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The use of high-performance liquid chromatography in the quality control of oxytocin, vasopressin and synthetic analogues*

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Abstract: Optimized C_{18} reversed-phase systems for oxytocin, desamino–oxytocin, lysine–vasopressin, ornithine–vasopressin and felypressin with gradient elution are discussed, focussing on precision, selectivity and ruggedness of the methods. Data from collaborative studies are presented, demonstrating the equivalence of high-performance liquid chromatography (HPLC) assays to bioassays.

The findings suggest that HPLC is an excellent alternative to the time-consuming and less reliable animal testing.

Keywords: *Reversed-phase chromatography; oxytocin, vasopressin and analogues; validation data; correlation with bioassays.*

Introduction

High-performance liquid chromatography (HPLC) can replace many of the classical methods (bioassays, amino acid analysis, adsorption chromatography, electrophoresis) nowadays, because of its superior separation power, high speed of analysis and reliability. For small peptides in pharmaceutical preparations, the predominant technique involves reversed-phase HPLC (RPLC), since these columns can be used with aqueous solvents. In RPLC, separation is based on hydrophobic interaction of the amino acid residues with the stationary phase. The mechanism of separation involves a once-only adsorption/desorption onto the column packing material, rather than partition.

As the desorption is controlled by the concentration of the organic modifier, isocratic elution is rarely used and gradients are almost universal.

Many test procedures taken from the literature [1–20] have been evaluated, applied to routine quality control and constantly improved over the years.

Materials and Methods

Table 1 shows the structures of the peptides investigated. The bulk products and the pharmaceutical preparations were aqueous buffer solutions with a suitable preservative.

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Table 1
Structures of the peptides investigated

Demoxytocin	Mps–Tyr–Ile–Gln–Asn–Cys–Pro–Lue–Gly–NH_2
Oxytocin	H–Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2
Lypressin	H–Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Lys–Gly–NH_2
Ornipressin	H–Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Orn–Gly–NH_2
Felypressin	H–Cys–Phe–Phe–Gln–Asn–Cys–Pro–Lys–Gly–NH_2

The concentrations were in the range of 1–200 International Units (IU) which correspond to about 2–400 μg ml^{-1}. Two HPLC systems which selectively separate by-products and degradation products, preservatives and ordinary impurities, were applied.

Tables 2a and 2b give the chromatographic conditions for the analysis of oxytocin and other nonapeptides.

Results and Discussion

Some of these validation data for quantitative analysis of nonapeptides in the bulk substances and in the pharmaceutical dosage forms are given below.

Precision

The precision was calculated from the results of at least seven determinations which were carried out by the same analyst under the same conditions. The relative standard

Table 2a
Chromatographic conditions for oxytocin

Mobile phase	A: 50% acetonitrile B: 0.1 M sodium dihydrogenphosphate
Column	RP-C18, 5 μm, 125 × 4.6 mm, e.g. Shandon Hypersil
Gradient	30% A to 60% A in 30 min
Flow	1 ml min^{-1}
Injection volume	Up to 200 μl
Temperature	Ambient
Detection	UV 220 nm

Table 2b
Chromatographic conditions for the other nonapeptides

Mobile phase	A: 0.02 M tetramethylammonium-hydroxide (TMAH) in 50% acetonitrile, adjusted to a pH 2.5 with *o*-phosphoric acid B: 0.02 M TMAH adjusted to a pH 2.5 with *o*-phosphoric acid
Column	RP-C18, 5 μm, 125 × 4.6 mm, e.g. Shandon Hypersil
Gradient	10% A to 60%A in 30 min
Flow	1 ml min^{-1}
Injection volume	Up to 200 μl
Temperature	60°C
Detection	220 nm

deviations (RSD) of these replicates, carried out with five bulk products and eight preparations, were between 0.2 and 1.3%, demonstrating excellent repeatability.

However, to provide a further indication of reproducibility and in a sense of ruggedness, data were elaborated to characterize day-to-day, laboratory-to-laboratory, analyst-to-analyst and column-to-column variability.

For oxytocin (10 IU ml^{-1}) for example, a 17-h automatic run was carried out on seven independent HPLC instruments with different pumps, samplers, detectors, columns and analysts. The RSDs from a total of 40 injections on each HPLC equipment were <1.3%.

Selectivity

The methods have to be free from significant interference by substances that are known to be present in the product as well as process-related substances, excipients and degradation products.

Figure 1 shows the separation of oxytocin from potential by-products. Further experiments like stress tests (degradation induced by high temperature and addition of trifluoroacetic acid), fresh and old placebos, demonstrated no interference with the active ingredient.

