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POST-COLUMN DERIVATIZATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING THE AIR SEGMENTATION PRINCIPLE: APPLICATION TO DIGITALIS GLYCOSIDES

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SUMMARY

The possibility of using post-column derivatization techniques to improve the detection in high-performance liquid chromatography (HPLC) has been suggested before, but a serious limitation to the choice of the reactions is the kinetics, as slow reactions require longer reaction times and correspondingly larger reaction units (long spirals) with the associated problems such as a high pressure drop and loss of resolution.

In this study it was attempted to overcome some of these drawbacks by applying the well known air segmentation principle used in connection with Auto-Analyzers. The studies were carried out on cardiac glycosides which, upon reaction with concentrated hydrochloric acid, yield highly fluorescent products. As this reaction is kinetically slow, with at least a 10-min reaction time for a reasonable fluorescence yield, it served ideally for the investigation.

The optimal reaction conditions and the influence of solvents, reagent composition, temperature and time on the kinetics and fluorescence yield were studied. With a 10-min reaction time the band broadening was only about 15%. The reproducibility of the derivatization step is 1.2% (relative standard deviation, $n = 10$) and the improvement in the detection limit was at least 100-fold in comparison with UV detection. For a non-retained glycoside the detection limit is 500 pg per injection at a signal to noise ratio of 4:1. The technique was applied successfully to members of the C-group of cardiac glycosides (digoxin, digoxigenin, lanatoside C and desacetyllanatoside C), which were separated on a reversed phase HPLC system. Many of the concepts studied are of general validity for post-column reactions with relatively slow kinetics.

INTRODUCTION

The merits and problems of post-column derivatization in high-performance liquid chromatography (HPLC) have been discussed earlier[1–3]. Although the use of such reaction techniques after column chromatographic separation has been known for more than a decade with classical column techniques (*e.g.*, amino acid analyzers),

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little has appeared in relation to modern HPLC. One reason is the many technical problems such as the dead volume and mobile phase compatibility that still have to be solved.

One of the earlier applications of the technique involving true HPLC was published by Muusze and Huber[4] on the oxidative fluorescence detection of thioridazine and its metabolites. Simpler and equally rapid reactions have been adopted with the use of *o*-phthalaldehyde[5] and Fluram[6] for primary amino groups in amino acids. The limitations of these post-column techniques with regard to dead-volume problems (band broadening) in mixing and reaction units have been discussed in detail with the Fluram system applied to nonapeptides[7,8]. It was concluded that for reactions with relatively slow kinetics (reaction times of 5 min and longer) it would be advisable to adopt the air segmentation principle used conventionally in AutoAnalyzers[1]. The coupling of an AutoAnalyzer as a reaction detector system to a HPLC unit was described as early as 1973[9], adapting the cerium(IV) → cerium(III) system as a indirect fluorescence detection principle for oxidizable compounds such as phenols and polythionates[10]. The coupling of a cholinesterase inhibition AutoAnalyzer to HPLC for the determination of carbamate and organophosphate pesticides was described by Ramsteiner and Hörmann[11]. All of these studies were carried out with earlier HPLC equipment and with ion-exchange or other chemically bonded supports of large particle size (35–50 μm) packed in 1-m columns. With such a system the coupling with a first-generation AutoAnalyzer causes only minor additional band broadening as dead-volume problems are less serious.

Moye and Wade[12] later studied the cholinesterase system in more detail and applied it to carbamates. They used modern HPLC equipment and supports and reported band broadening in the vicinity of 35% for total reaction times of about 4 min. Obviously dead-volume problems in the reaction detector unit are dominant if such a loss in resolution is encountered. An improved situation was presented by Schwedt[13], who discussed an ethanol–diamine reaction detector for the fluorescence detection of catecholamines. A band broadening of 30% was reported and reaction times of 10 min were used. Again 10-μm particles (Nucleosil 10-SA and 10-C_{18}) were used in shorter columns, whereas the AutoAnalyzer equipment was of the earlier generation, using mixing spirals of 2.4 mm I.D.

In this study, we attempted to pay more attention to band broadening for kinetically slow reactions and to miniaturize the reaction unit so that it would fit more appropriately to a modern HPLC unit (5-μm particles, 10- or 15-cm columns).

