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QUANTITATIVE ANALYSIS OF NONAPEPTIDES IN PHARMACEUTICAL DOSAGE FORMS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method is described for determining oxytocin, lypressin and other nonapeptides and their by-products in liquid and solid pharmaceutical dosage forms. The use of injection volumes up to 750 μl permits accurate determination of, *e.g.*, oxytocin in injection solutions containing only 1 I.U. (international unit) per ml. Reproducibilities between 1.0 and 1.5% (relative S.D.) have been obtained for liquid dosage forms and up to 3% (relative S.D.) for tablets. The correlation between results obtained by bioassay and by the proposed method is highly significant and suggests the use of HPLC as an alternative technique for stability and content-uniformity tests on dosage forms and concentrates.

INTRODUCTION

The chromatographic behaviour of the nonapeptides oxytocin, demoxytocin, lypressin, ornipressin and felypressin investigated in this study has already been discussed[1]. Our intention was to develop quantitative physico-chemical techniques based on high-performance liquid chromatography (HPLC) as an alternative to bioassay. The use of reversed-phase materials has been most promising, and further studies were carried out with the optimal systems suggested earlier[1].

In this paper, the application of HPLC to liquid and solid pharmaceutical forms has been critically investigated, and the results are compared with data obtained by bioassay.

EXPERIMENTAL

Apparatus

A Hewlett-Packard UFC-1000 liquid chromatograph equipped with an Orlita pump, and a "low-cost" instrument with a Haskel pump (Haskel Engineering, Burbank, Calif., U.S.A.) were used in conjunction with a Perkin-Elmer LC-55 variable-wavelength detector set at 210, 215 or 220 nm. The samples were introduced by

means of a loop injector (Altex, Berkeley, Calif., U.S.A.). A laboratory-data system 3352 B (Hewlett-Packard) was used for integration and data treatment.

Reagents

The reversed-phase column materials Nucleosil C_8 and C_{18} (5 μm and 10 μm average particle size) (Macherey-Nagel, Düren, G.F.R.) and RP 8 (10 μm average particle size) (Merck, Darmstadt, G.F.R.) were used. The 10 μm material was packed by a balanced-density slurry technique[2], in columns of length 25 cm and I.D. 3 or 4 mm. The 5 μm material was packed by a dynamic slurry procedure[3] in columns of length 15 cm and I.D. 4 mm. Uvasol grade acetonitrile (Merck) and doubly distilled water, buffered at pH 7 with phosphate buffer according to Soerensen[4], were used as mobile phase.

Chromatography

Elution was effected isocratically using the previously recommended[1] mobile phase, *i.e.*, water buffered at pH 7–acetonitrile (4:1). Before use, the mobile phase was de-gassed for 5 min in an ultrasonic bath. Separations were carried out at room temperature (thermostatic control was not necessary in an air-conditioned laboratory). Liquid pharmaceutical formulations (34–750 μl) were injected directly; tablets were ground and shaken for 45 min in 4 ml of mobile phase, and after filtration, 120 μl were injected. Further details are given with the appropriate chromatograms. The columns were checked for shrinkage daily before use.

RESULTS AND DISCUSSION

Detection limits and injection volumes

It is well known that detection limits depend on the injection volume, the retention time and on the signal-to-noise ratio (which, in turn, is dependent on the wavelength of detection). In Table I, the detection limits for four peptides are reported for defined conditions.

For the determination of peptides in low-dosage forms (oxytocin ampoules, 1 I.U./ml) and for the investigation of by-products, it was desirable to use an injection volume as high as possible in order to inject samples of low concentration. The influence of a large injection volume on band-broadening (loss of separation efficiency)

TABLE I

DETECTION LIMITS FOR PEPTIDES

Injection volume, 34 μl (aqueous solution); signal-to-noise ratio, 3:1.

Substance	*k' (capacity factor)*	*Detection limit ng/injection*	*Detection wavelength nm*
Oxytocin	3.6	30	210
		40	215
Demoxytocin	9.9	75	215
Ornipressin	1.3	30	215
Lypressin	1.3	30	215

was therefore investigated for samples dissolved in an aqueous solution and for samples dissolved in the mobile phase (see Table II); the band-broadening was measured indirectly as variation in peak height.

