Journal of Chromatography, 126 (1976) 665–677

CHROM. 9424

POST-COLUMN FLUORESCENCE DERIVATIZATION OF PEPTIDES

PROBLEMS AND POTENTIAL IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Some critical parameters such as the pumping system, mixing devices and detector design in instrumentation for post-column derivatization in high-performance liquid chromatography are discussed. The derivatization was studied with pharmaceutically important nona-peptides containing primary amino groups which react with Fluram® and reaction parameters such as pH, solvent and reagent concentration were investigated.

Both adsorption systems and reversed-phase systems were used to separate the peptides prior to the post-column reaction. Reversed-phase chromatography has the advantage of simpler sample preparation, better reaction control and optimization of solvent conditions. As a result, detection limits of between 5 and 10 ng per injection can be obtained and the reproducibility of the results is better than ±2% (relative standard deviation).

The method has been applied to the analysis of injection solutions (ampoules).

INTRODUCTION

The nona-peptides oxytocin (Ox), lysine^8-vasopressin (Lyp) and ornipressin (Orp) which were investigated in this study are important pharmaceutical substances and some of the techniques used for their physico-chemical determination have been discussed elsewhere[1]. None of these techniques is sufficiently accurate and sensitive to permit the analysis of these peptides and their by-products and degradation products in pharmaceutical formulations except for high-performance liquid chromatography (HPLC)[1,2]. Otherwise bioassay techniques are used to test the activity of these substances.

Direct separation on reversed-phase material and detection at 210–220 nm was shown to be very helpful for many problems in the analysis of pharmaceutical preparations. The limitation of this technique, however, is the critical detection conditions under low-wavelength UV radiation, which renders the quantitation of low dosage forms (<1 international unit) and particularly of the by-products difficult.

In this study, we therefore attempted to investigate the possibilities of carrying out chemical reactions after the chromatographic separation in order to render the peptides fluorescent prior to detection. The advantages and disadvantages of these post-column reaction techniques have been discussed elsewhere[3,4]. An ideal and selective reagent for this purpose has been developed by Udenfriend *et al.*[5]; the reagent Fluram® is selective for primary amino groups and has been used for amino acid analysis in conjunction with HPLC[4,6].

Preliminary results on adsorption systems have shown the potential of this reagent for post-column derivatization of the nona-peptides containing a primary amino group[3]. It was therefore decided to use Fluram as a fluorigenic reagent after reversed-phase separation according to Krummen and Frei[1] and to investigate extensively the various parameters involved in such a post-column reaction.

EXPERIMENTAL

Reagents

The structures of the peptides investigated are listed in Table I. Acetonitrile (Uvasol; Merck, Darmstadt, G.F.R.), tetrahydrofuran (THF; analytical grade, Merck) and doubly distilled water were used as chromatographic solvents.

Buffers for pH 7, 8 and 9 (Titrisol, Merck) and a 0.05 *N* solution of acetic acid were used. The reagent solution consisted of Fluram (Hoffman-La Roche, Nutley, N.J., U.S.A.) at various concentrations in acetonitrile (Uvasol quality). Other solvents used were dioxan, acetone and THF (analytical grade, Merck). The chromatographic supports were packed by slurry techniques[7,8]. Reversed-phase material RP 8 (Merck) of average particle size 10 μm was packed in a column of 25 cm length and 0.3 cm I.D.

For the adsorption system a similar column, packed with silica gel SI 60 (Merck), particle size 10 μm, was used. The exact batch conditions and chromatographic conditions are given in the legends of the appropriate figures and under Results and discussion.

Apparatus

A schematic diagram of the apparatus constructed for this work is given in Fig. 1. A Perkin-Elmer Model MPF 3 spectrofluorimeter was used for recording of spectra and some batch investigations.

RESULTS AND DISCUSSION

The general reaction of the primary amino group with Fluram has been discussed elsewhere[3,5]. An excitation maximum $\lambda_{ex} = 390$ nm and an emission maximum $\lambda_{em} = 470$ nm have been recorded. The spectra were not corrected.

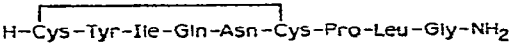
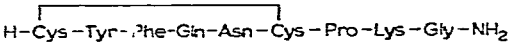
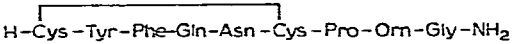
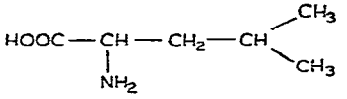
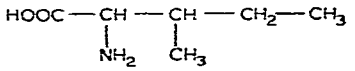
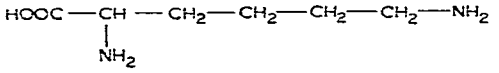
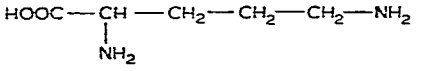
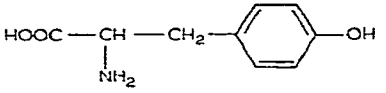
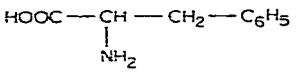
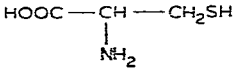
As virtually no information is available on the reaction of nona-peptides with Fluram, extensive *in vitro* studies of the reaction conditions were first undertaken, the effects of pH, solvent and Fluram concentration being investigated.