The reaction of cardiac glycosides with concentrated hydrochloric acid to form highly fluorescent products[14] was chosen as a model system. The cardiac glycosides of the cardenolide type (digitalis glycosides) possess only a relatively weak chromophore with λ_{max} at *ca.* 220 nm. Direct UV detection is possible and useful for certain analytical applications[15] but for real trace analysis, including biological samples, for by-products or metabolites better detection properties are needed. Nitrobenzoylation of these molecules has been shown to improve the detection limits[16] but the sample preparation step and the need to derivatize in an anhydrous environment impose some limitations on this method. It was therefore of interest to develop an alternative technique which modifies the glycosides only after the separation step and which permits adaptation of the already known separation systems and sample preparation techniques.

EXPERIMENTAL

Reagents

The cardiac glycosides were obtained internally; their structures have been given elsewhere[15]. The reagents used for the fluorogenic reaction were doubly distilled water, hydrogen peroxide (30% for synthesis, Merck, Darmstadt, G.F.R.), ascorbic acid, concentrated hydrochloric acid and absolute ethanol (all Merck, analytical grade). Occasionally the surfactant Brij® 35 (Merck, Cat. No. 801962) was added in order to reduce flow resistance. Dehydroascorbic acid was prepared by adding 100 mg of ascorbic acid to 200 ml of water; 5 ml of peroxide solution (1 ml of H_2O_2 in 200 ml of water) were added dropwise followed by stirring for 2 h.

Solvents, reversed-phase chromatographic materials and procedures for the HPLC separation of the glycosides are described in the figure legends and elsewhere[15].

Apparatus

A typical apparatus for HPLC separation and post-column derivatization without air segmentation has been described earlier[7]. The pneumatic Haskel pump was replaced with an Altex Model 100 pump (Altex Scientific, Berkeley, Calif., U.S.A.). The reagent pumping and mixing module of this apparatus was replaced with the reaction module shown in Fig. 1.

The mixing and reaction spirals had to be miniaturized using 1-mm I.D. Portex tubing (Portex Ltd., Hythe, Great Britain). Other parts, such as fittings, mixing units and the peristaltic four-channel pump, were conventional Technicon accessories. The reaction spiral was thermostatted at 45° and the mixing spirals and the fluorimeter cell at 20°. The apparatus was laid out for an eluent flow-rate of 0.4 ml/min and a total reagent flow-rate of 0.58 ml/min (see Fig. 1). Other details of the conditions are given under Results and Discussion.

RESULTS AND DISCUSSION

Optimization of the reaction

The optimization studies were carried out with the AutoAnalyzer reaction system (not in batch) without coupling to an HPLC system. The reagent solution was 800 ng/ml of lanatoside C in methanol.

The fluorigenic reaction is based on the interaction between hydrochloric acid and the steroid portion of the cardiac glycoside. The reaction mechanism is not known exactly but a dehydration phenomenon is probably responsible. A hydrogen peroxide–ascorbic acid mixture was added for enhancement of the fluorescence. The influence of these reagent parameters plus the reaction temperature and time of reaction were studied.

Hydrochloric acid. From previous experience, ideally the concentrated acid is added undiluted to yield a high fluorescence signal. With the decrease in tubing diameter to 1 mm I.D. the pressure drop when pumping pure concentrated hydrochloric acid becomes too high for the peristaltic pump used. The addition of ethanol with a corresponding decrease in viscosity solved this problem but resulted in a drop in the fluorescence intensity, as can be seen in Fig. 2. The pressure drop can also be reduced by adding a surfactant to the hydrochloric acid (0.5 g of Brij 35 was added to