It is interesting to observe that, in the first instance, no band-broadening was observed even with a k' value of 1.4, while in the second instance, significant band-broadening occurred at k' values below 5.

TABLE II

EFFECT OF A LARGE INJECTION VOLUME ON PEAK HEIGHT

The amount injected is kept constant for each substance. Column: RP 8 (10 μm), 25 cm × 3 mm.

Substance	k'	*Peak height* (n = 3) (%)*	
		340-µl injection of aqueous solution	*340-µl injection of mobile phase solution*
Lypressin and Ornipressin	1.4	100 ± 3	78 ± 3
Oxytocin	3.9	101 ± 2	94 ± 1
Felypressin	5.6	100 ± 2	98 ± 1
Demoxytocin	10.8	101 ± 1	101 ± 1

* The peak height is expressed as a percentage of that given by a 34-µl injection of a solution of 10-fold higher concentration (taken as 100%).

Thus, there is obviously a concentrating effect at the top of the column, since water does not elute the substances of interest, and elution begins only when the injected water has been displaced by the mobile phase. It is important to use an injection device with a low dead-volume in order to avoid mixing of the sample with mobile phase at the top of the column. The benefits that can be derived from this concentrating effect are best demonstrated by a chromatogram for a dilute injection solution of oxytocin.

By comparing Figs. 1a and 1b, it can be seen that there is no detectable increase in band-broadening if the injection volume is increased from 50 to 750 µl. Thus, by increasing the injection volume, lower detection limits and better accuracy can be achieved without significant loss in separation efficiency. With an injection volume of 750 µl, accurate determination of the active ingredient and an estimate of some of the by-products present in relatively small amounts have been achieved; for the 50-µl sample, the same by-products are barely visible on the chromatogram.

Calibration curves

Calibration curves for peak area *vs.* concentration were rectilinear for the five peptides studied up to concentrations of 100 µg per injection. Correlation coefficients were better than 0.9998 for $f = 4$ degrees of freedom. The curves could be extrapolated through zero with the exception of that for ornipressin; by measuring peak height instead of peak area for ornipressin, this deviation from zero was eliminated.

Analysis of pharmaceutical formulations

The results for the determinations of different peptides and some of their by-products in liquid concentrates, injection solutions and tablets are shown in Table III and in Figs. 2–4.