Effect of the solvent system

From data reported by other workers[9], it is known that the fluorescence of the Fluram derivatives and other derivatives[4] is strongly dependent on the type of organic

TABLE I

STRUCTURES OF THE NONA-PEPTIDES STUDIED

Nona-peptide	*Structure*
Oxytocin	
Lysine^8-vasopressin	
Ornipressin	
Leucine	
Isoleucine	
Lysine	
Ornithine	
Tyrosine	
Phenylalanine	
Cystine	

solvent used in the mobile phase of a chromatographic system or in the reagent solution. In addition to dielectric effects and heavy atom effects (with halogenated solvents), the kinetics of the derivatization reaction and the rate of hydrolysis of the Fluram also account for this dependence. Different solvents that are of interest as reagent solvents or mobile phases in adsorption or reversed-phase chromatography were tested. Fig. 2 shows the influence of organic solvents added in various amounts to an aqueous solution buffered at pH 9. In the batch experiments the volume was maintained constant in order to eliminate dilution effects. In Fig. 3 the net fluorescence (sample minus blank) has been plotted based on the results in Fig. 2.

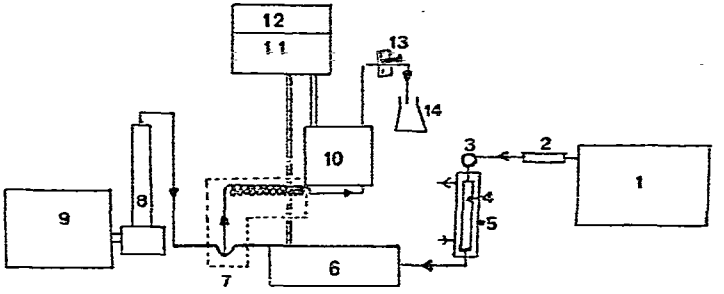


Fig. 1. Schematic diagram of the instrument used. 1 = Haskel pump (Haskel Engineering Co., Burbank, Calif., U.S.A.); 2 = pre-column, Si 60, 30 μm, 5 cm length, to remove impurities in solvent stream; 3 = injection system (loop, 20–150 μl) (Rheodyne Co., Berkeley, Calif., U.S.A.); 4 = analytical column; 5 = mantle for thermostating with water; 6 = PE LC 55 UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.); 7 = mixing device; 8 = Isco pump (Isco, Lincoln, Nebr., U.S.A.); 9 = electronic control for Isco pump; 10 = Aminco fluorescence detector (American Instrument Co., Silver Spring, Mo., U.S.A.); 11 = W + W two-pen recorder (W + W Electronic Inc., Basle, Switzerland); 12 = Hewlett-Packard HP 3352 integration system (Hewlett-Packard, Cupertino, Calif., U.S.A.); 13 = metal clamp for back-pressure; 14 = reagent reservoir (waste).

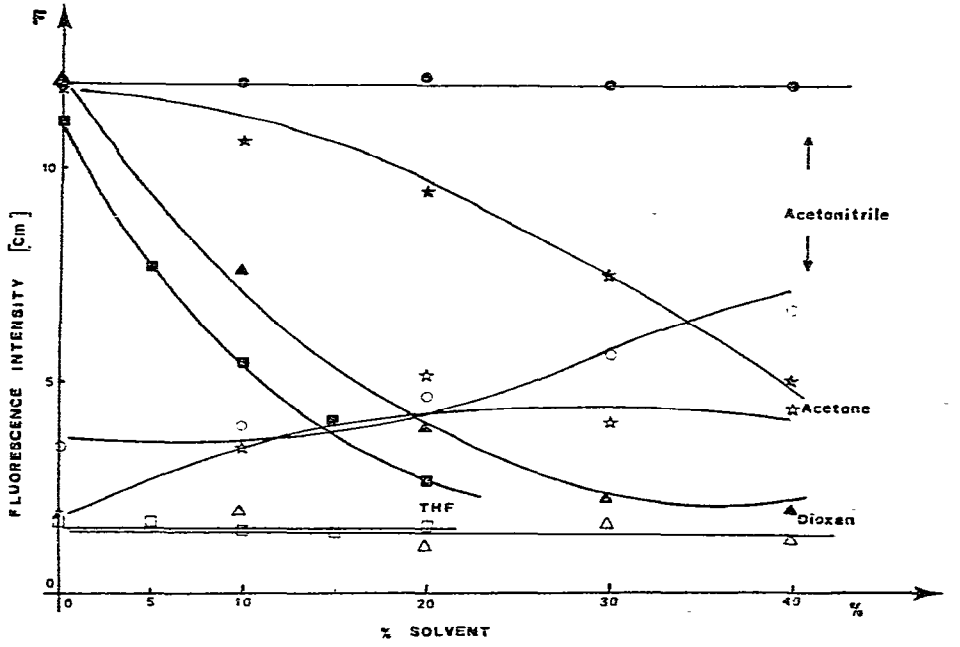


Fig. 2. Influence of different solvents on the fluorescence reaction of oxytocin at pH 9. Condition at 0%: 5 ml of buffer (pH 9) + 4 ml of water + 5 μl of oxytocin (200 I.U./ml) + 1 ml of Fluram (30 mg per 100 ml of acetonitrile). Closed symbols: assay, open symbols: blank. $\lambda_{excitation} = 390$ nm, $\lambda_{emission} = 470$ nm.