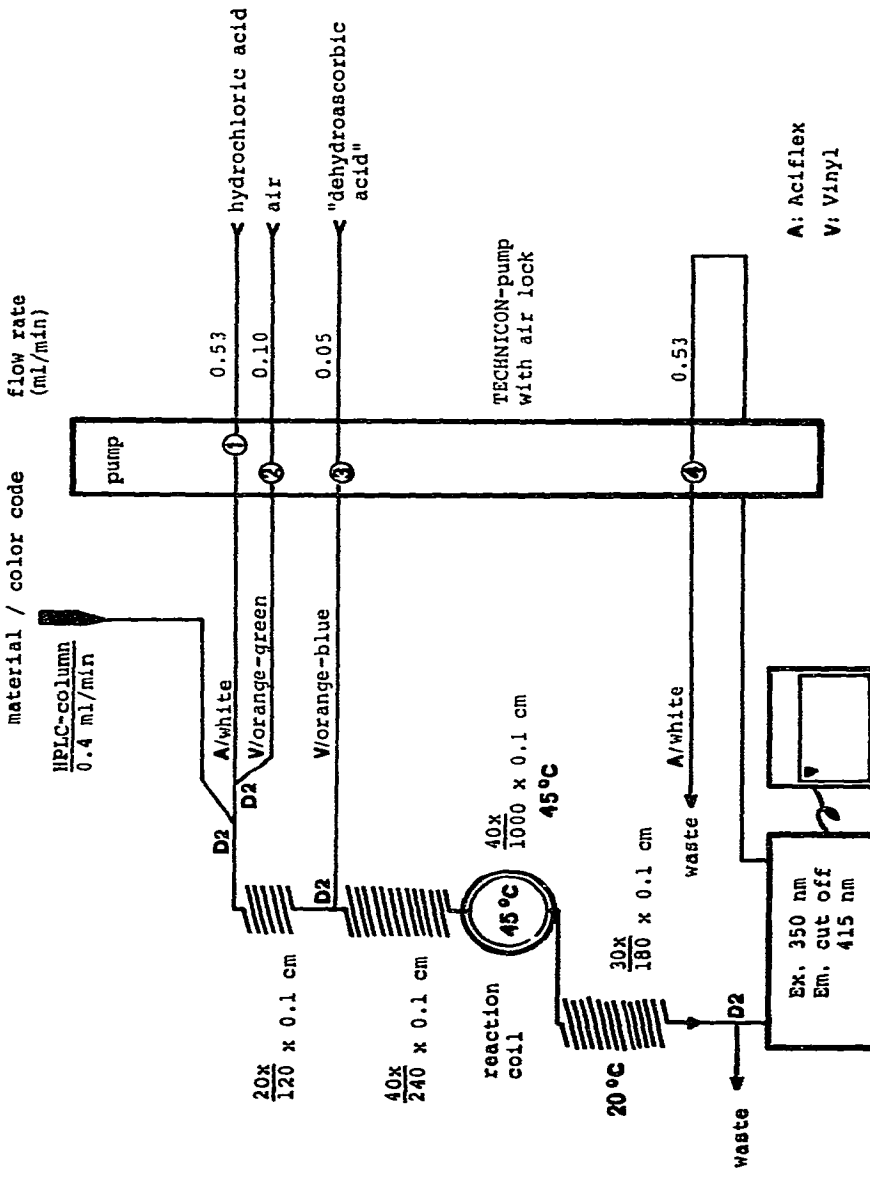

Fig. 1. Schematic diagram of the reaction detector unit for fluorescence detection.

