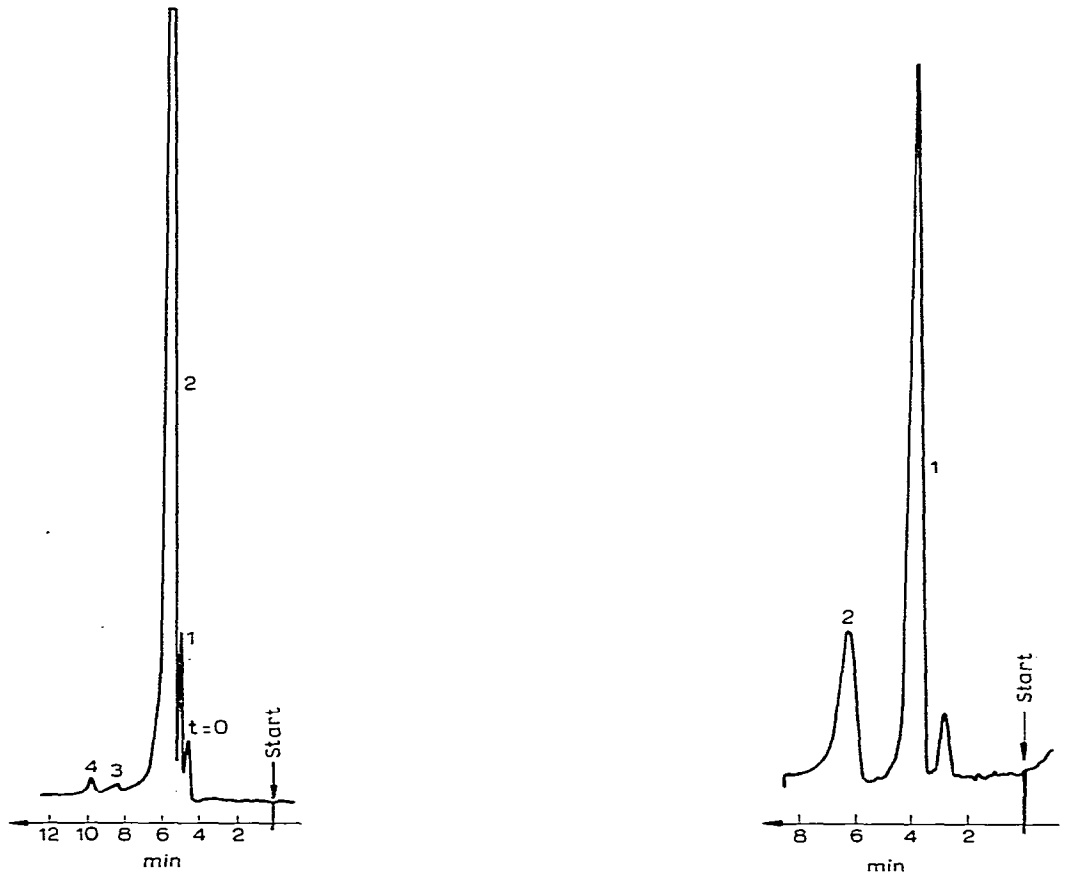


Fig. 5. Separation of components in Cafergot PB®. Column, SI-100 (5 μm), 15 cm × 3 mm I.D. Mobile phase, chloroform saturated with stationary phase, 0.06 *M* picric acid at pH 6. Flow-rate, 0.2 ml/min. Detection at 254 nm (1.0 a.u.f.s.). Peaks: 1 = butalbital; 2 = caffeine; 3 = hyoscyamine; 4 = ergotamine[12] (components 3 and 4 separated as ion pairs).

Fig. 6. Separation of Cafergot PB® components as ion pairs. Detection at 345 nm (0.05 a.u.f.s): 1 = hyoscyamine; 2 = ergotamine[12].

