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NEW ASPECTS OF POST-COLUMN DERIVATIZATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A nonapeptide–Fluram reaction system has been further investigated in order to gain a general insight into the problems of post-column derivatization. Some kinetic aspects and their influence on reaction volumes, design of reaction units and mixing devices have been studied. As a result, reaction times up to several minutes can be tolerated with the present system without appreciable band broadening.

The coupling of sample enrichment phenomena with post-column reactions for further improvement of detection properties was also studied. Several millilitres of sample solution can be injected without serious band broadening. The combination of step gradients with post-column reactions and pre-concentration techniques has been shown to be feasible and opens up interesting possibilities for trace analysis in complex matrices.

The reproducibility of retention times was less than 1.5% relative standard deviation and of peak areas less than 4% relative standard deviation for all of these operations, thus permitting the characterization and quantitation of substances.

INTRODUCTION

In a recent paper[1], some of the problems and possibilities of using post-column fluorescence derivatization for peptide analysis by high-performance liquid chromatography (HPLC) were discussed. Since then some new aspects of this subject have emerged. In order to be able to generalize some of the data, some kinetic aspects of the post-column reaction and their influence on reaction volumes, design of reaction units and mixing devices have been investigated.

The coupling of pre-concentration techniques on reversed-phase systems[2] with post-column reactions for further enhancement of the sensitivity and particularly for increasing in selectivity was also studied. The combination of a simple step gradient system[3] with such derivatization techniques and possibly combinations of all three operations (pre-concentration, step gradient and derivatization) can yield the powerful methodology that is needed for solving complex trace analytical problems.

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The investigations were carried out with the nonapeptide–Fluram system used in the earlier study[1]. Applications are presented for low-dosage ampoule solutions.

EXPERIMENTAL

Reagents

The peptides oxytocine, lysine-vasopressine and ornipressine were obtained internally (Sandoz); their structures have been listed earlier[1]. Acetonitrile (Uvasol; Merck, Darmstadt, G.F.R.) and phosphate buffer (Titrisol; Merck) in doubly distilled water were used as chromatographic solvents. The fluorescence reagent Fluram (Hoffmann-La Roche, Nutley, N.J., U.S.A.) was dissolved in acetonitrile. Titrisol buffer solution (Merck) was used to adjust the pH.

The columns were packed according to a dynamic slurry procedure[4] with the reversed-phase material RP 8 (Merck). Other detailed conditions are given in the legends of the figures.

Apparatus

A Perkin-Elmer MPF 3 spectrofluorimeter was used for batch investigations. A schematic diagram of the apparatus used for this study has been described earlier[1].

Occasionally the pneumatic amplifier pump (Haskel Engineering) was replaced with a Model 100 feedback-controlled reciprocating pump (Altex, Berkeley, Calif., U.S.A.) with success. For very low flow-rates of reagent addition (0.02 ml/min), a Predicor Model 5003 medicinal syringe-type pump (Infors, Basle, Switzerland) with an upper pressure limit of 10 bar and equipped with a Glenco Model 99929 50-ml syringe was used. Peak-area integration was carried out with a Hewlett-Packard Model 3352 B data system.

The unit designed for step gradients and large-volume injections has been described by Erni *et al.*[3].

Chromatography

The chromatographic conditions were as in earlier work[5] with minor modifications. Details are given in the legends of the figures.

RESULTS AND DISCUSSION

Consideration of reaction kinetics

From previous batch investigations[1], the optimal conditions were established to be acetonitrile as most suitable organic system (20%, v/v) in phosphate buffer for the reversed-phase separation and detection, with a reagent solution consisting of 30 mg of Fluram in acetonitrile added at one tenth of the volume of the mobile phase. Under these conditions the reaction kinetics were studied batchwise at room temperature (Fig. 1). It can be seen that the plateau of the reaction is reached after about 50 sec and that a small decrease in fluorescence of *ca.* 5% occurs after about 5 min.