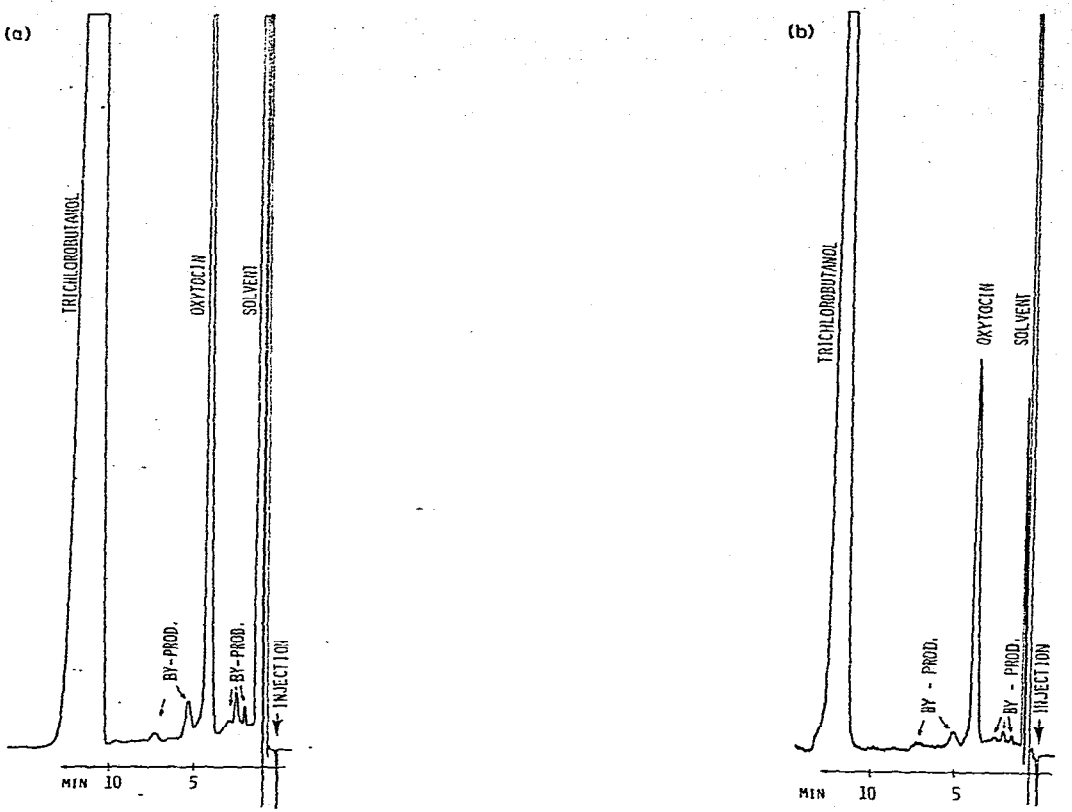


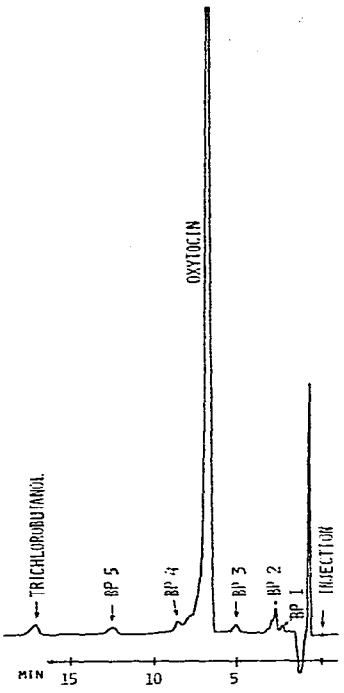
Fig. 1. Chromatograms of oxytocin injection solution (10 I.U./ml) on a column (25 cm × 4 mm) of Nucleosil C_{18} (10 μm). Mobile phase, 20% acetonitrile in phosphate buffer (4/15 *M*) of pH 7; flow-rate 4.0 ml; pressure at column inlet, 200 atm. (a) Injection volume 750 μl, UV monitor at 210 nm (sensitivity 0.1 a.u.f.s.); (b) injection volume 50 μl; UV monitor sensitivity 0.02 a.u.f.s.

As can be seen for the liquid formulations, it is possible to determine the active ingredient with a relative S.D. between 1.0 and 1.5%. The same is true for ampoules containing up to 5 I.U., except that, with these samples, 340 (or even 750) μl were injected (see also Fig. 1). For the determination of demoxytocin in tablets, reproducibility was somewhat inferior (2.9% relative S.D.); this can be attributed to the sample-preparation step.

The simultaneous determination of active ingredient and by-products was possible for ampoules containing 5 or more I.U. of oxytocin per ml; the relative S.D. for the by-products was generally less than 10%.

For ampoules containing less than 5 I.U. of oxytocin per ml, analysis for the by-products was not possible, owing to the interfering solvent peak (a large injection volume was needed).

The preservatives Nipagin and trichlorobutanol are well separated from the active ingredient and, despite their much higher concentration, do not interfere with determination of the nonapeptides (see, especially, Figs. 1 and 3).



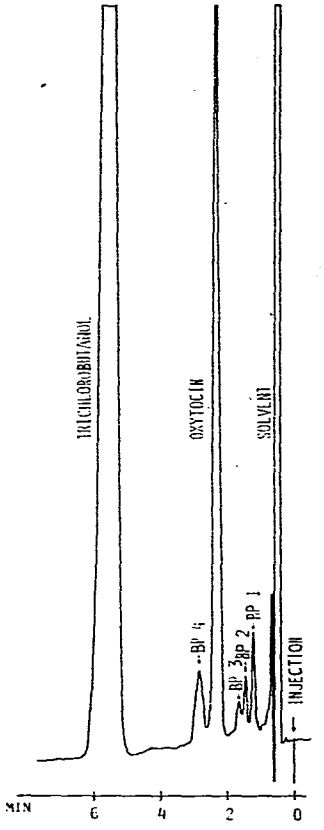


Fig. 2. Chromatogram of 50 μl of oxytocin concentrate (200 I.U./ml) on a column (15 cm × 4 mm) of Nucleosil C_8 (5 μm). Mobile phase, 20% acetonitrile in phosphate buffer (1/15 *M*) of pH 7; flow-rate 1.8 ml/min; pressure at column inlet, 200 atm; UV monitor at 220 nm (sensitivity 0.5 a.u.f.s.). BP = by-product.