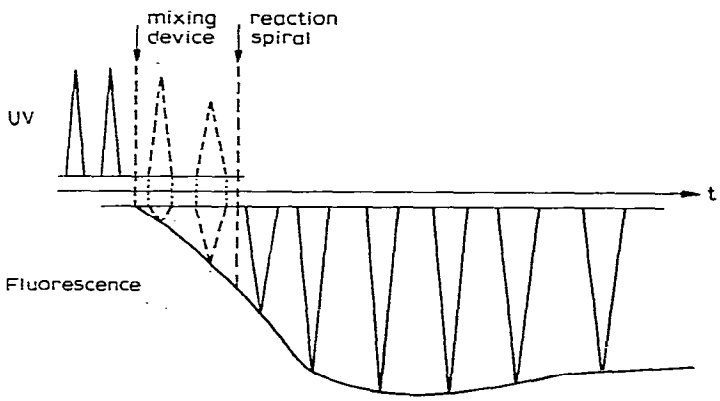


Fig. 7. Schematic representation of peak behaviour in a post-column reactor[16].

3.1. Tubular reactors

As in any other post-column reactor, the major problem is to avoid excessive band broadening during the reaction. As shown schematically in Fig. 7, this band broadening can occur in the mixing device(s) during addition of reagent and in the reaction spiral. An optimal design is therefore essential in order to avoid substantial decreases in the chromatographic resolution. The theoretical aspects of band broadening in tubular reactors have been discussed recently[14]. Based on classical flow dynamic principles, it is relatively simple to predict band broadening in tubular reactors.

A typical example of the use of a tubular reactor is briefly discussed below. It involves the reaction of nonapeptides with Fluram® [15,16].

The rapid kinetics of this fluorigenic reaction are shown in Fig. 8. A plateau for this reaction is reached in *ca.* 50 sec. It is now possible to use a much shorter reaction time, because at 10 sec there is close to 90% of the total fluorescence. Plotting peak heights against signal will result in a maximum (see Fig. 8), which can give an indication of the optimal reaction time as at this point band broadening will offset any further gains in fluorescence.

The design of the mixing unit is also of utmost importance, particularly when solutions of different densities are mixed and when the flow-rates of the mobile phase and the reagent stream differ considerably. In such instances mixing against the eluent stream causes more turbulence and radial mixing and hence less band broadening. Some useful designs are shown in Fig. 9.

An actual application of this principle is shown in Fig. 10, representing a duplicate injection of a peptide mixture, derivatized with Fluram and detected by fluorescence. It can be seen that the reproducibility of the chromatographic pattern is good and the reproducibility of peak areas for major peaks is such that one can truly speak of a quantitative technique. The band broadening measured for the oxytocine peak due to the mixing device and tubular reactor is of the order of 4% for a 10-sec reaction time. Theoretical calculations give a lower value (3.5%), but do not include the influence of the mixing device. Experimental results have shown that with the conditions used (see Fig. 10) one obtains about 7% peak broadening for each 500-μl reaction volume (spiral volume). If we now assume a 1-min reaction time with a total flow-rate (reagent solution + eluent) of, say, 2 ml/min we would have 28% band broadening due to the reaction unit, which for many applications would not be acceptable. Clearly in such a case one might have to consider the use of a bed reactor.

3.2. Bed reactors

The use of bed reactors consisting of columns packed with glass beads of various sizes has been developed primarily by two groups[14,17]. Such a bed reactor can be considered as a chromatographic column essentially used under t_0 conditions (no retention) and the theory for predicting band broadening phenomena in a bed reactor is consequently based on parameters such as packing geometry, average particle size, tortuosity, fluid velocity and diffusion coefficients[14,17]. Taking again the above example in which with a tubular reactor a band broadening of 28% was obtained and calculating the expected band broadening with the equations proposed earlier[14], one would obtain well below 5% band broadening. Hence clearly for reaction times of 1 min or longer one would preferably use bed reactors in spite of the complication introduced.