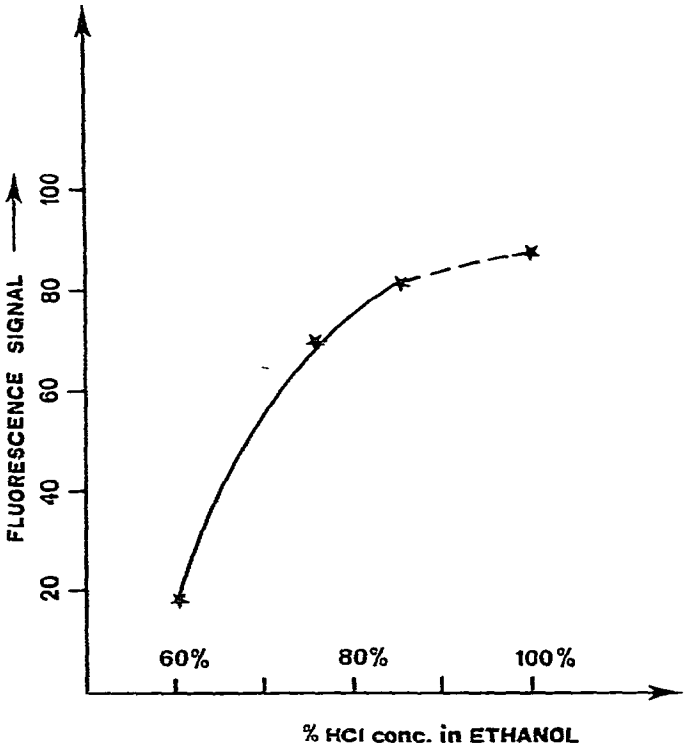

Fig. 2. Influence of ethanol on the fluorescence yield. At the 100% mark a surfactant (Brij 35, 0.5 g per 500 ml of conc. HCl) was added instead of ethanol to reduce the pressure drop.

500 ml of acid) without a decrease in sensitivity. The reagent flows indicated in Fig. 1 were found to be near optimal with regard to the fluorescence yield and dilution effects with the present system.

Hydrogen peroxide–ascorbic acid mixtures. When adding hydrogen peroxide and ascorbic acid, dehydroascorbic acid is formed, which then continues to react with the peroxide to form a complex mixture of by-products. It has been observed that this mixture produces a considerable fluorescence enhancement for the hydrochloric acid–glycoside reaction. The mechanism of this phenomenon is not known.

The influence of the relative concentrations of peroxide and ascorbic acid was investigated. The ratio of hydrochloric acid to glycoside was kept constant. In the first test, various amounts of peroxide solution were added to a solution containing 100 mg of ascorbic acid in 200 ml of water. The results are given in Table I. An optimum is reached at a peroxide concentration of $1.1 \cdot 10^{-3}$ *M* (5 ml added).

In a second test the influence of the ascorbic acid concentration was studied, and the results are given in Table II. It can be seen that about a 15-fold enhancement of the fluorescence results when the hydrochloric acid–glycoside mixture is treated with dehydroascorbic acid. Enhancement of the fluorescence can also be observed if hydrogen peroxide solution alone is added. Previous results showed, however, that it is difficult to control and reproduce the reaction if only peroxide is present. The

TABLE I

FLUORESCENCE YIELD AS A FUNCTION OF HYDROGEN PEROXIDE CONCENTRATION IN THE "DEHYDROASCORBIC ACID" MIXTURE

Amount of peroxide in "dehydroascorbic acid"		*Fluorescence (relative)*
Volume of peroxide solution in 200 ml of ascorbic acid solution (ml)	*Concentration of peroxide (M)*	
1	$2.2 \cdot 10^{-4}$	11.0
3	$6.6 \cdot 10^{-4}$	42.0
5	$1.1 \cdot 10^{-3}$	53.0
10	$2.2 \cdot 10^{-3}$	51.0
20	$4.4 \cdot 10^{-3}$	46.0

TABLE II

FLUORESCENCE YIELD AS A FUNCTION OF THE ASCORBIC ACID CONCENTRATION IN THE "DEHYDROASCORBIC ACID" MIXTURE

Composition of the dehydroascorbic acid solution		*Fluorescence (relative)*
Peroxide solution (ml)	*Ascorbic acid (mg)*	
0	0	4.0
5	0	51.0
5	50	59.0
5	100	60.0
5	200	53.5

optimal conditions chosen from these studies were therefore those given under Experimental (100 mg of ascorbic acid in 200 ml of water and 5 ml of peroxide solution added).

Reaction temperature. Investigations showed that an endothermic reaction is involved. The fluorescence signal increased significantly at higher temperatures, being optimal at *ca.* 45°. Thermostatting at $45 \pm 0.5°$ was therefore used for the reaction spirals. The flow-through fluorescence cell and the mixing spirals were maintained at 20°.

Reaction time. Reaction spirals of different lengths were constructed in order to be able to vary the time of reaction. All other conditions were kept constant at the previously mentioned values. The results are shown in Fig. 3. Under the flow and reaction conditions chosen, it was not possible to investigate longer reaction times owing to the high pressure drop across the reaction spirals.