Fig. 3. Chromatogram of 120 μl of oxytocin injection solution (10 I.U./ml) on a column (25 cm × 3 mm) of RP 8 (10 μm). Mobile phase, as in Fig. 2; flow-rate 3.0 ml/min; pressure at column inlet, 200 atm; UV monitor at 215 nm (sensitivity 0.04 a.u.f.s.).

Correlation between results by HPLC and bioassay

As already mentioned, the main reason for undertaking this study was to find a physico-chemical method for determining the nonapeptides in pharmaceutical preparations and to compare it with the bioassay techniques which, at present, are the sole means of control. It was therefore imperative to study the correlation between results by these two techniques on the same production batches. Because of the inherently large scatter of bioassay data, samples with different biological activities were analyzed in order to increase the significance of such a comparison. Most of the samples were taken from the stability-testing programme for pharmaceutical dosage forms.

The results are shown in Fig. 5. The correlation between the two techniques ($r = 0.91; f = 17$) was significant at the 99.9% level[5]. The square root of the variation about the regression line was computed as $s_{y \cdot x} = 9.9\%$ (regression of y on x) or

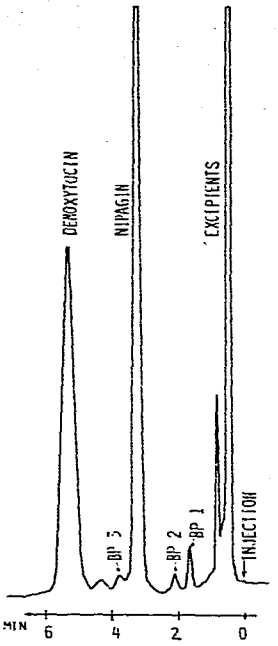
TABLE III

ANALYSIS OF PHARMACEUTICAL FORMULATIONS

S.D. = relative standard deviation for $n = 8$.

	Active ingredient		*By-product**										*Comments*
			1		*2*		*3*		*4*		*5*		
	Concn. (%)	*S.D. (%)*	*Concn. (%)*	*S.D. (%)*	*Concn. (%)*	*S.D. (%)*	*Concn. (%)*	*S.D. (%)*	*Concn. (%)*	*S.D. (%)*	*Concn. (%)*	*S.D. (%)*	
Oxytocin concentrate (200 I.U./ml; 50 µl injected)	100	1.3	0.5	10.8	2.5	3.6	0.9	8.5	0.7	5.9	1.4	11.0	Fig. 2
Oxytocin injection solution (10 I.U./ml; 120 µl injected)	100	1.4	7.5	2.5	3.4	19.6	3.0	6.8	12.4	5.6	–	–	Fig. 3
Ornipressin concentrate (150 I.U./ml; 50 µl injected)	100	1.0	6.8	7.3	6.7	7.2	3.5	8.0	0.5	41.3	–	–	
Demoxytocin tablet (50 I.U./tablet; 120 µl injected)	100	2.9	4.1	8.6	3.1	24.8	3.0	12.5	–	–	–	–	Fig. 4

* The concentration of each by-product is based on that of the active ingredient (taken as 100%); the numbers of the by-products correspond to those in Figs. 2-4.