Conversion of these results into dynamic, *i.e.*, actual chromatographic conditions shows good agreement with the batch results. The peak areas reach a plateau after about 50 sec, which would correspond to a capillary volume of 1.4 ml (length 20 m, I.D. 0.3 mm) with the flow conditions mentioned for Fig. 2. With the previous

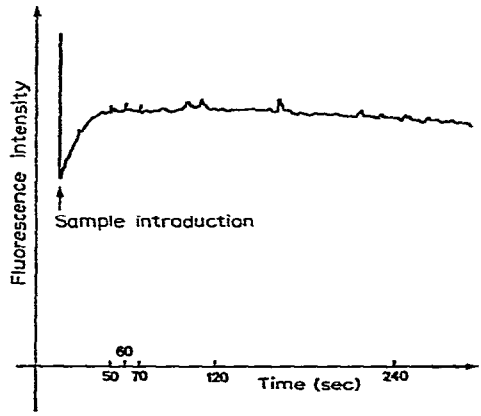

Fig. 1. Investigation of the kinetics of the oxytocine–Fluram reaction. Conditions: 3 ml of acetonitrile–water (20:80, v/v) buffered to pH 7 + 100 μl of oxytocine solution (5 I.U./ml) + 300 μl of Fluram (30 mg per 100 ml of acetonitrile). Apparatus: Perkin-Elmer MPF 3 fluorimeter, λ_{ex} 390 nm, λ_{em} 470 nm.

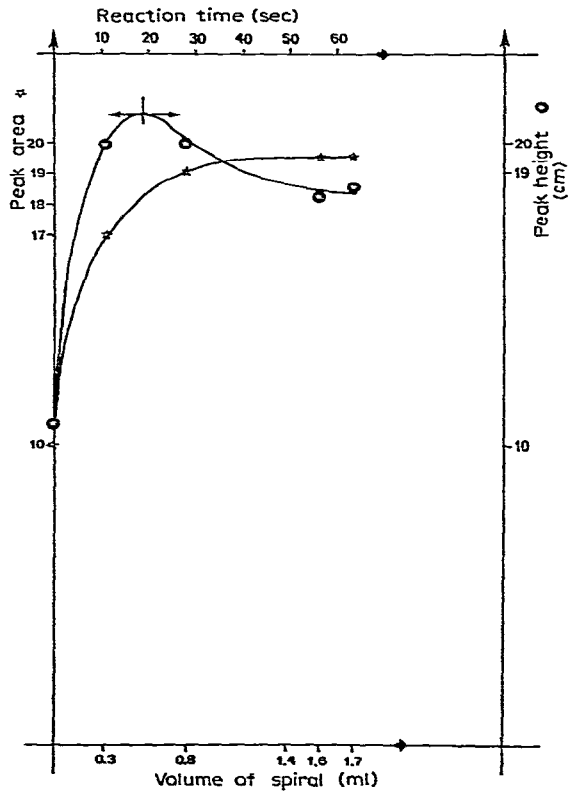

Fig. 2. Influence of length of spiral (reaction volume and time) on the fluorescence signal. Conditions: column, RP 8, 10 μm, length 10 cm, I.D. 0.4 cm; eluent, acetonitrile–water, 20:80 (v/v), pH 7; reagent, Fluram, 30 mg per 100 ml of acetonitrile; flow-rate, 0.15 ml/min; injection, Loop, 100 μl of oxytocine (5 I.U./ml); detection, fluorescence (Aminco fluoromonitor).

experimental conditions only a 0.3-ml reaction volume (≡ 4.4-m capillary length) was used which, according to Fig. 2, corresponds to a 10-sec reaction or to 85% of the maximal fluorescence yield. It is interesting that a five-fold longer reaction time and consequently a five-fold longer reaction spiral will yield only a 15% higher fluorescence.

As a general conclusion, one can say that in post-column derivatization, unlike in pre-column techniques, it is not necessary to have a complete reaction. The most important aspect is the reproducibility of the system, and this has been shown earlier[1] to be better than ±2% relative standard deviation. If peak height is plotted against reaction time or capillary length (Fig. 2), a maximum is observed at about 15–20 sec, followed by a decrease due to increased peak broadening with a longer capillary (this is also shown schematically in Fig. 3).