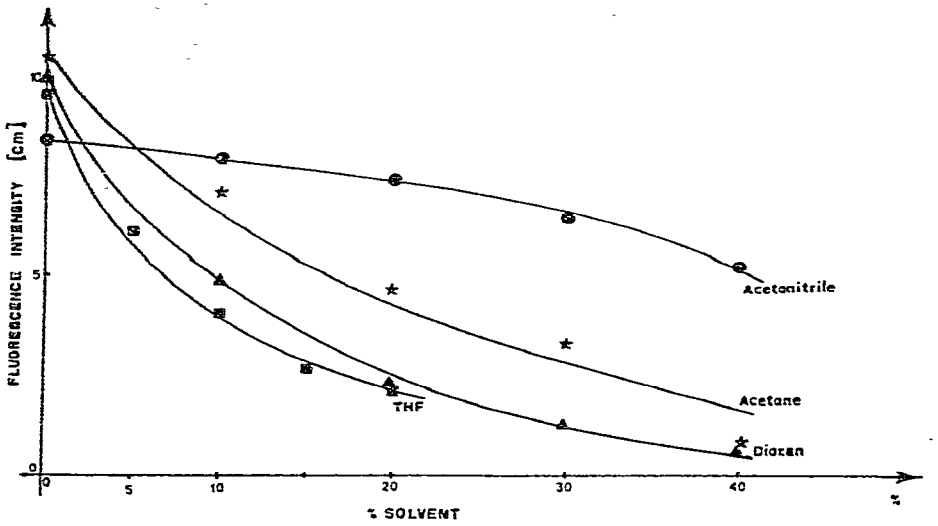


Fig. 3. Influence of different solvents on the fluorescence reaction of oxytocin at pH 9. Conditions as in Fig. 2. Net values (blank subtracted from assays).

In Figs. 2 and 3 a decrease in net fluorescence is observed with increasing proportions of organic solvent. This phenomenon is not simple to explain as it can be due to a mixed effect of solvent polarity (dielectric constant) and hydrolysis of Fluram. It is interesting that the net fluorescence decreases with a decrease in polarity in the order acetonitrile, acetone, dioxan and THF.

From Fig. 2, it is obvious that acetonitrile gives the best net fluorescence despite having also the largest increase in blank fluorescence (Fig. 2). This result is fortunate, as acetonitrile also happens to have very useful solvent properties for reversed-phase separations of these peptides and for the reagent solution[1].

Influence of pH

Studies on the effect of pH were carried out in water–acetonitrile (80:20) as this system was found to be the most promising for chromatographic purposes (see above). Such an investigation in the pH range 5–10 was carried out with Ox under the conditions that would occur in an actual analysis (for details, see legend to Fig. 4). From this study, an optimal pH range between 6 and 8 is obtained when considering the net fluorescence curve in Fig. 4. The effect of the percentage of acetonitrile added to the aqueous phase buffered to pH 7 is shown in Fig. 5. Results at pH 8 were similar or slightly inferior owing to a higher blank, resulting in higher noise (see also Fig. 4).

From these results, one can conclude that one should work at as low an acetonitrile content as possible in the mobile phase and add as little reagent solution as possible.

Effect of Fluram concentration

To study the effect of reagent concentration, solutions of various concentrations of Fluram in acetonitrile (0.1 to 30 mg per 100 ml) were added to aqueous solu-

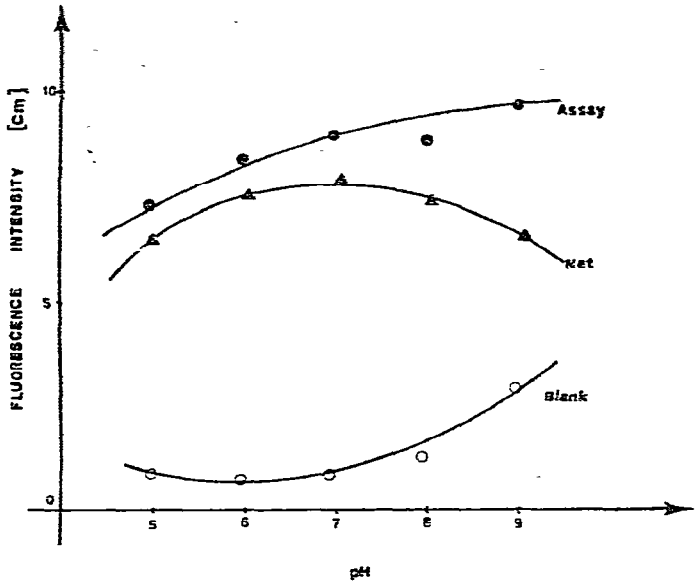


Fig. 4. Influence of pH on the fluorescence reaction with oxytocin. Conditions: phosphate buffer adjusted to appropriate pH with H_3PO_4 or NaOH; 3 ml of buffer mixture–acetonitrile (80:20, v/v) + 100 µl of oxytocin (5 I.U./ml) + 300 µl of Fluram (30 mg per 100 ml of acetonitrile).