In earlier work[8] we were able to show that it is not necessary to reach the completion of a reaction in post-column derivatization; all that is necessary is to have reproducible conditions. In fact, it has been shown that optimal conditions are somewhere between one quarter and half of the total reaction time (plateau). In this study, a 10-min reaction time at 45° (13.2-m spiral) was chosen as optimal for fluorescence yield and pressure drop. The roughly extrapolated kinetics curve (Fig. 3)

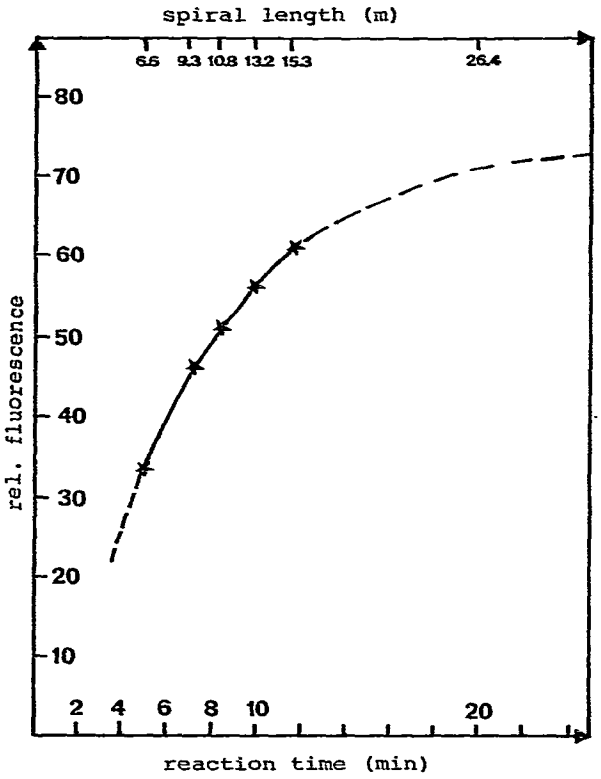

Fig. 3. Extrapolated kinetic curve for the fluorescence reaction with desacetyllanatoside C.

shows that this optimal range is reached within 10 min. The actual time of reaction in Fig. 3 may be slightly longer as the total residence time of the glycoside was 20 min. A time of 10 min was used in the mixing units, the mixing spirals, the flow cell and the connections that were maintained at 20°. As the reaction at 20° proceeds at a much slower rate it can reasonably be assumed that only a minor portion of the reaction has taken place outside of the reaction coil (see Fig. 1).

Coupling with HPLC

The first series of tests were carried out with methanol as the mobile phase and lanatoside C as the test substance. A column of 12.5 cm length and 0.4 cm I.D. was packed with Merck RP-8 reversed-phase material, particle size 5 μm, by a packing technique described elsewhere[17]. Under these conditions the test substance is not retained but it permits band broadening phenomena and other quantitative parameters to be studied.

Peak broadening. Peak broadening has a direct impact on loss of resolution. It was tested by detecting first with a UV detector directly after the column and then with a fluorescence detector after the reaction. The peak broadening therefore gives an indication for the quality of the design of the reaction unit. Contrary to previous designs[9–13] tubing of smaller diameter was used for the reaction spirals and the

flow-rate for the chromatography was slower than usual (0.4 ml/min). With the short column used (12.5 cm), the separation time was still reasonably short.

The results are given in Table III as the average of four replicate tests. The peak broadening was found to be 15% for a system tailored to give a 20-min residence time. The peak broadening for a comparable situation reported earlier[13] with a 10-min reaction time was 30%. It is certain that this situation can be further improved by adapting mixing units and reaction spirals with even better dead-volume performances such as are now available, for example with the third-generation Technicon AutoAnalyzer equipment.

TABLE III

PEAK BROADENING AS A RESULT OF THE POST-COLUMN REACTION

Parameter	*HPLC system*	*Derivatization system (see Fig. 1)*
Flow-rates (ml/min)	0.40	0.53
Peak width at half-height (in.)	46.5	40.5
Peak volume (μl)	310	358
Peak broadening	$\Delta V = 48\ \mu l\ (= 15\%)$	

Reproducibility. The repeatability of this derivatization step was tested by multiple injection ($n = 10$) of the same sample (20 ng of lanatoside C in a 20-μl injection volume). A relative standard deviation of 1.2% was found, indicating the quantitative usefulness of the technique. Calibration graphs for the 2–20 mg per injection concentration range of the glycosides were linear, with a regression coefficient of 0.9998.

