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TRACE ENRICHMENT TECHNIQUES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P. SCHAUWECKER

Groupe de Recherche Analytique des Materiaux (GRAM), Département de Chimie, Université de Montréal, Montreal, P.Q. (Canada)

and

R. W. FREI* and F. ERNI

Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., 4002 Basle (Switzerland)

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SUMMARY

A trace enrichment technique is described that permits the use of large injection volumes of up to several hundred millilitres. Some of the parameters were studied with ergot alkaloids. An application of this method to urine samples containing ergot alkaloids and peptides demonstrated the feasibility of the application of this technique to complex matrices.

Considerable enhancement of sensitivity and selectivity can be achieved by coupling trace enrichment with step-gradient elution and with post-column derivatization techniques. The latter effect was studied with a nonapeptide.

The reproducibility of these techniques when applied to quantitation is usually below 4% (relative standard deviation) and is further enhanced (2–3%) by using a timer-controlled valve and a continuous-displacement pumping device for injection of very large volumes (> 2 ml).

INTRODUCTION

The importance of detection limits in trace analysis is clearly recognized. If chromatographic separations are necessary prior to signal generation, then one has to realize that the chromatographic parameters will strongly influence the detection limit attainable, as discussed by Huber *et al.*[1] several years ago. A critical discussion on this subject was also given by Karger *et al.*[2]. Some of the parameters discussed were the selectivity factor (α) and the use of short columns with small diameters, packed with small particles.

Karger *et al.*[2] pointed out that the use of large injection volumes is even more important for improving detection limits because in many instances further concen-

* To whom correspondence should be addressed. Present addres: Department of Analytical Chemistry, Free University of Amsterdam, De Boelelaan 1085, Amsterdam, The Netherlands.

tration of samples becomes difficult owing to sample loss and/or solubility problems. The model presented by Karger *et al.* did not consider the situation of strongly or completely retained compounds under injection conditions but, nevertheless, with their test system (alumina, benzene and pyrene in water-saturated *n*-hexane) they used injection volumes of 100 μl without serious band broadening. Similar results were reported by Strubert[3] with some polynuclear hydrocarbons on silica gel and by Huber *et al.*[1].

Recently, Little and Fallick[4] reported the use of very large injection volumes (up to 200 ml) for relatively non-polar organic compounds on C_{18} reversed-phase materials, in which large concentration effects and only minor band broadening were found. Similar observations were made with peptides[5] and ergot alkaloids[6] injected as aqueous samples on peptide systems. One obviously encounters a concentration effect at the top of the column due to complete retention of the injected components in the injection medium.

It was the aim of this study to explore further this concentration phenomenon, to study injection problems when using such high volumes and to investigate the problems and potential of this approach in conjunction with gradient elution and post-column derivatization. These studies were carried out with the aim of improving detection limits.

EXPERIMENTAL

Reagents

The mobile phase consisted of acetonitrile (Uvasol grade, Merck, Darmstadt, G.F.R.) and pH 7 buffer solution (Titrisol, Merck) in doubly distilled water. Degassing was carried out on a water aspirator vacuum for 1 min while stirring.

Reversed-phase material RP-8, 5 μm (Merck) and Nucleosil C_{18} (Macherey-Nagel, Düren, G.F.R.) were used and packed in 3-, 5- and 10-cm columns according to a previously published dynamic slurry procedure[7]. The ergot alkaloids and the peptides used as model substances were provided by Sandoz (Basle, Switzerland). Fluram (Hoffmann-La Roche, Nutley, N.J., U.S.A.) was used for post-column derivatization.

Apparatus

An HPLC-pump Waters Model M 6000 (Waters Assoc., Milford, Mass., U.S.A.) with a recycle unit was used for some experiments. The apparatus used for injection of very large volumes and for step-gradient work has been described elsewhere[6].

For sample volumes up to 4.38 ml, an Altex loop injector (Altex, Berkeley, Calif., U.S.A.) with exchangeable loops was used. Detection was carried out with a Model L.C. 55 spectrophotometric detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). The apparatus for post-column derivatization has been described elsewhere[8,9].

Deproteination of urine

A procedure according to Graffeo and Karger[10] was adapted, a 10-ml volume of urine being treated with 1 ml of zinc sulphate solution (100 g/l) and 0.5 ml of 0.1 *M* sodium hydroxide solution for precipitation of urinary proteins. After cen-

trifugation, the sample was filtered through a 5-μm Millipore filter (Millipore, Bedford, Mass., U.S.A.).