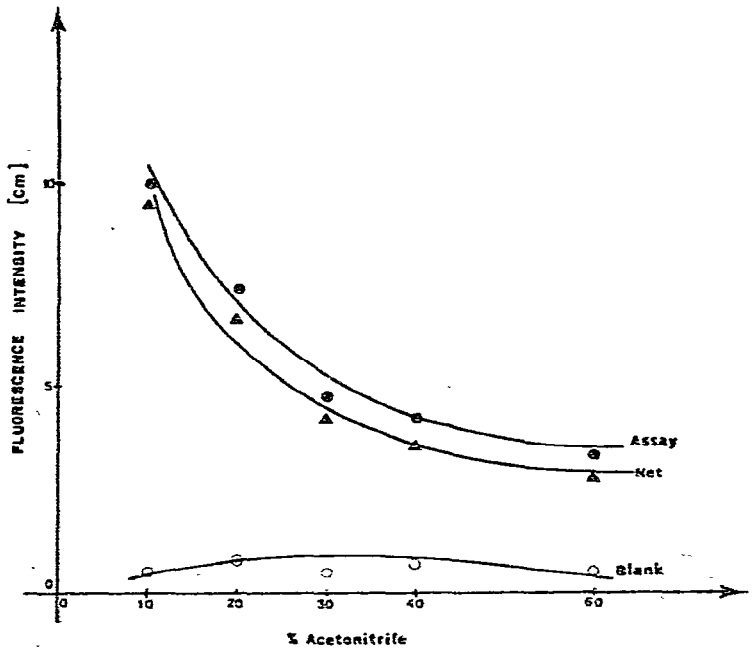


Fig. 5. Influence of acetonitrile concentration on the fluorescence reaction at pH 7 (phosphate buffer) with oxytocin. Conditions as in Fig. 4.

tions of Ox buffered to pH 7 with a phosphate buffer (Fig. 6) and to pH 8 with a borate buffer (Fig. 7). The optimal concentration seems to be reached with about 20 mg of Fluram per 100 ml of acetonitrile at both pH 7 and 8. The net fluorescence yield is inferior at pH 8 owing to the higher blank mentioned previously. The time needed for complete reaction under these conditions and at room temperature is about 50 sec.

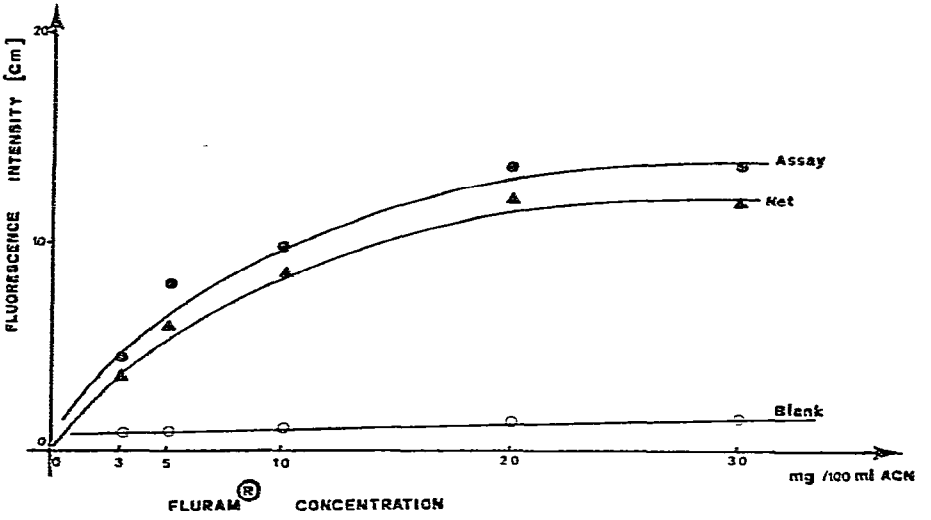


Fig. 6. Influence of Fluram concentration on the fluorescence reaction at pH 7 (phosphate buffer) with oxytocin. Conditions as in Fig. 4.

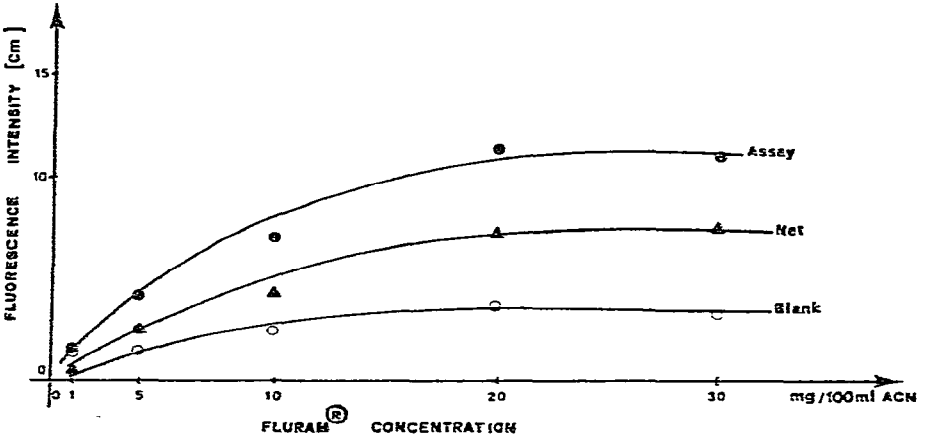


Fig. 7. Influence of Fluram concentration on the fluorescence reaction at pH 8 (borate buffer) with oxytocin. Conditions as in Fig. 4.