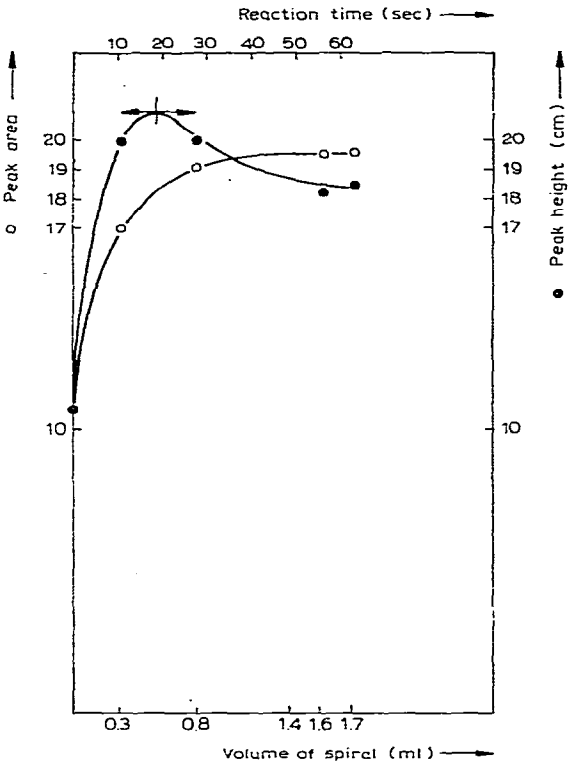


Fig. 8. Influence of length of spiral (reaction volume and time) on the fluorescence signal. Conditions column, RP-8 (10-μm), 10 cm × 0.4 cm I.D.; eluent, acetonitrile–water (20:80), pH 7; reagent Fluram (30 mg per 100 ml of acetonitrile). Eluent flow-rate, 1.6 ml/min; reagent flow-rate, 0.1 ml/min; injection of 100 μl of oxytocine (5 I.U./ml) via loop; fluorescence detection with Amince fluoromonitor[16].

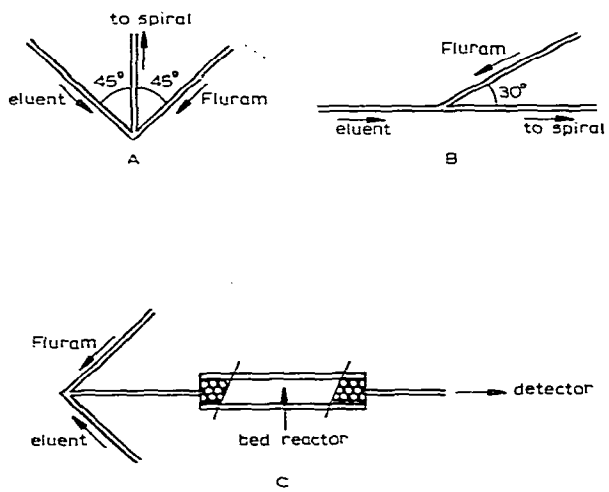


Fig. 9. Different mixing units used for the Fluram–peptide reaction[16].

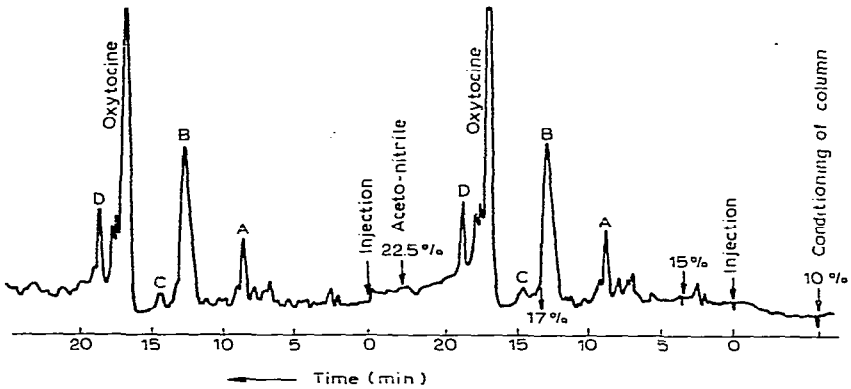


Fig. 10. HPLC of a peptide mixture (purification fraction for oxytocine). Duplicate chromatogram. Step gradient: acetonitrile, 10 to 22.5% (pH 7). Other conditions as in Fig. 8.