RESULTS AND DISCUSSION

General investigations

Some basic parameters were investigated, using aqueous solutions of dihydroergocristine mesilate as a model system. Injection volumes were increased from 50 to 4384 μl using loops of different size and up to 165 ml using the low-cost step-gradient device described by Erni *et al.*[6] with timer control for accurate and reproducible dosage. The overall concentration per injection was kept constant by appropriate dilution. The following parameters served for a judgement of the quality of the injection procedure:

$$A_K = \frac{A}{cI}$$

and

$$H_K = \frac{H}{cI}$$

where A_K = corrected peak area, A = measured peak area (integration), H_K = corrected peak height, H = measured peak height (manual), c = concentration and I = injection volume. In addition, w_v was measured and compared [w_v = signal width (μl) measured at 60% of the peak height].

The results are given in Table I. The values for A_K should remain constant even with changes in separation efficiency (α). It can be seen, however, that peak areas decrease significantly by about 10% with injection volumes up to 59 ml and, as found in another experiment, up to 20% for 165 ml in comparison with 50-μl injection volumes. An explanation of this phenomenon is difficult at present.

TABLE I

INFLUENCE OF THE INJECTION VOLUME ON CORRECTED PEAK AREA (A_K) CORRECTED PEAK HEIGHT (H_K) AND PEAK WIDTH (w_v) FOR DIHYDROERGOCRISTINE MESILATE

For chromatographic conditions, see Fig. 1.

Volume injected	*Corrected retention volume** (ml)*	A_K		H_K		w_v (μl)
		Arbitrary units	%*	*Arbitrary units*	%*	
50 μl	12.44	28.0	100	21.1	100	345
343 μl	12.59	26.9	96.1	20.2	95.7	330
4384 μl	13.50	23.9	85.4	17.7	83.9	330
~7 ml (pump)	—	25.0	89.3	15.6	73.9	340
59 ml (pump)	—	25.0	89.3	14.5	68.7	485
165 ml (pump)	—	22.5	80.3	12.7	59.6	546

* Based on the smallest injection volume (50 μl) being taken as 100%.

** Retention volume corrected by the volume injected.

As expected, the corrected peak heights (H_K) experience a significant reduction of up to 40% for 165-ml injection volumes; a noticeable increase in peak width (w_v) was observed only for larger injection volumes (> 59 ml). Table I indicates that there is an increase in corrected retention volumes with increasing injection volume. It is reasonable to assume that the large amounts of water injected on to the column influence the retention properties of the reversed-phase material.

In general, one can say, however, that the changes in chromatographic conditions due to these large injection volumes are not very drastic and warrant the use of such techniques for trace enrichment. The reproducibility of the method is also satisfactory. Multiple injection of 165-ml samples using a timer and a low-volume pump[6] resulted in reproducibilities of between 2% and 3% (relative standard deviation).

Similar results to those given in Table I were also observed for the nonapeptides s udied previously[5].

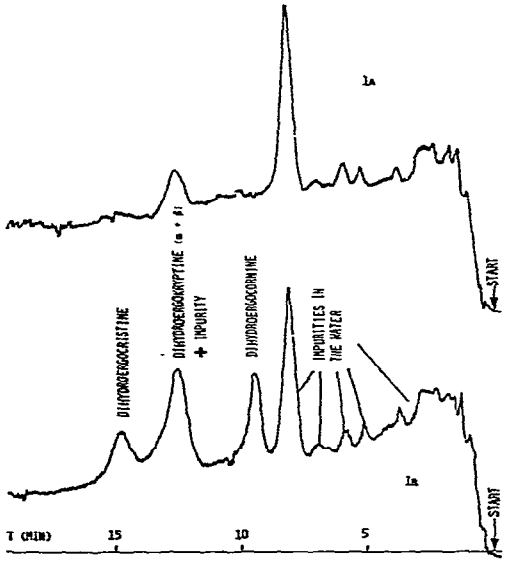

Fig. 1. Chromatogram for dihydroergotoxine mesilate. (A) Injection of 165 ml of distilled water; (B) injection of 165 ml of distilled water containing 6.7 ppb of dihydroergotoxine mesilate. Chromatographic conditions: Nucleosil C_{18}, 5 μm; column, 10 cm × 3 mm I.D.; acetonitrile–0.1 *M* ammonium carbonate solution (40:60); flow-rate, 0.97 ml/min; detection, 280 nm.

