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Animal test or chromatography?

Validated high-performance liquid chromatographic assay as an alternative to the biological assay for ornipressin

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ABSTRACT

Ornipressin is a peptide drug which is usually assayed by a test on live rats. In order to reduce the animal experiments an alternative method was developed which uses gradient high-performance liquid chromatography (HPLC) on reversed-phase. The HPLC method was validated and shown to be selective and precise. Correlation studies were performed on samples of different dosage strengths and on thermally degraded samples, showing good correlation with the results obtained by the biological assay. The HPLC method was applied on various batches of ornipressin in bulk and in pharmaceutical preparations. HPLC is a rapid and inexpensive method which can replace the animal assay. A new quality control concept is proposed which uses HPLC for the analysis of ornipressin in bulk and in pharmaceutical preparations. With this concept animal testing can be reduced by 90%.

INTRODUCTION

Ornipressin is a polypeptide drug which exhibits a vasopressor effect when injected. It is used, *e.g.*, in the case of minor surgery to produce a local ischaemia. The drug is presently assayed by a biological assay which is performed on live rats. In this test, drug solutions are injected into the animals and the blood pressure is monitored. The resulting change in blood pressure is compared with the change produced by a standard preparation. The potency of the drug is expressed in terms of I.U. (approximately 2.4 μg of peptide corresponds to 1 I.U.). The bioassay is very time-consuming and expensive, and shows an assay variation which is higher than that of physicochemical assays. Such assay variations are often inadequate for potency estimations of bulk substance as any analytical inaccuracy on the bulk material automatically leads to a systematic deviation in the final product.

In order to improve the assay precision and to reduce the animal experiments alternative assay methods were evaluated. Reversed-phase high-performance liquid chromatography (HPLC) is a technique which is nowadays predominant in the field of peptide analysis [1–11], so this methodology was applied for the quantitative analysis of ornipressin in bulk material and pharmaceutical preparations.

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EXPERIMENTAL

Chemicals and equipment

Ornipressin and the other nonapeptides were produced by Sandoz Pharma (Basle, Switzerland). The concentrations of the solutions ranged from approximately 2 to 10 I.U./ml. The peptides were about 95% pure. Acetonitrile and water were of HPLC grade, all other chemicals were of analytical grade. An HPLC system was used equipped for automated sample injection, gradient elution, column thermostatisation, UV detection and automated peak integration.

HPLC assay for ornipressin

Gradient chromatography was used for the separation and quantification of ornipressin [11]. Mobile phase A consisted of a 0.02 *M* solution of tetramethylammonium hydroxide in water, mobile phase B was a 0.02 *M* solution of tetramethylammonium hydroxide in a mixture of water–acetonitrile (50:50, v/v). The pH of both mobile phases were adjusted to pH 2.5 with concentrated orthophosphoric acid. The mobile phases were degassed prior to use.

As stationary phase, columns of 125 × 4.6 mm or 100 × 4.6 mm filled with octadecylsilanised silica gel of 5 μm mean particle size were used, *e.g.* Shandon Hypersil ODS or Spheri 5 from Brownlee Labs. (Santa Clara, CA, USA). Injection volume was 100 μl, flow-rate was 1.0 ml/min, a linear gradient was run from 10 to 60% of mobile phase B in 25 min. Column temperature was set to 60°C if not stated otherwise. Detection was by UV at 220 nm.

Biological assay for ornipressin

The biological assays for ornipressin were done in analogy to the biological assay for lypressin described in The British Pharmacopoeia [12]. As reference, an internal reference standard of ornipressin was used which had previously been standardised against the first international standard for lysine-vasopressin.

Collaborative study

A collaborative study was performed to compare and to validate the two methods with respect to accuracy, precision and laboratory-to-laboratory reproducibility. The study involved three different laboratories on the biological assay and three different laboratories on the HPLC assay. Each laboratory analysed each sample in duplicate or triplicate.

Solutions of ornipressin in the dose range 2.75–8.25 I.U./ml were used for a linearity test. The solutions were prepared in aqueous buffer of pH 4, sterilised by filtration and sealed in ampoules. For a degradation test ampoules of 5 I.U./ml were taken and artificially degraded by exposure to a temperature of 50°C for up to 2 months.