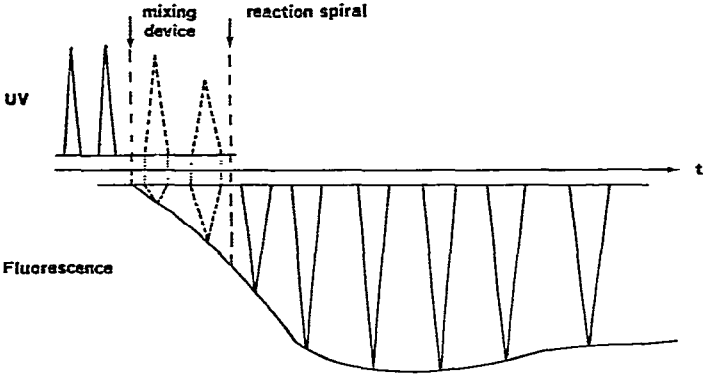

Fig. 3. Schematic representation of peak behaviour in the detection and reaction part.

The maximum (Fig. 2) would correspond to the area of the best signal-to-noise ratio and hence to the optimal reaction conditions.

There are two main reasons for keeping the reaction spiral as short as possible: pressure drop and band broadening. With a capillary of length 20 m and I.D. 0.3 mm, the pressure drop is between 30 and 40 bar at flow-rates of <1.5 ml/min, so that one has to use costly motor-driven syringe pumps.

The peak broadening increases linearly with increasing length of the capillary, by about 7% for each 0.5 ml of capillary volume (flow-rate *ca.* 1.5 ml/min); this has been tested up to 1.8 ml. From these data one can conclude that with the apparatus described earlier[1], reactions with similar kinetics and lasting up to several minutes could be handled reasonably well.

Eventually one would have to cope with a rather large pressure drop. One can also conclude that the band broadening in a regular capillary spiral of a relatively wide spiral diameter (<10 cm) is not too serious. It is important that the spiral should be free of irregularities and bends; the influence of a bend in the spiral is shown in Fig. 4. The peak width of oxytocine increased by 50% after the reaction spiral had been twisted accidentally, even though this minor bend did not result in a significant change in pressure drop.

Other than that, it was observed that the most serious band broadening occurs

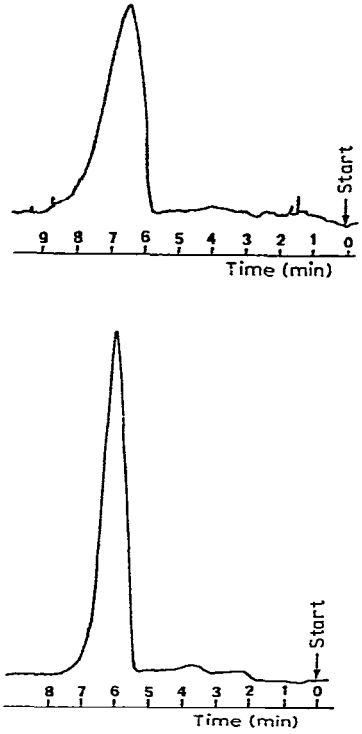

Fig. 4. Influence of capillary irregularity on band broadening. Conditions as in Fig. 2.

in the actual mixing unit. This is shown schematically in Fig. 3. The design of a good mixing unit is therefore of major importance.

Some of the mixing units tested have been briefly mentioned previously[1]. Adding the reagent through a regular T-piece causes layering effects owing to the different densities of the mobile phase and the reagent solution, and the result is considerable band broadening. A design such as that shown in Fig. 5B, whereby the reagent solution enters at a 30° angle against the eluent stream, causes enough turbulence and good radial mixing to reduce band broadening by more than 30%. A design developed by Zech and Voelter[1,6] gives a similar performance and was used for these studies. In another experiment the mixing unit was coupled to a glass bead reactor containing 1- or 0.28-mm diameter beads instead of a spiral (Fig. 5).