The great potential of this technique is demonstrated in Fig. 1. Fig. 1A shows the chromatogram of a blank distilled water sample. The peaks corresponding to some organic impurities, possibly extracted from container walls, were not identified. The use of a very careful cleaning process for the containers resulted in a reduction of the impurities, particularly of the two larger peaks. Another phenomenon observed is the increase in the baseline following the concentration step. Nevertheless, useful chromatograms can be obtained for specific trace components.

Fig. 1B shows the chromatogram obtained from 6.7 ppb of dihydroergotoxine

mesilate. The peak of dihydroergokryptine* overlaps with an impurity peak but the others could well be analyzed quantitatively.

The use of the multi-step gradient for sample introduction proved advantageous owing to the automatic control possibilities. As only the first valve position is used for the trace enrichment step, one can use the subsequent positions for step-gradient elution and hence significantly enhance the benefit of large-volume injections (see also the next section). The advantage of combining large-volume injection with gradient elution has also been noted by other workers[4].

Study of urine samples

Once the feasibility of this approach with water samples had been established, it was of interest to investigate a more complex matrix such as urine, the need for trace enrichment in drug analysis, metabolism studies, etc., in biological fluids being well known. In dealing with urine, it was obvious that smaller samples would cause problems and that isocratic operation would be ineffective. The sample volume was therefore set to an upper limit of 1.78 ml (loop injection). Step-gradient elution[6] was used throughout for the subsequent separation.

The complexity of a blank urine sample (human urine from a healthy male) is demonstrated in Fig. 2. It can be seen that a large number of water-soluble polar substances are eluted during the concentration step, then a series of fractions of compounds of decreasing polarity are gradually eluted by increasing the proportion of acetonitrile or another solvent of lower polarity. During injection, one has to be careful that mobile phase (*i.e.*, acetonitrile), already present in the chromatographic system, does not start the movement of the compounds to be concentrated. The

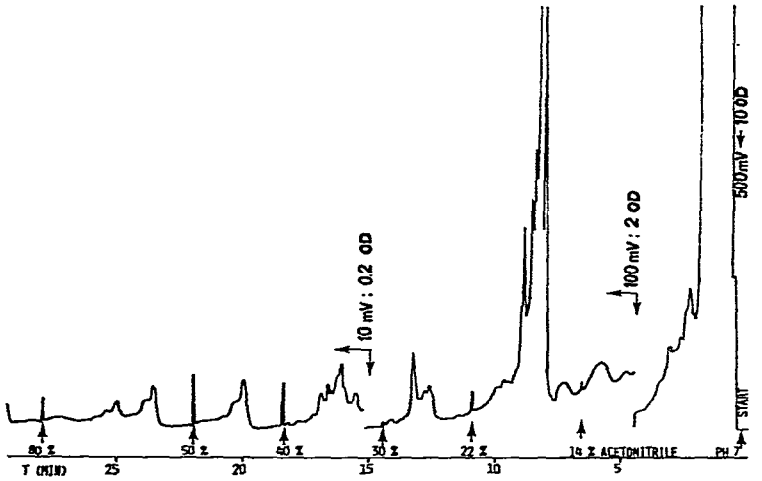

Fig. 2. Chromatogram of an urine blank using a step gradient. Injection: loop, 1.777 ml. Chromatographic conditions: Nucleosil C_{18}, 5 μm; column, 15 cm $\times$ 4 mm I.D.; step gradient with acetonitrile and 0.1 *M* ammonium carbonate solution; flow-rate, 3 ml/min; detection, 237 nm.

* Dihydroergokryptine consists of two structured isomers, dihydro-α-ergokryptine and dihydro-β-ergokryptine[11].

following general rules were therefore developed in order to avoid some of these pitfalls. The first step consists in flushing with water in order to replace acetonitrile remaining from the previous step; then the sample is introduced and another water flush is applied for elution of all water-soluble components in urine. Now one can initiate the step gradient (with our apparatus[6], up to six steps), depending on the problem. The final step consists in flushing the column with acetonitrile for removal of residual non-polar impurities.

The following problems were then investigated.

Dihydroergotoxine mesilate in urine. Dihydroergotoxine mesilate (24 ppm) was first analyzed in an aqueous solution by injecting 1.77 ml by loop. The separation is shown in Fig. 3, and the same separation from a urine sample but under otherwise identical conditions is shown in Fig. 4. A similar separation pattern can be obtained but in the urine sample matrix interferences would render the quantitation of the ergot alkaloids difficult. The solution of such a complex problem should, however, be possible eventually.