Instrument optimization

Mixing devices (Fig. 8). Modification c (Fig. 8) was optimal for the Fluram reaction, resulting in lowest band broadening. The instrument used is shown in Fig. 1. Such an apparatus is still relatively simple to construct from the different parts mentioned in the legend. It is approximately the same for adsorption and reversed-phase

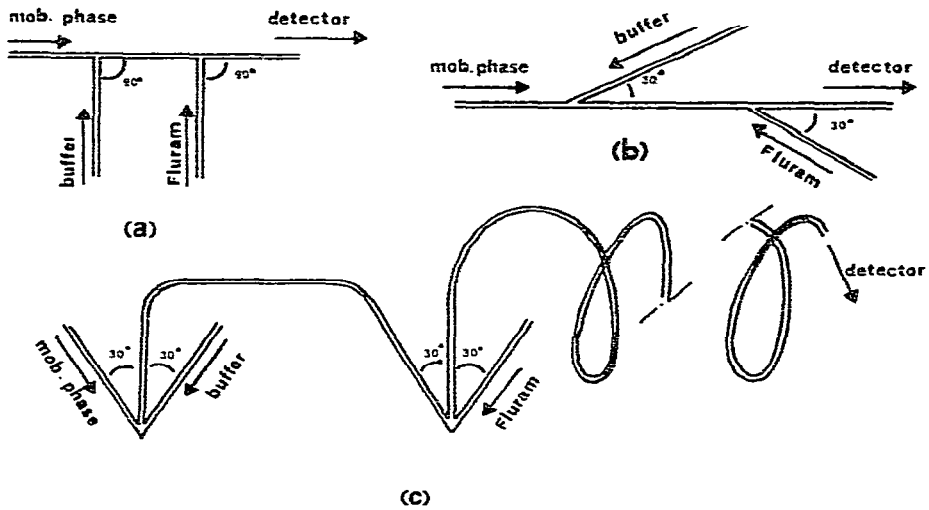


Fig. 8. Mixing devices studied for derivatization after the column. I.D. of tubes = 0.25 mm. Length of spiral (system c) ≈ 4.4 m.

systems except that only one mixing block is required on the latter, because the buffer is part of the mobile phase. Some rules have to be observed in the assembly of the instrument in order to avoid any pitfalls. Some of the more important parts are described below.

Pumping systems. As it has been observed that small variations in the organic solvent–water composition can strongly influence the fluorescence, the blank and the reaction conditions, it is not surprising that constancy of the flow-rate of the chromatographic effluent and of the reagent mixing is of prime importance. Piston pumps, even with good damping systems, are therefore not suitable for this work and pneumatic or syringe-type pumps have to be used. As can be seen in Fig. 1, a Haskel pneumatic amplifier pump and an Isco syringe-type pump were used in the optimal systems. Cheaper constant-displacement devices are currently being investigated.

Fluorescence detectors. Suitable commercial fluorescence detectors are still difficult to find. The modification of a Turner 111 fluorimeter has been described earlier[10] but its drawback is a relatively long response time. The LDC fluoro-monitor has also been used. The best results to date have been obtained with the Aminco fluoro-colorimeter and later the fluoro-monitor with cell volumes varying between 70 and 12 μl. These detectors permit the detection of, for example, quinine sulphate in the higher picogram concentration range. Fluorescence detector design is still far from optimal and as new and more efficient detectors (cell design, energy sources, focusing) become available, the detection limits might be decreased[11].

Chromatography and derivatization

Adsorption systems. The peptide derivatization after separation on an adsorption system has been mentioned briefly in an earlier paper[3]. The following conditions were used for the separation and derivatization of the peptides: mobile phase, THF–0.05 *M* acetic acid (2:1, v/v), flow-rate 0.7 ml/min; column, SI 60, 5 μm, length 25 cm, I.D. 3 mm, packed by a slurry technique[7]; buffer solution, pH 9 (Merck), flow-

rate 1.3 ml/min; Fluram solution, 30 mg per 100 ml acetonitrile, flow-rate 0.4 ml/min. Under these conditions, k' values of 0.4 for Ox, 3.8 for Orp and 4.0 for Lyp were obtained.

The flow-rates indicated are optimal with regard to fluorescence yield and reproducibility of fluorescence signals. The ratio of organic phase to water is about 0.6.

From the previous discussion, it has been shown that the proportion of organic solvent should be kept to a minimum. An increase in the flow-rate of the mobile phase containing THF therefore resulted, as expected, in a rapid decrease in the fluorescence signal.