Separation. The separation of the C-group of digitalis glycosides was carried out according to a previously published technique[15] by reversed-phase chromatography (see legend to Fig. 4). A comparison of the corresponding chromatogram with UV detection (Fig. 4a) directly after separation and with fluorescence detection (Fig. 4b) following the reaction is shown. The effect of the band broadening on the resolution is small.

The stabilizing effect of the air segmentation principle on diffusion phenomena is clearly demonstrated in Fig. 5, where the same mixture of compounds was separated with and without segmentation. All other conditions were kept the same except for a reduction of the reaction volume by the volume occupied by air bubbles, in the bottom chromatogram.

Application

The potential of the technique was tested by applying it to the analysis of ampoule solutions containing desacetyllanatoside C. The excellent sensitivity of this post-column reaction technique is further enhanced by the very low background fluorescence encountered in this derivatization procedure. The detection limit for desacetyllanatoside C is <0.5 ng per injection (signal to noise ratio 4:1). The resolution from possible by-products is satisfactory considering that the sample has to go through a 20-min residence time in the mixing and reaction modules of the reaction detector.

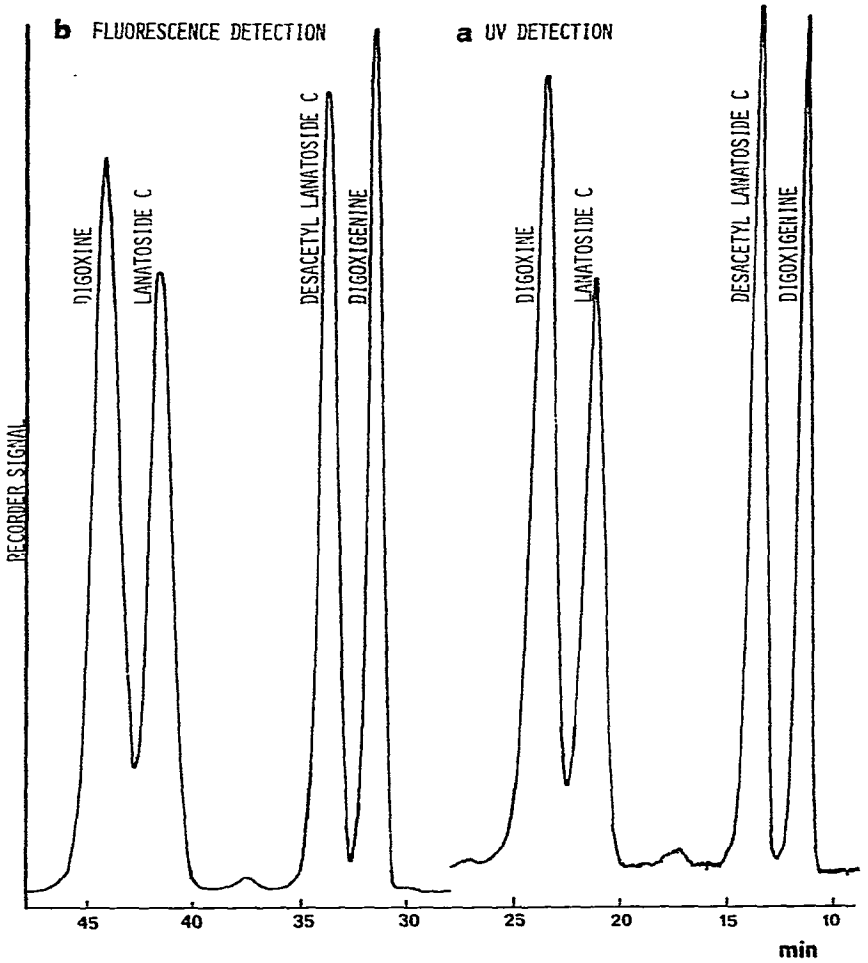

Fig. 4. Comparison of UV and fluorescence detection for the separation of the C-group of lanatosides. Column: reversed phase, Nucleosil C_{18}, 10 μm (Macherey-Nagel, Düren, G.F.R.); length, 25 cm; I.D., 0.4 cm; mobile phase, 40% (v/v) acetonitrile–dioxane (1:1) in water at a flow-rate of 0.4 ml/min. Detection: UV at 220 nm (Perkin-Elmer LC-55). Fluorescence: $\lambda_{ex} = 350$, $\lambda_{em} = 485$ nm. Secondary 7-2 A Wratten filter with Aminco Fluoromonitor. Injection volume, 20 μl (Valco loop).