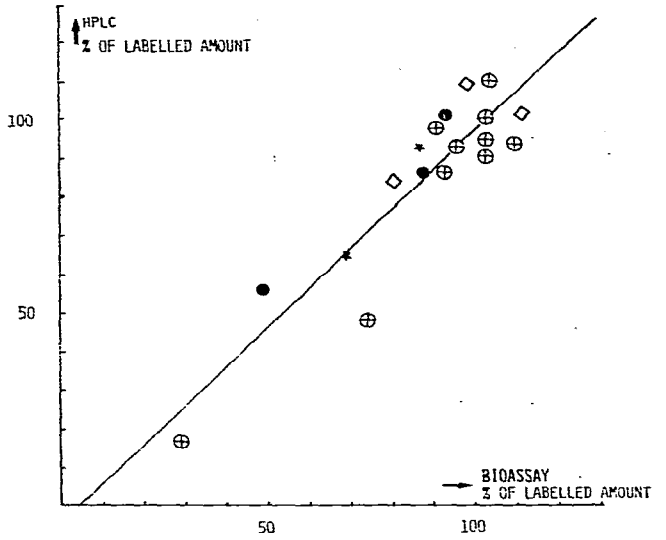


Fig. 4. Chromatogram of demoxytocin in 50-I.U. tablets. Conditions as in Fig. 3.

Fig. 5. Correlation between HPLC and bioassay results. ⊕, oxytocine; ◊ demoxytocin, ● ornipressin, ★ lypressin. Regression line found: (Y = HPLC, X = bioassay), $Y = A + BX$ [$A = -4.5$ (± 21.4); $B = 1.01$ (± 0.24)] (95% confidence interval).

$s_{x \cdot y} = 9.0\%$ (regression of x on y). These values are within the range of variation expected for bioassay tests.

There was also good correlation between the two most commonly used bioassay techniques for oxytocin (rat-uterus contraction, and blood pressure in the cock) and the HPLC values (see Table IV). The large variations that can occur between bioassay techniques are shown for sample No. 5. A significant difference between bioassay and HPLC results was observed only for sample No. 2; in our experience, however, the HPLC result was the more reliable.

CONCLUSIONS

The HPLC method described here is sufficiently reproducible and sensitive to determine quantitatively the active ingredients and their by-products in liquid and solid pharmaceutical formulations. For low-dosage forms (*i.e.*, 1-I.U. injection solutions), it is necessary to use large injection volumes, and up to 750 μl of sample solution have been injected without any loss in resolution. The injection volume could be increased even further in order to analyse samples of lower concentration. In this study, the limit has not yet been reached; the chosen injection volumes were large enough to determine the active ingredient in all the dosage forms investigated.

The use of larger injection volumes (> 100 μl) was proposed by Karger *et al.*[6] to improve detection limits. It was assumed, however, that the samples were dissolved

TABLE IV

COMPARISON OF RESULTS FOR OXYTOCIN BY BIOASSAY AND HPLC

Sample No.	*Sample type*	*Percentage of labelled concn. found by*			
		HPLC		*Bioassay (cock)*	*Bioassay (rat (uterus)*
1	Concentrate 200 I.U./ml (batch a)	99.5			
		100.3			
		100.4	Av. 100.1	101.5	99.8
2	Concentrate 200 I.U./ml (batch b)	116.6			
		119.0			
		120.5	Av. 118.7	108.8	101.5
3	Ampoule 1 I.U./ml (batch c)	120.7			
		122.9			
		122.5	Av. 122.0	122.0	130.3
4	Ampoule 1 I.U./ml (batch d)	122.6			
		124.9	Av. 123.0	131.1	124.6
5	Ampoule 10 I.U./ml	128.0			
		128.9	Av. 128.5	112.1	151.8

in media with a elution power similar to that of the mobile phase. In this study, a concentrating effect was observed when injecting aqueous samples on to reversed-phase columns; this concentrating effect has been used to advantage for the determination of by-products. The same effect has also been used for trace enrichment in river water[8].

In low-dosage forms (< 5 I.U. of oxytocin per ml injection), determination of by-products becomes difficult, owing to the poor signal-to-noise ratio and to interference from the excipients (solvent peak). The use of an alternative method based on post-column derivatization and fluorescence detection[7] might then become necessary.

The excellent agreement between results for a series of samples tested by bioassay and by HPLC suggests that stability and content-uniformity tests could be carried out by HPLC; the time- and cost-saving factors would be appreciable. The long-term stability of the chromatographic system is sufficiently good to render this method useful for routine investigations with automatic injection devices and computer control and data handling.

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