The advantage is the lower pressure drop (about 10 times lower than for capillaries for equal reaction volumes). However, they do exhibit additional band broadening of the order of 20% or higher. Some further investigations will have to be carried out on this aspect as the system design shown here does not seem to be optimal.

There are obviously other possibilities for solving problems concerned with reaction times. The kinetics can often be modified by working at a higher temperature, which at the same time will reduce the viscosity of the reaction medium and hence the pressure drop which often increases the fluorescence. In the peptide–Fluram system, however, the fluorescence decreased by *ca.* 1% for each degree of increase in tem-

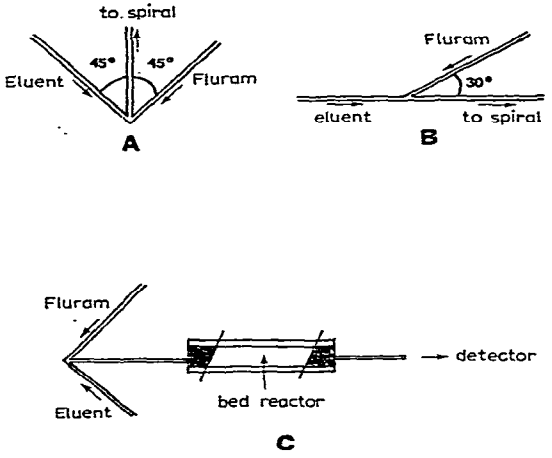

Fig. 5. Different mixing units tested: bed reactor, length 5 cm, 4 mm I.D.; steel column filled with glass beads of 1 mm or 0.28 mm diameter; metal frit at inlet and outlet.

perature (Fig. 6). This is attributed to a more rapid rate of hydrolysis for the Fluram. For this system, room temperature was therefore recommended.

Exposure of the mixing and reaction unit to an ultrasonic bath can sometimes help by increasing the reagent–reactant interaction. For the water–acetonitrile eluent used for the peptides it caused problems because dissolved air was released, which disturbed the detection.

Catalytic and photochemical effects were not investigated.

Coupling with gradient techniques

The use of solvent gradient techniques for resolving difficult separation problems has been widely adopted in HPLC. The use of post-column derivatization following gradient elution of complex samples would also be useful. As the solvent

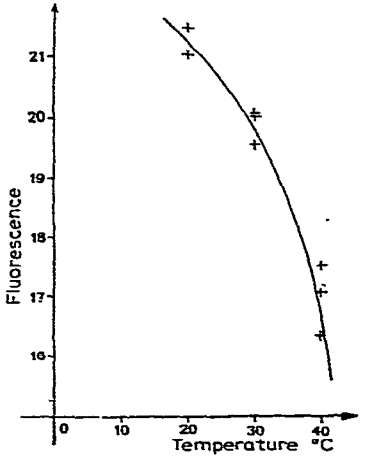

Fig. 6. Influence of reaction temperature on fluorescence signal. Conditions as in Fig. 2. Reaction spiral thermostated to appropriate temperature in a water-bath.

composition of the chromatographic eluent tends to influence most post-column reactions strongly, it is obvious that difficulties can occur when coupling a continuous gradient with post-column derivatization. By using a step gradient such as that described by Erni *et al.*[3] one can eliminate some of these problems, as shown below.

Fig. 7 shows a blank run with Fluram addition using a step gradient programme frequently applied in peptide separations. At acetonitrile concentrations below 20% (v/v) in water the fluorescence background remains constant and then increases for each subsequent step. This agrees well with batch studies made earlier[1].

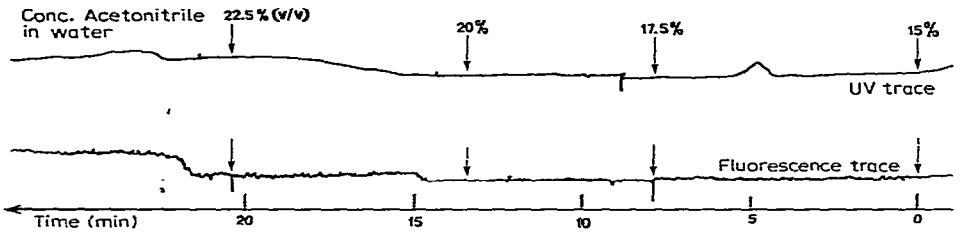

Fig. 7. Baselines for a blank run with step gradient[3] and flluorescence derivatization. Conditions as in Fig. 8. Step gradient: Acetonitrile 15%, 17.5%, 20%, 22.5% (v/v) buffer (pH 7).