3.3. Segmented-flow reactors

For even longer reaction times (*i.e.*, longer than 5 min), one would have to resort to the air-segmentation principle in order to overcome excessive band broadening. The theoretical aspects of air-segmented flows are the most complex and least understood. A semi-empirical approach to achieve a better understanding of the principle was made by Snyder[18].

Band broadening in the reaction unit can be attributed in part to leakage between the segments (spaced by air bubbles) due to wetting of the capillary wall. This effect, however, is small in comparison with the band broadening effects introduced by mixing tees, debubblers or phase separators. Much effort is therefore necessary to achieve better designs of these parts or possibly electronic debubbling techniques.

A typical example of such a longer reaction is given here and involves the dehydration of cardiac glycosides with concentrated hydrochloric acid to form a fluorescent product. This process is enhanced by adding a mixture of hydrogen peroxide and ascorbic acid, the exact mechanism being unknown. The extrapolated kinetic curve for this reaction is shown in Fig. 11. A plateau is reached after about 0.5 h and, following our previous theory, an actual reaction time of 10 min was chosen. Use of the air-segmentation principle is the only feasible approach from the point of view of both reaction time and agressiveness of the reagents. An all-glass–PTFE AutoAnalyzer unit was used.

A schematic diagram of the apparatus is shown in Fig. 12. The system corresponds to the standard Technicon second-generation AutoAnalyzer unit except for the reaction spirals, which where constructed with 1 mm I.D. Portex tubing. It was possible with such a system to keep the band broadening due to the AutoAnalyzer reactor unit below 10%, which is surprising when it is considered that a mechanical debubbler was used. The corresponding decrease in chromatographic resolution compared with the UV signal can be seen for the two pairs of glycosides in Fig. 13. The improvement in detectability, which is about 100 times better than for direct UV detection, and the gain in selectivity compensate fully for this decrease in resolution.

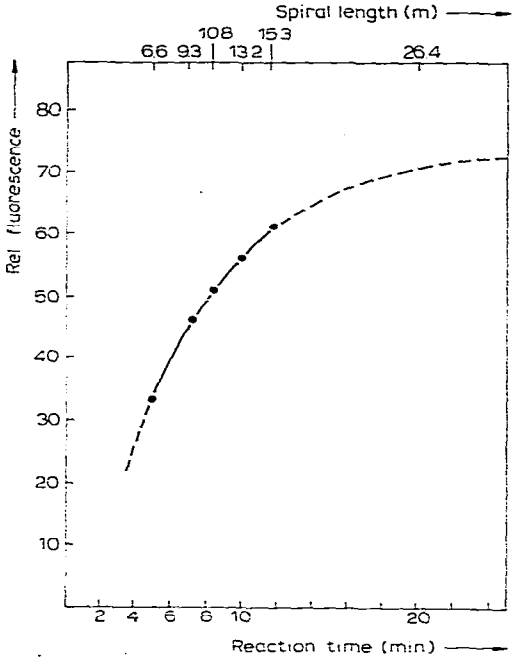


Fig. 11. Extrapolated kinetic curve for the fluorescence reaction of desacetyllanatoside C' with concentrated hydrochloric acid[19].