CONCLUSION

The results demonstrate that post-column derivatization using reactions with relatively slow kinetics can be adapted successfully with the use of the air segmentation principle. The relatively high losses in resolution reported in earlier studies can be significantly reduced if attention is paid to the design of the reaction module. Excessive dead volumes in the mixing units (the mixing T) and mixing and reaction spirals have to be avoided and the design should be compatible with the current state of development in HPLC. With newer generation AutoAnalyzers becoming available this problem will be more easily overcome.

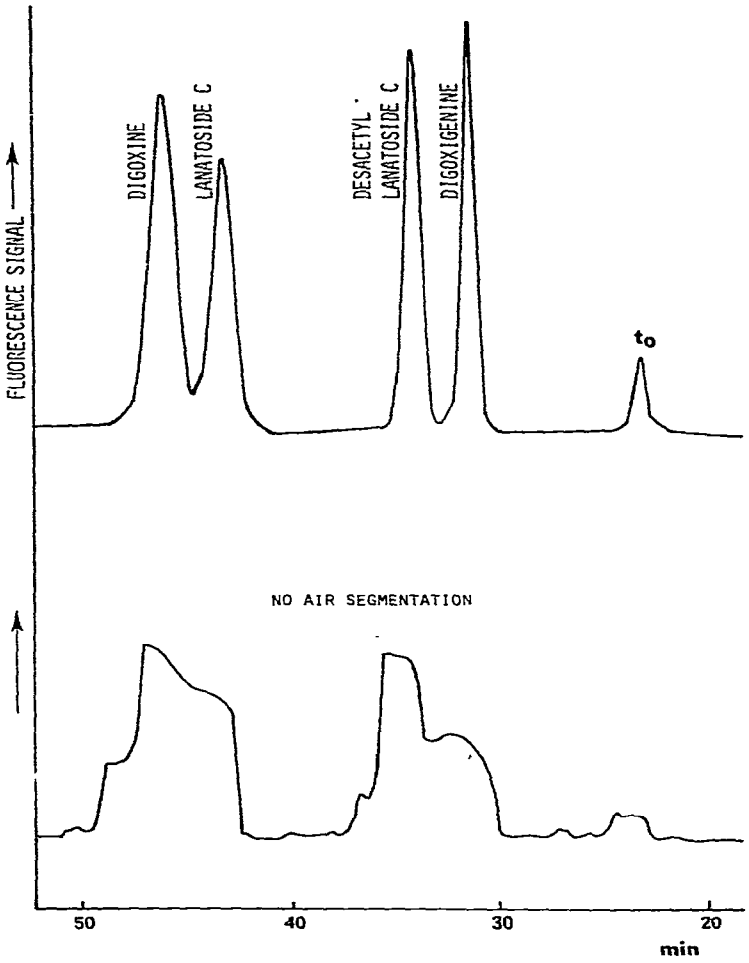

Fig. 5. Comparison of the fluorescence signals obtained with and without air segmentation. Chromatographic conditions as in Fig. 4.

For the method at present it can be said that this detection mode is at least 100 times more sensitive than UV detection without derivatization[15], while still maintaining the relative ease of sample preparation. The all-glass and polypropylene design of the reaction detector unit permits one to work with materials as corrosive as the concentrated hydrochloric acid used in this study. The adoption of the instrumentation used for non-segmented derivatization techniques[7,8] would also be rather problematical from this point of view.

The reproducibility of the technique is such that the method can be recommended as a truly quantitative approach to trace analytical problems.

The knowledge gained in this study should be applicable to many other reaction systems and groups of compounds and it is hoped that it will generate more studies in this area.

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