The same phenomenon is observed with an increase in flow-rate of the reagent solution (acetonitrile). The decrease in signal intensity is of the same magnitude when increasing the flow-rate of Fluram solution from 0.4 to 0.7 ml/min or the eluent flow-rate from 0.3 to 1 ml/min. An increase in the rate of addition of buffer in order to optimize the ratio of water to organic solvent results in a decrease in the signal due to a dilution effect.

The chromatogram for Ox is shown in Fig. 9. Simultaneous detection by UV and, following derivatization, by fluorescence clearly demonstrates the advantage of post-column derivatization. Very little information can be obtained from the UV detection. The sensitivity is good enough (detection limit 40 ng at a signal-to-noise ratio of 3:1) to permit the analysis of injection solutions containing 5 international units (I.U.) of Ox (25 μl injected) and to see by-products at the 1% level (Fig. 10).

In principle, one can say that post-column Fluram derivatization of peptides is possible in an adsorption chromatographic system. There are several disadvantages, however. The need for mixing two solutions into the eluent stream makes the system more complicated and harder to control. The eluent composition containing acetic acid and particularly THF is less favourable for a good fluorescence yield, and finally sampling and removal of matrix interferences are more difficult with adsorption systems. The modification of the mobile phase to alter separation patterns and good reproducibility of retention data are more difficult to achieve.

As a result, the reproducibility of the data is inferior to 3% (relative standard deviation), for example for the analysis of samples as shown in Figs. 9 and 10, and by-products are difficult to separate and quantitate.

Reversed-phase systems. Based on the previous experience with reversed-phase separations of these peptides by Krummen and Frei[1] and based on the batch experiments discussed previously, separations were carried out with water–acetonitrile mixtures (Fig. 5) buffered to pH 7. The instrumental arrangement is depicted in Fig. 1. The formation of air bubbles in the detector can be eliminated by applying a small back-pressure (<5 atm). The optimal conditions with regard to separation and fluorescence signal were water (buffered at pH 7)–acetonitrile in the proportions 80:17.5 for Ox and 80:15 for Orp and Lyp.

From Fig. 6, it can be seen that reagent solutions of concentration ≈20–30 mg per 100 ml of acetonitrile are optimal. This concentration corresponds to a 300–400-fold excess of reagent and corresponds to previously reported data for amino acids[6]. In order to keep the organic solvent content low, this Fluram solution is added at relatively slow flow-rates. Fig. 11 shows the influence of flow-rate of the reagent solution on the detection signal for Lyp injection solution (10 I.U.). In going from a flow-rate of 0.16 to 1.4 ml/min, the signal is reduced 8-fold. A 3 mg per 100 ml con-