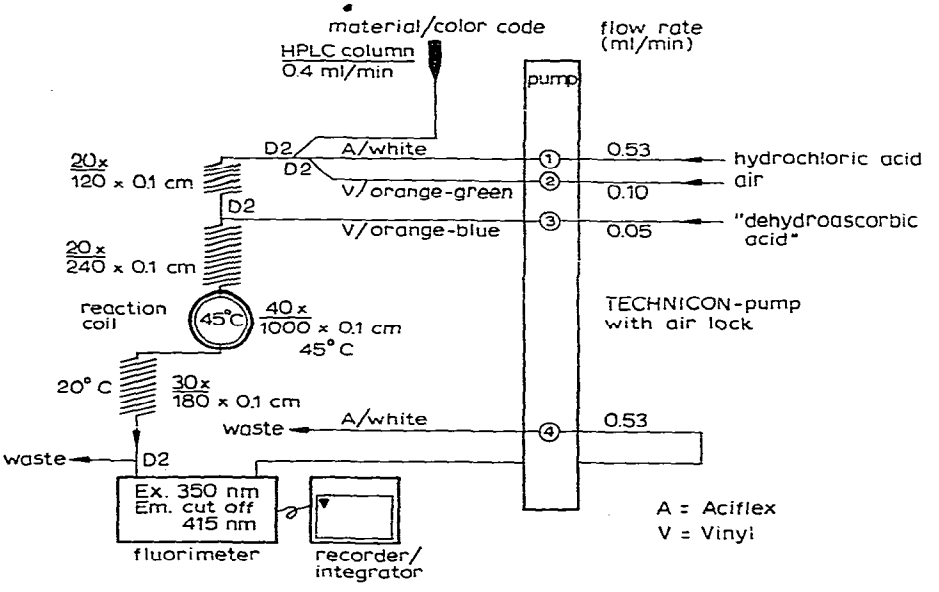


Fig. 12. Schematic diagram of the reaction detector unit for fluorescence detection[19].

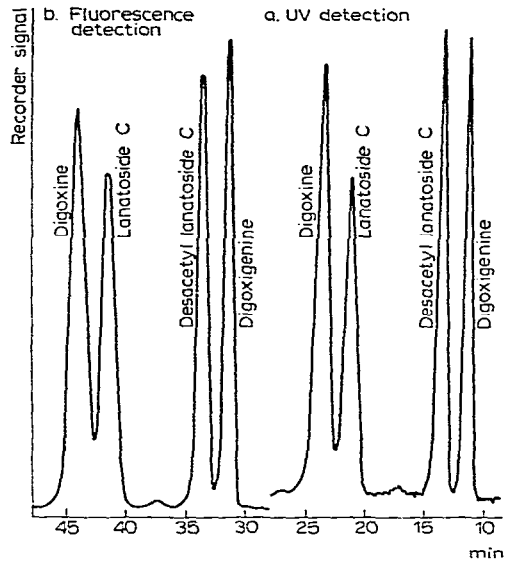


Fig. 13. Comparison of the resolution of the cardiac glycosides obtained with UV detection right after the column and with fluorescence detection after the post-column reaction[19].

4. CONCLUSION AND PROSPECTS

Derivatization techniques can be a very powerful means of enhancing the effectiveness of modern liquid chromatographic techniques and detectors. In order to make full use of them, it is important to recognize the limitations and pitfalls and to make the proper choice of the derivatization mode. As more reagents become known and commercially available for practically all feasible functional groups, it should be possible to adapt these techniques to a wide range of problems.

In post-column techniques, many groups are still working on optimization of technical designs to reduce band broadening further; however, as the theoretical aspects of these reactors become better known one could expect good advances in this area. In 1977 significant advances in this direction could be discerned.

The possibilities of adapting thermal[17], photochemical[20] and catalytic[21] processes to initiate, accelerate and simplify post-column reactions are being actively pursued by many groups. Coupling of reaction detectors with step gradients[16] (see Fig. 10) and with large volume injections[16,22] will greatly enhance their usefulness. Even though the examples discussed in this paper were restricted to UV and fluorescence detection, there is no doubt that this philosophy can be extended to other detection modes. In electroanalytical detection this has in fact already been done[14,23] and work in this direction is also in progress in our laboratories.

The possibilities of using chemiluminescence techniques has been discussed by Neary *et al.*[24]. Metal chelation phenomena have been used to adapt atomic absorption spectrophotometry to HPLC detection[25] and can also be interesting for electroanalytical detectors or flame and plasma emission techniques. The use of flame emission spectroscopy for HPLC has been demonstrated by Kirkland[26] and Freed[27].