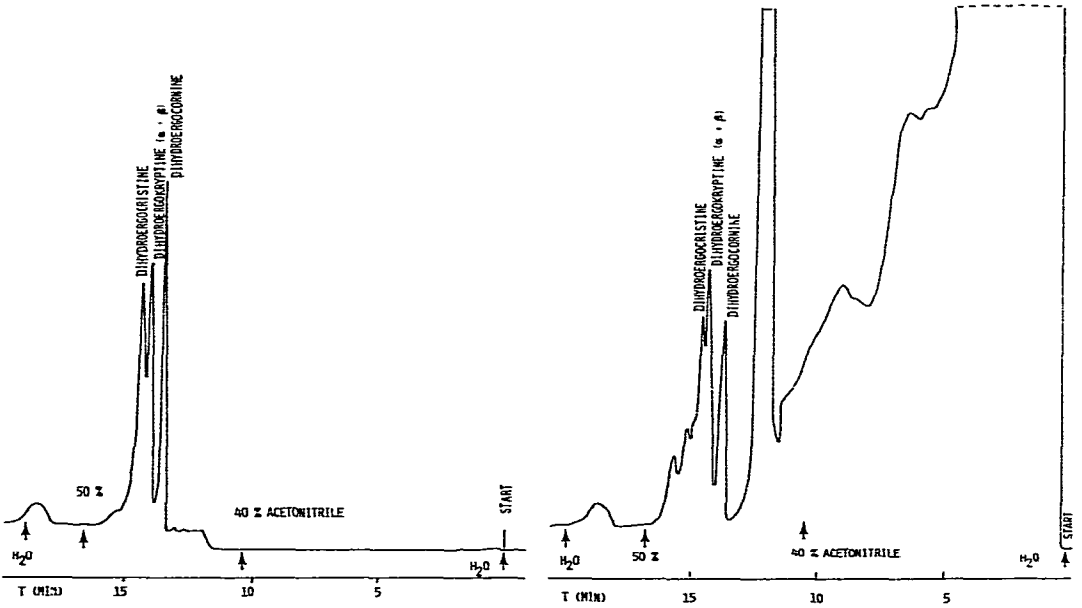

Fig. 3. Chromatogram of a water sample containing 24.2 ppm of dihydroergotoxine mesilate. Conditions as in Fig. 2; detection, 280 nm.

Fig. 4. Chromatogram of a urine sample containing 24.2 ppm of dihydroergotoxine mesilate. Conditions as in Fig. 3.

Dihydroergocristine mesilate in urine. In order to simplify the conditions and to study some quantitative parameters, dihydroergocristine was chosen. The separation of 2.74 ppm of dihydroergocristine mesilate from all other interferences in a urine sample and without using preliminary extraction or urine treatment techniques is shown in Fig. 5. The total separation time until the system was ready for a re-run was 13–14 min. The reproducibility of peak areas of this chromatogram was $<2\%$ (relative standard deviation; $n = 9$). The reproducibility of retention volumes was

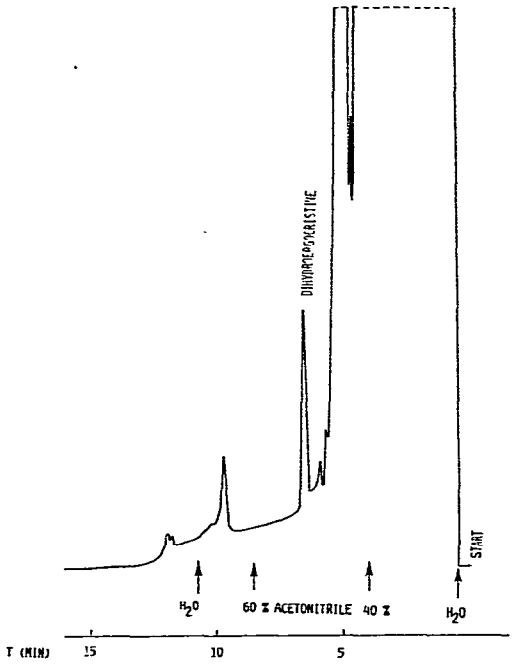

Fig. 5. Chromatogram of 2.74 ppm of dihydroergocristine mesilate in urine. Conditions as in Fig. 3; column temperature, 70°.

excellent. The detection limit under the conditions used (Fig. 5) was 100 ppb (3:1 signal-to-noise ratio), but can easily be improved by further optimization, *i.e.*, the use of a larger injection volume, a different gradient programme and better column efficiency.