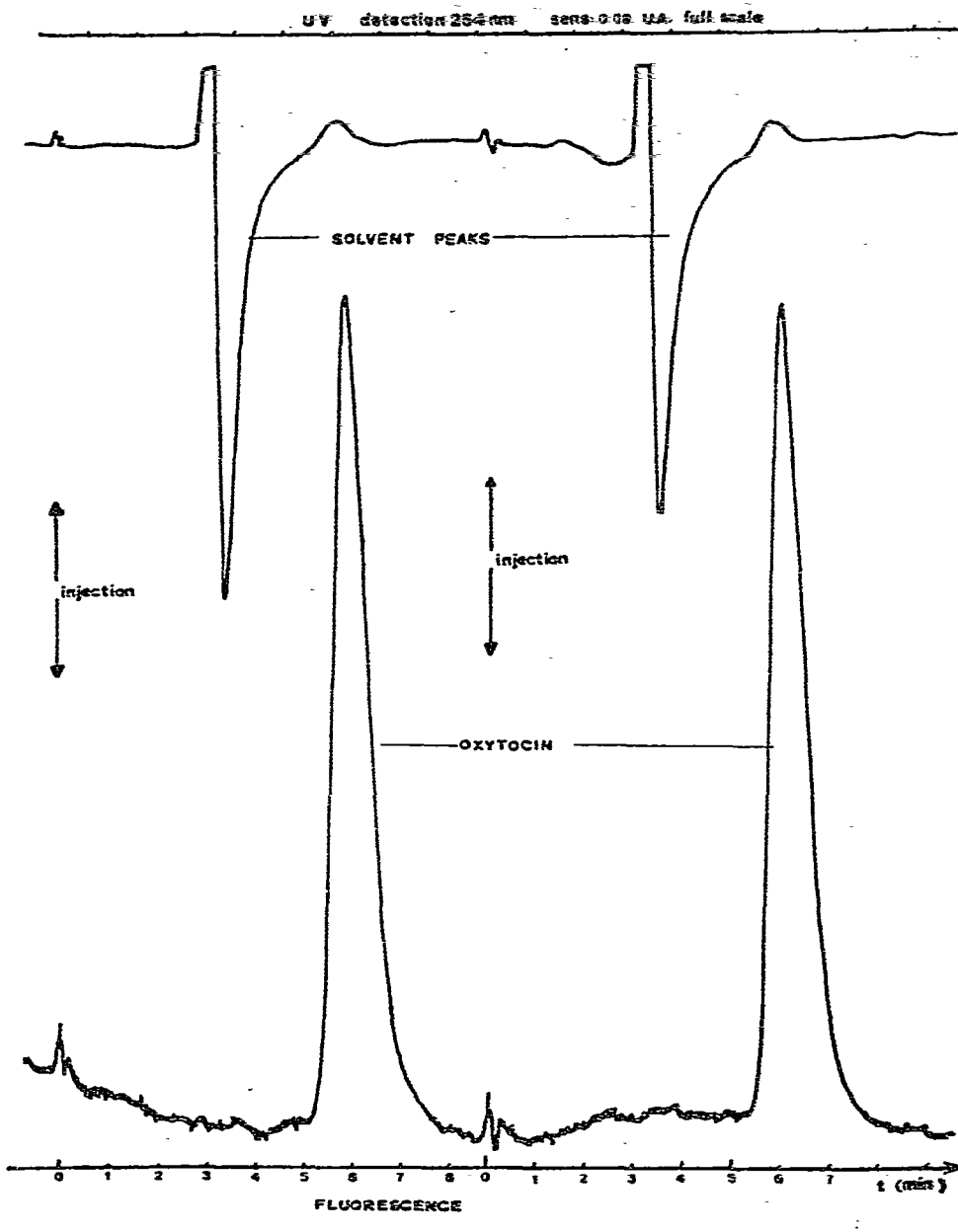


Fig. 9. Chromatogram of oxytocin (0.88 μg per injection of 2 μl). Comparison of UV (254 nm) and fluorescence after mixing with buffer and Fluram. Column, Si 60, 10 μm, I.D. 0.3 cm, length 25 cm; mobile phase, THF–0.05 *M* acetic acid (2:1, v/v), flow-rate 0.28 ml/min, Δp = 46 kg/cm^2; borate buffer (pH 9), flow-rate 0.85 ml/min; Fluram (30 mg per 100 ml of acetonitrile), flow-rate 0.43 ml/min.

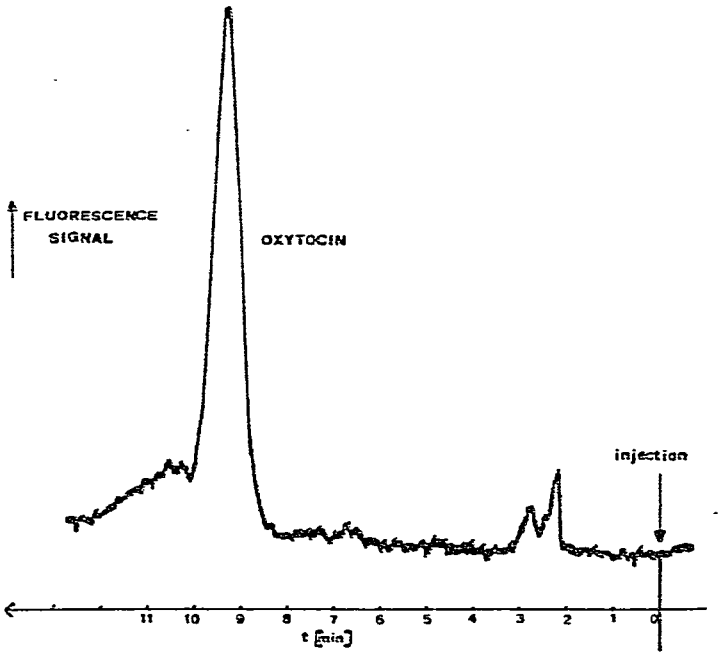


Fig. 10. Chromatogram of 25 μl of Syntocinon injection solution (5 I.U./ml). Stationary phase, Si 60 (Merck), 10 μm, length 25 cm, reaction with Fluram after the column; mobile phase, as in Fig. 9 but flow-rate 0.7 ml/min and $\Delta p = 92$ kg/cm^2; borate buffer (pH 9), flow-rate 1.3 ml/min; Fluram, (30 mg per 100 ml of acetonitrile), flow-rate 0.43 ml/min.

centration was used in this instance, but a similar decrease in signal can be observed for a 30 mg per 100 ml solution.

Fig. 11 also gives a comparison of the sensitivity of UV and fluorescence detection at comparable background noise. For optimal reaction conditions (*i.e.*, a 30 mg per 100 ml Fluram solution) this improvement in sensitivity is about 5-fold (in Fig. 11 it is 2-fold).

The detection limits for the three peptides Orp, Lyp and Ox at a signal-to-noise ratio of 3:1 and using an Aminco fluoro-colorimeter detector with a 30-μl flow cell are 5.5, 6.4 and 9.0 ng per injection, respectively. About a 3-fold improvement can be expected by using the Aminco fluoro-monitor with a 12-μl cell. The reproducibility at concentrations higher than 100 ng per injection is better than $\pm 2\%$ (relative standard deviation).

This reproducibility and sensitivity of the method permit an accurate analysis of these peptides in pharmaceutical formulations, for example injection solutions, down to 1 I.U. as well as the quantification of trace amounts of by-products containing a primary amino group.

A chromatogram for an injection solution of Ox (5 I.U. $\approx$11 μg/ml) is shown in Fig. 12. Comparison with Fig. 10 demonstrates the superior performance with the reversed-phase system.