As a result of this study, one can say that for each gradient step a good equilibrium is rapidly obtained and no difficulties are expected unless the peaks appear during the short mixing intervals for the transition from one step to another.

Coupling with trace enrichment techniques

The possibilities of concentrating relatively non-polar substances on a reversed-phase column have been studied[2]. Provided that the compounds are contained in a solvent which is less polar than the mobile phase, they will be collected on top of the column in a very narrow zone. A disadvantage of this trace enrichment effect when dealing with complex samples is that many interfering compounds will be equally concentrated and may be difficult to separate in the subsequent chromatographic step. This situation can be improved by using powerful separation systems and/or gradient elution, but in many instances it can be solved by combining trace enrichment with post-column derivatization. Not only will the detection limit be enhanced even further, but also the selectivity can be improved by choosing a more or less selective reagent. This possibility was investigated with the oxytocine–Fluram model and has been briefly mentioned in an earlier paper[2].

The separations were carried out on Merck RP-8 10-μm material, packed in a 25-cm column of I.D. 4 mm and a flow-rate of 1.2 ml/min, and also on a 5-cm column with a flow-rate of 0.2 ml/min. The conditions were maintained such that the overall amount of oxytocine was constant for 20-μl and 1.777-ml injection volumes. Detection was carried out by UV absorption followed by derivatization and fluorescence detection. The results are presented in Table I and Fig. 8.

In the chromatograms in Fig. 8 the advantage of the derivatization technique for eliminating interferences which at high injection volumes obstruct the UV signal for oxytocine can clearly be seen. What actually happens with large injection volumes is a gradual displacement of the injected aqueous sample solution (1.777 ml) con-

TABLE I

INFLUENCE OF INJECTION OF LARGE SAMPLE VOLUMES OF OXYTOCINE

Conditions		*Amount injected*	*Peak area (fluorescence)*	*Peak height*		*Retention time (min)*		$w_{1/2}$ *(sec)* ** *(fluorescence)*
Column length (cm)	*Flow-rate (ml/min)*			*Fluorescence*	*UV*	*Fluorescence**	*UV**	
25	1.21	20 µl (25 I.U./ml)	1773	14.0	4.9	9.5	9.0	17
		1.777 ml (0.28 I.U./ml)	1821	14.0	5.8	11.9	11.4	18
5	0.2	20 µl (25 I.U./ml)	3598	12.6	4.5	14.4	12.0	42
		1.777 ml (0.28 I.U./ml)	3452	12.08	4.3	25.9	23.5	43

* The difference between the values in these two columns is also partly attributed to the volume of the connections of the UV detector to the mixing unit and the mixing unit itself (*ca.* 400 µl for both columns).