Cyclosporine A (peptide) in urine. Cyclosporine is a peptide of molecular weight 1202 and of lower polarity than the ergot alkaloids tested. The detection was carried out at 215 nm. At this wavelength, more serious interferences are encountered in urine. The gradient programme was selected such that a clear isolation of the cyclosporine was possible in an urine sample. The separations were carried out on a column of 3 cm length at a column temperature of 70°. Fig. 6 shows the chromatogram of the appropriate gradient section for a urine blank and Fig. 7 the separation of a sample to which 360 ppb of cyclosporine had been added. For comparison, aqueous samples containing the same concentrations of cyclosporine were run under otherwise identical conditions. These and some other quantitative data are in Table II.

It can be seen that the retention times vary considerably in going from water to urine samples, but nevertheless the agreement between the peak areas for the same concentrations of 18.1 and 0.36 ppm is good. As would be expected, the peak heights vary considerably. Deproteination of the urine results in a loss of cyclosporine which, as a surface-active component, would tend to be partially adsorbed on the precipitate formed.

The reproducibility of the peak areas for quantitation purposes of $<1\%$ (relative standard deviation) seems to be better than the 2–3 % for peak heights ($n = 3$).

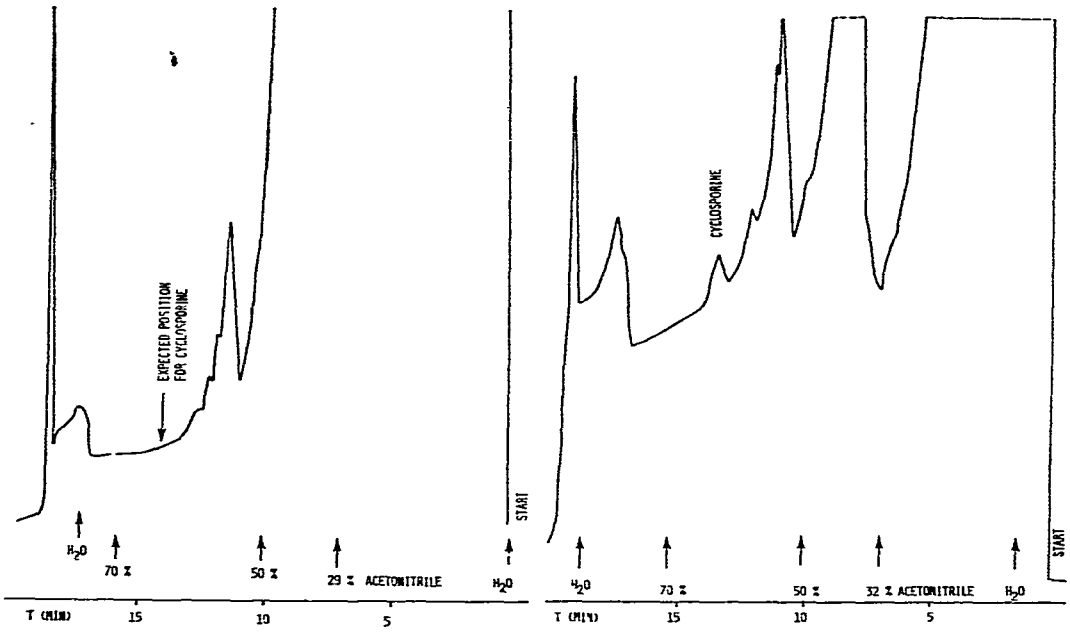

Fig. 6. Chromatogram of a urine blank under conditions suitable for analysis of cyclosporine. Injection: loop, 1.777 ml. Chromatographic conditions: Merck RP-8, 5 μm; column, 3 cm × 4 mm I.D.; step gradient with acetonitrile and 0.1 *M* ammonium carbonate solution; flow-rate, 4 ml/min; detection, 215 nm; column temperature, 70°.

Fig. 7. Chromatogram of 360 ppb of cyclosporine in urine. Conditions as in Fig. 6.

From Fig. 7, it can be concluded that detection limits could easily be in the lower ppb range.