Setting up in-house standards

In establishing an individual in-house standard, usually a batch is taken from the pharmaceutical production and calibrated with reference to the International Standard by means of bioassays and HPLC in several independent laboratories. The weighted

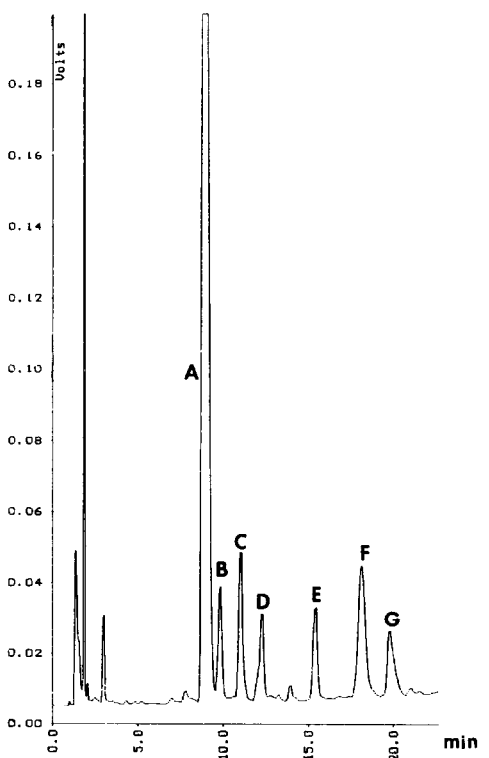

Figure 1
Separation of oxytocin from potential by-products. Key: A, oxytocin; B, [D-Gln^4]-oxytocin; C, [D-Tyr^2]-oxytocin; D, [D-Tyr^2-D-Gln^4]-oxytocin; E, oxytocin-α-dimer; F, oxytocin-β-dimer; G, chlorbutol (preservative).

mean of these determinations is considered when using this in-house standard for bioassays and HPLC determinations.

Correlation of bioassays with HPLC

A comparative study of the different methods (chicken blood pressure, rat uterus and HPLC) was investigated by means of two experiments.

The first experiment — called the linearity test — included eight samples of a dilution series of oxytocin injection from 1 to 8 IU ml^{-1}; 5 IU ml^{-1} was defined as 100%. The data found are given in Tables 3 and 4.

The results of the different methods were plotted against the theoretical values. The parameters from the regression calculation are shown in Table 4. The scatter of the individual bioassay results (standard error of estimates, approx. 7%) was considerably wider than for the HPLC method (<1%).

A second experiment — called the stress experiment — included six samples of oxytocin ampoules (10 IU ml^{-1}), subjected to various heat treatments. The bioassay

Table 3
Linearity test

Test solution	Amount of oxytocin in theory (%)	Amount of oxytocin found (%) Chicken blood pressure method	Rat uterus method	HPLC method
A	26	23	24	29
B	51	66	55	53
C	82	80	85	83
D	92	94	93	93
E	102	97	103	102
F	112	115	127	112
G	122	112	114	124
H	153	143	154	152

Table 4
Parameters calculated from the linearity test

Parameter	Chicken blood	Rat uterus	HPLC
Slope	0.880	0.998	0.965
Intercept	9.63	2.04	4.59
Regression coefficient	0.983	0.988	1.000
Standard error of estimate (%)	7.07	6.92	0.75

Table 5
Stress experiment

Sample	Treatment	Amount of oxytocin found (%) Chicken blood pressure method	Rat uterus method	HPLC method
588C3 A	No	98	110	102
588C3 B	5 min/121°C	98	103	95
588C3 C	20 min/121°C	95	93	81
588C3 D	120 min/121°C	27	28	25
588C3 E	Sample A + D (1:1)	61	59	62
440H9 —	4 years at 30°C	50	50	48

Table 6
Parameters calculated from the stress experiment

Parameter	Chicken blood versus HPLC	Rat uterus versus HPLC
Slope	1.013	1.116
Intercept	0.33	−4.46
Regression coefficient	0.972	0.985

values found were plotted against the HPLC values. Data from the regression calculation are illustrated in Table 6. In this stress experiment the slopes of the straight lines did not differ significantly from the theoretical value. The regression coefficient was 0.97 for chicken blood pressure versus HPLC, and 0.98 for rat uterus versus HPLC. The HPLC method was therefore concluded to be suitable for the assay of oxytocin and equivalent to the biological methods.

Apart from this special comparative study 342 batches of oxytocin injections have been assayed by bioassay and HPLC for release and stability testing. The results obtained by HPLC have been found to be in accordance with the bioassays.

Specification

The author's goal is to meet the pharmacopoeial requirements. However, the precision of bioassays is inadequate for the manufacturer when deciding the potency which should be used as the basis for calculating the quantity of peptide to be incorporated in the final preparation. Therefore, one must use additional methods and apply specifications which are more exacting than those laid down in the Pharmacopoeia. For all the company's bulk products HPLC test procedures are carried out in addition to the bioassays for assays and purity determination. For the finished dosage forms both bioassays and HPLC are currently carried out. For oxytocin injection only we were allowed to switch to a reduced bioassay testing in agreement with some authorities.

Conclusion

The authors conclude from more than 10 years' experience and from the elaborated validation data that it is very well justified to reduce or replace the pharmacopoeial bioassays by HPLC assays for these small peptides, which are nowadays manufactured synthetically.

The information obtained from HPLC is much more accurate and reliable. Only the activity of bulk products measured by bioassays seems to some extent valuable in proving biological effectiveness.

Today it is of public interest to reduce experiments with animals. Especially for routine product control, preference should be given to alternative assays. The authors believe such assays should be incorporated quickly into the Pharmacopoeias.

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