Other similar areas of development may be seen in the future for radiochemical[28], mass spectroscopic and phosphorescence detection.

There are many possibilities in this area for the imaginative analytical chemist. The enhanced sensitivity and selectivity that can be gained will often be essential for solving some of the complex trace analytical problems that we are confronted with in modern problem-solving processes.

5. SUMMARY

Some of the advantages and also pitfalls and limitations of chemical derivatization techniques are discussed. For post-column derivatization, different reactor designs are discussed and compared. Finally, prospects are surveyed for further development in this field.

REFERENCES

1 R. W. Frei, *Res./Develop.*, Feb. (1977) 42.
2 J. F. Lawrence and R. W. Frei, *Chemical Derivatization in Liquid Chromatography*, Elsevier, Amsterdam, Oxford, New York, 1976.
3 K. Blau and G. S. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden & Son, London, 1977, pp. 346 and 500.
4 G. Schwedt and H. Bussemas, *Chromatographia*, 9 (1976) 57; *Z. Anal. Chem.*, 283 (1977) 233.
5 F. Nachtmann, H. Spitzy and R. W. Frei, *Anal. Chim. Acta*, 76 (1975) 57.
6 R. W. Frei, M. Thomas and I. Frei, *J. Liquid Chromatogr.*, 1 (1978) 443.
7 F. Nachtmann, H. Spitzy and R. W. Frei, *J. Chromatogr.*, 122 (1976) 293.
8 F. Nachtmann, H. Spitzy and R. W. Frei, *Anal. Chem.*, 48 (1976) 1576.
9 F. Nachtmann, *Z. Anal. Chem.*, 282 (1976) 209.
10 G. Schill, in J. A. Marinsky and Y. Marcus (Editors), *Advances in Ion Exchange and Solvent Extraction*, Vol. 6, Marcel Dekker, New York, 1974, p. 10.
11 W. Santi, J. M. Huen and R. W. Frei, *J. Chromatogr.*, 115 (1975) 423.
12 J. M. Huen, R. W. Frei, W. Santi and J. P. Thevenin, *J. Chromatogr.*, 149 (1978) 359.
13 J. Crommen, B. Fransson and G. Schill, *J. Chromatogr.*, 142 (1977) 283.
14 R. S. Deelder, M. G. F. Kroll, A. J. B. Beeren and J. H. M. van den Berg, *J. Chromatogr.*, 149 (1978) 669.
15 R. W. Frei, L. Michel and W. Santi, *J. Chromatogr.*, 126 (1976) 665.
16 R. W. Frei, L. Michel and W. Santi, *J. Chromatogr.*, 142 (1977) 261.
17 K. M. Jonker, *Thesis*, University of Amsterdam, Amsterdam, 1977.
18 L. R. Snyder, *J. Chromatogr.*, 125 (1976) 287.
19 J. C. Gfeller, G. Frey and R. W. Frei, *J. Chromatogr.*, 142 (1977) 271.
20 P. J. Twitchett, P. L. Williams and A. C. Moffat, *J. Chromatogr.*, 149 (1978) 683.
21 F. Nachtmann, G. Knapp and H. Spitzy, *J. Chromatogr.*, 149 (1978) 693.
22 P. Schauwecker, F. Erni and R. W. Frei, *J. Chromatogr.*, 136 (1976) 63.
23 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 447A.
24 M. P. Neary, R. Seitz and D. M. Hercules, *Anal. Lett.*, 7 (1974) 583.
25 D. R. Jones and S. E. Manahan, *Anal. Chem.*, 48 (1976) 502.
26 J. J. Kirkland, *J. Chromatogr.*, 112 (1975) 443.
27 J. D. Freed, *Anal. Chem.*, 47 (1975) 186.
28 G. Gübitz, R. W. Frei, W. Santi and B. Schreiber, *J. Chromatogr.*, 169 (1979) 213.