Internal reference standard

Ampoules containing injection material from a pharmaceutical production batch were used for this purpose. The reference standard was calibrated against the first international standard for lysine-vasopressin by means of biological assays in three different laboratories. The mean of the assay results was taken as the potency

for the internal reference standard. This standard was used for all further calibrations by HPLC and bioassay.

RESULTS AND DISCUSSION

Reversed-phase HPLC of ornipressin and some structure-related peptides

The selectivity of the separation system was investigated by studying the retention behavior of ornipressin and four other nonapeptides. The nonapeptides all had structures very similar to that of ornipressin and most of them differ in one amino acid only. The sequences are given in Table I. The samples also contained methyl 4-hydroxybenzoate and chlorobutanol which served as preservatives in the bulk solutions.

TABLE I

STRUCTURES OF THE PEPTIDES INVESTIGATED

Peptide	Structure
Demoxytocin	Mps-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-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

With a mobile phase pH of 2.5 and a temperature of 80°C three of the five nonapeptides are baseline-separated, the peptide pair lypressin/ornipressin being separated with a resolution of $R_s = 0.9$. A chromatogram of the nonapeptide separation is shown in Fig. 1. A very important parameter for selectivity was found to be the temperature. At room temperature the peptide pairs lypressin/ornipressin and felypressin/oxytocin are not separated. Separation improves with increasing temperature and at 80°C a resolution of $R_s = 7.0$ is achieved for the felypressin/oxytocin pair and the lypressin/ornipressin pair is partially separated. Separation is also influenced by the pH of the mobile phase. The critical peptide pair lypressin/ornipressin can only be resolved at very low pH or at high pH: $R_s = 0.9$ at pH 2 and $R_s = 1.4$ at pH 9.

The HPLC method was validated for quantitative determination of ornipressin in bulk and in pharmaceutical preparations. Precision, accuracy, linearity and sample stability were studied. A good linearity was found in the tested range 2–10 I.U./ml: the slope of the linear regression line was 0.94 ± 0.04, the intercept was 0.15 ± 0.24. The correlation coefficient was calculated as $r = 0.9997$. The 95% confidence interval

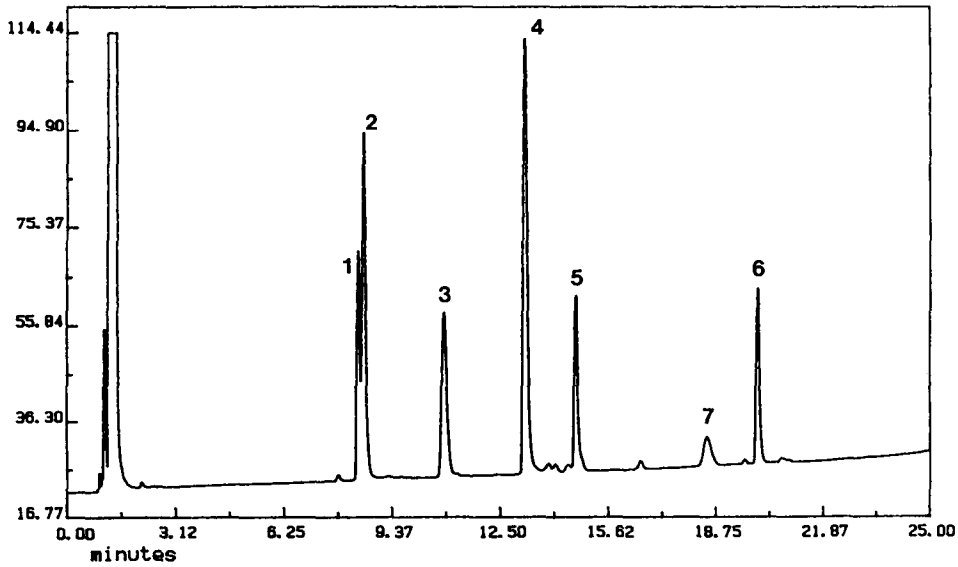

Fig. 1. Separation of various nonapeptides on reversed-phase HPLC. Peaks: 1 = lypressin; 2 = ornipressin; 3 = methyl 4-hydroxybenzoate (preservative); 4 = felypressin; 5 = oxytocin; 6 = demoxytocin; 7 = chlorobutanol (preservative). Chromatographic conditions: stationary phase, Spheri 5 ODS-MP 5 μm, 100 × 4.6 mm from Brownlee Labs; mobile phase A: 0.02 *M* tetramethylammonium hydroxide in water, adjusted to pH 2.5 with orthophosphoric acid; mobile phase B: 0.02 *M* tetramethylammonium hydroxide in a mixture of water–acetonitrile (50:50, v/v), adjusted to pH 2.5 with orthophosphoric acid; gradient programme: 10–60% B in 25 min; flow-rate 1.0 ml/min; injection volume: 20 μl; column temperature: 80°C; detection: UV at 220 nm; concentration of nonapeptides: 2–5 I.U./ml each.