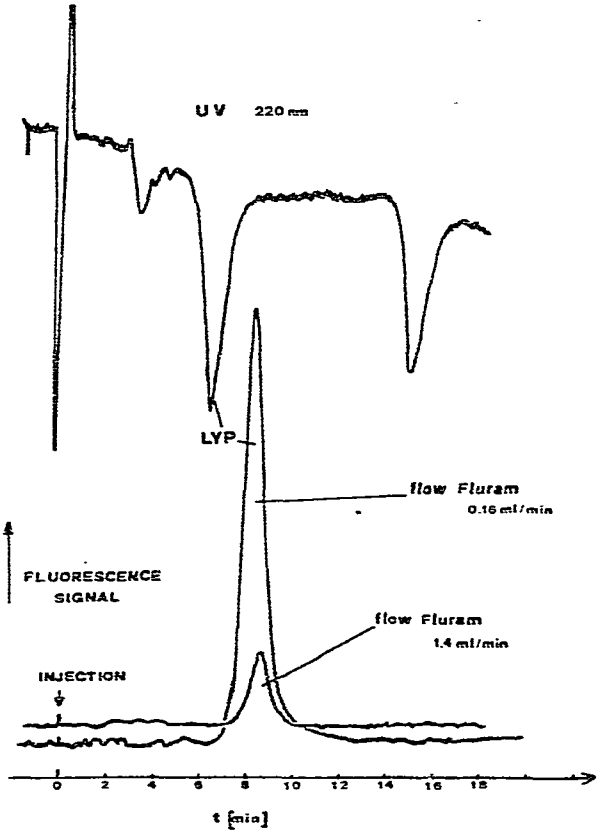


Fig. 11. Chromatogram of 25 μi lysine^8-vasopressin (10 I.U./ml). Influence of the flow-rate of Fluram on the fluorescence detection. Stationary phase, RP 8 (Merck), 10 μm, column I.D. 0.3 mm, length 25 cm; mobile phase, acetonitrile–phosphate buffer (pH 7) (15:80, v/v), flow-rate 1.58 ml/min, Δp = 130 kg/cm^2; Fluram, 3 mg per 100 ml of acetonitrile.

CONCLUSIONS

With proper instrument design and control it is possible to carry out quantitative determinations of the nona-peptides investigated and their by-products in 1 h. This was not possible previously owing to the lack of physico-chemical analytical techniques with sufficiently good selectivity and sensitivity. The bioassay techniques utilized for such systems are tedious, time consuming and costly and their replacement by physico-chemical methods could prove advantageous. The method developed by Krummen and Frei[1] gives results that are in agreement with these; the limitations of their method, however, become apparent when complicated matrices have to be handled or when trace amounts of by-products have to be quantitated.

The difficulties encountered with Fluram post-column reactions in conjunction with adsorption systems suggest that reversed-phase systems should be used whenever possible.

Further studies on the influence of dead volumes and reaction volumes, reaction temperatures, salt and buffer concentrations, and longer residence times due to

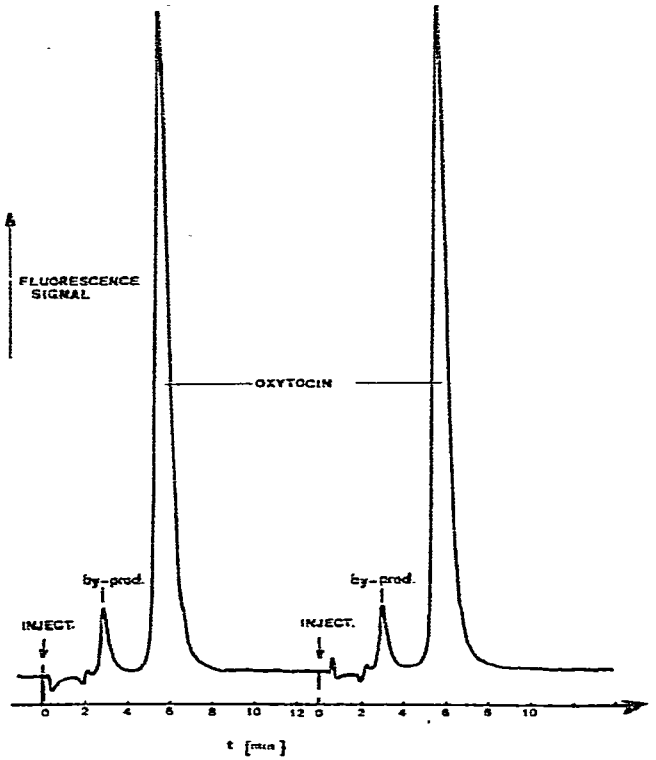


Fig. 12. Chromatogram of 100 μl of Syntocinon injection solution (5 I.U./ml). Stationary phase, RP 8 (Merck), 10 μm, column I.D. 0.3 mm, length 25 cm; mobile phase, phosphate buffer (pH 7)–acetonitrile (80:20, v/v), flow-rate 1.47 ml/min, Δp = 132 kg/cm^2; Fluram, 30 mg per 100 ml of acetonitrile, flow-rate 0.16 ml/min.

lower elution rates and pressures have to be investigated with regard to fluorescence yield and resolution.

Further instrumental improvements can be expected with pumping systems and fluorescence detectors to lower the cost and improve the sensitivity. Some of these studies are currently in progress.

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