** $w_{1/2}$ = peak width measured at half-height.

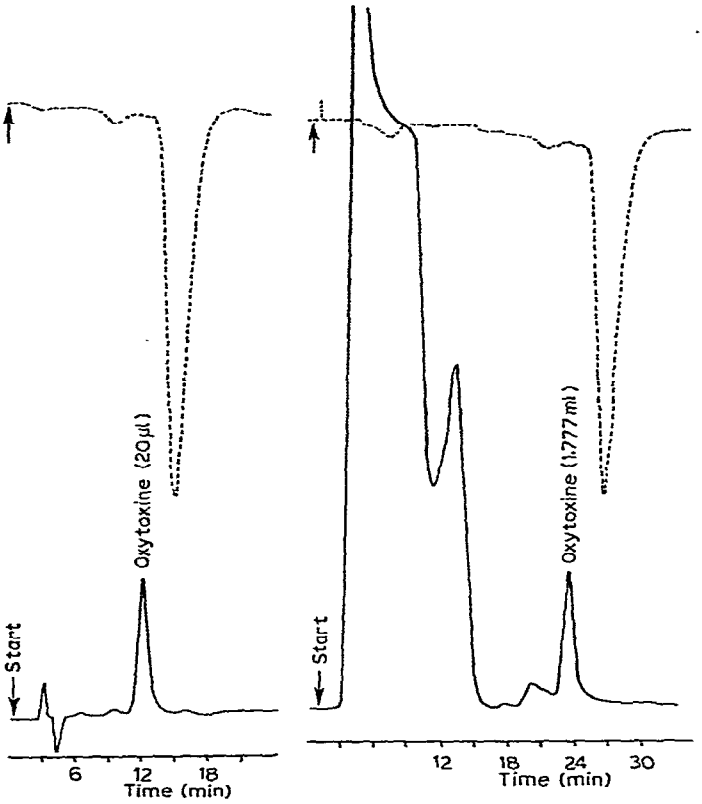

Fig. 8. Chromatograms of oxytocine in buffered solutions at different injection volumes (loop 20 µl and 1.777 ml) on a column of 5-cm length. ———, UV detection at 210 nm prior to derivatization; — — —, fluorescence detection after derivatization with Fluram. Conditions: solution of 30 mg of Fluram in 100 ml of acetonitrile; flow-rate, 0.02 ml/min; detection conditions, λ_{ex} *ca.* 360, λ_{em} *ca.* 470. Chromatographic conditions: Merck RP-8, 5 µm; column, 4 mm I.D.; mobile phase, acetonitrile–water 20:80 (pH 7); flow-rate, 0.2 ml/min; column thermostated at 24°. The absolute amounts of oxytocine injected are the same for both injection volumes (0.5 I.U.).

taining UV-active compounds with good water solubility (*ca.* 9 min; see Fig. 8), followed by re-conditioning of the column and elution and separation of oxytocine from interfering compounds with similar chromatographic properties.

From Table I it can be seen that on the 25-cm column the recovery of the oxytocine after the injection of a large volume is 100% (within experimental error).

CONCLUSIONS

This study has shown the validity of batch results when transferred into dynamic conditions. This can greatly facilitate preliminary studies of new post-column reaction systems. If properly designed capillaries are used as the reaction volume in the post-column mode, very little band broadening occurs and it should be possible to handle reaction times of up to 5 min. The replacement of capillaries by bed reactors has the advantage of a lower pressure drop hence inexpensive reagent

delivery pumps can be used. The design of optimal bed reactors was further investigated. For reactions that take longer than 5 min, the AutoAnalyzer air segmentation principle[7] can be adopted to reduce band broadening. Some studies on this subject are under way with other reactions.

The most critical part of the derivatization method is still the proper design of a mixing device. Most of the band broadening is encountered in improperly designed mixing units, particularly when low flow-rates are used (0.02–0.2 ml/min). This problem has to be further studied. The use of higher temperature or ultrasonic mixing did not bring advantages to the Fluram reaction.

The coupling of step gradient techniques with post-column reactions and/or trace enrichment is feasible and opens up many interesting possibilities for trace analysis in complex matrices. With the examples in this work and using a four-step gradient, the reproducibility of retention times was better than 1.5% relative standard deviation and peak areas integrated electronically were reproducible to $< 4\%$ relative standard deviation.

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REFERENCES

1 R. W. Frei, L. Michel and W. Santi, *J. Chromatogr.*, 126 (1976) 665.
2 P. Schauwecker, R. W. Frei and F. Erni, *J. Chromatogr.*, 136 (1977) 63.
3 F. Erni, R. W. Frei and W. Lindner, *J. Chromatogr.*, 125 (1976) 265.
4 H. R. Linder, H. P. Keller and R. W. Frei, *J. Chromatogr. Sci.*, 14 (1976) 234.
5 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27 and 429.
6 K. Zech and W. Voelter, *Chromatographia*, 8 (1975) 350.
7 L. R. Snyder, *J. Chromatogr.*, 125 (1976) 287.