Combination with post-column derivatization

The advantages of post-column derivatization as a means of improving detectability and selectivity have been discussed earlier[8]. An extensive study was carried out with nonapeptides using Fluram as a fluorigenic reagent for derivatization prior to fluorimetric detection[9]. The advantages, *viz.*, lower detection limit in com-

TABLE II

QUANTITATIVE RESULTS FOR TRACE ENRICHMENT OF CYCLOSPORINE A

Sample cyclosporine	*Retention time (min)*	*Peak area (arbitrary units)*	*Peak height (arbitrary units)*
18.1 ppm (H_2O)	6.33 ± 0.02	333.4 ± 0.5	180 ± 4
18.1 ppm (urine)*	11.04 ± 0.04	328.4 ± 1.0	403 ± 3
360 ppb (H_2O)	6.34 ± 0.05	3.32 ± 0.14	2.6
360 ppb (urine)**	13.43 ± 0.03	3.14 ± 0.05	2.2
314 ppb (urine)***	13.22 ± 0.08	1.36 ± 0.32	~1.5

* 55% acetonitrile for separation step.
** Deproteinated before addition of cyclosporine.
*** Deproteinated after addition of cyclosporine.

parison with UV detection and less matrix and other interferences, seemed obvious. A combination of the trace enrichment approach with post-column reaction techniques should be particularly attractive from the selectivity point of view as interferences from undesirable pre-concentrated species can become very serious. The pre-concentration of nonapeptides using large injection volumes has been discussed before[5].

The results of this study with oxytocine are shown in Fig. 8. Comparing the 20-μl injection with a 1.777-ml injection on a 25-cm column in Fig. 8 one can notice very little band broadening with higher injection volumes. The reduction in peak height is to be expected as the corrected retention volume increases with increased sample size as observed earlier (Table I). From Fig. 8 it is apparent on comparing the UV trace with the fluorescence trace, that enhanced selectivity is a major argument for combining the two techniques. Most of the interferences encountered with the larger injection volume are suppressed in the derivatization mode. The improvement in detection limit is at least 50-fold and can be further enhanced by using even larger injection volumes and/or optimized fluorescence detectors[12]. The loss in reproducibility caused by the combination of the two techniques is minor (2–4% relative standard deviation).

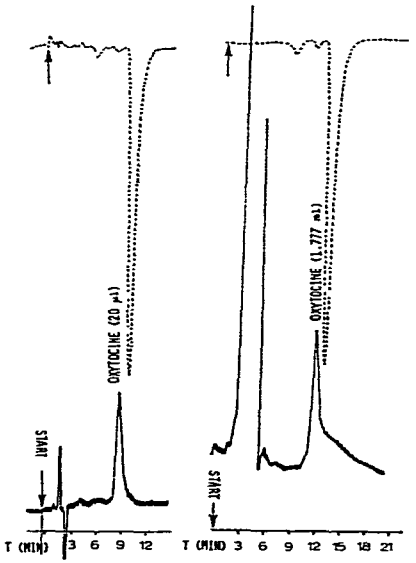

Fig. 8. Chromatograms of oxytocine in buffered solutions with different injection volumes (loop, 20 μl and 1.777 ml) on a column of 25-cm length. (———), UV detection at 210 nm prior to derivatization; (– – –), fluorescence detection after derivatization with Fluram. Conditions: solution of 30 mg Fluram in 100 ml acetonitrile; flow-rate 0.19 ml/min; detection conditions: excitation wavelength, *ca.* 360 nm; emission wavelength, *ca.* 470 nm. Chromatographic conditions: Merck RP-8, 5 μm; column 4 mm I.D.; mobile phase acetonitrile–water (pH 7) (20:80); flow-rate, 1.2 ml/min; column thermostatted at 24°. The absolute concentrations of oxytocine injected were the same for both injection volumes.

CONCLUSIONS AND FURTHER STUDIES

It has been shown that trace enrichment of organic compounds on reversed-phase supports can be a feasible approach to overcoming detection problems in the trace and ultra-trace analysis of different types of matrices. Combining the technique with gradient elution and post-column derivatization techniques will further enhance the sensitivity and add to the selectivity needed for a full exploration of the concentration effect. The reproducibility of all of these methods is sufficiently good for quantitative work.

The range of applications is, of course, not restricted to the few examples studied in this work. The only constraint is that the compounds be fully retained when injected as a solution that is distinctly more polar than the mobile phase used for separation. The following aspects are also of interest and currently under investigation: the use of very short pre-columns or filters for the concentration step and coupling of these concentration devices to an analytical column for separation and quantitation — such a device would have good potential in field work; and the combination of pre-concentration, step-gradient elution and post-column derivatization by adding reagent (possibly automatically controlled) during one or several gradient steps.

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