of the linear regression calculation includes the origin, thus proving the accuracy of the method. The assay precision was tested with seven consecutive assays in one laboratory and was found to be S_{rel} = 0.48%. Sample stability was tested over a period of 24 h in order to validate the method for use with autosampling systems. Sample solutions were found to be stable at room temperature over the whole testing period and thus samples can be analysed in overnight runs.

Correlation between HPLC and biological assay

The correlation between the two methods was established in a collaborative study where a number of samples were analysed in various laboratories by both techniques. In one experiment, termed the linearity test, samples of various dosages were analysed to compare the two methods with respect to precision and accuracy in a certain dosage range. The results found are given in Table II. A linear regression equation and 95% confidence limits were calculated to compare the two methods. The slope of the regression line was found to be 0.99 ± 0.04 which includes the theoretical value of 1.00; the intercept was found to be −0.11 ± 0.20, which includes the theoretical value of zero. The correlation coefficient was 0.9998, which is close to the theoretical value of 1.0000. All these parameters of the regression equation prove that the HPLC results are in good agreement with the biological results. Consequent-

TABLE II

LINEARITY TEST

Samples of different dosage strengths (2.75, 4.40, 5.50, 6.60 and 8.25 I.U./ml) were analysed with both assay methods in a collaborative study.

Laboratory	Found (I.U./ml)				
	2.75	4.40	5.50	6.60	8.25
HPLC laboratory 1	2.76	4.35	5.46	6.54	8.11
HPLC laboratory 2	2.68	4.18	5.38	6.40	7.80
HPLC laboratory 3	2.75	4.21	5.35	6.22	7.73
HPLC mean	2.73	4.25	5.40	6.39	7.88
Bioassay laboratory 4	2.65	4.04	5.26	6.20	7.61
Bioassay laboratory 5	2.53	4.04	5.07	6.17	7.80
Bioassay laboratory 6	2.64	4.20	5.16	6.31	7.67
Bioassay mean	2.61	4.09	5.16	6.23	7.69
Standard deviation (%)					
HPLC S_{rel}	1.60	2.14	1.05	2.51	2.75
Bioassay S_{rel}	2.55	2.26	1.84	1.18	1.26

ly HPLC can be used as alternative to the bioassay for the estimation of potency of ornipressin.

In a second experiment, termed the degradation test, we tested whether both methods also correlate when thermally degraded samples are analyzed. This is a prerequisite if the HPLC is to be used for stability testing. The results of this experiment are given in Table III. Two of the three laboratories, No. 4 and No. 5, showed a

TABLE III

DEGRADATION TEST

Thermally degraded samples (without heat treatment and with heat treatment at 50°C for 7, 14, 30 and 60 days) were analysed with both assay methods in a collaborative study.

Laboratory	Found (I.U./ml)				
	None	7	14	30	60
HPLC laboratory 1	5.37	5.07	4.86	4.48	3.90
HPLC laboratory 2	5.33	5.14	5.02	4.57	3.90
HPLC laboratory 3	5.39	5.25	5.03	4.63	3.92
HPLC mean	5.36	5.15	4.97	4.56	3.91
Bioassay laboratory 4	4.90	4.91	5.26	5.00	4.38
Bioassay laboratory 5	5.25	5.23	5.18	4.98	3.97
Bioassay laboratory 6	5.30	4.99	4.71	4.41	3.65
Bioassay mean	5.15	5.04	5.05	4.80	4.00
Standard deviation (%)					
HPLC S_{rel}	0.56	1.76	1.98	1.60	0.37
Bioassay S_{rel}	4.23	3.32	5.94	7.05	9.15

trend to higher values for samples which had been heat-treated for 2 weeks or longer, whereas laboratory 6 obtained results below those of the HPLC laboratories. A reason for these differences could not be found and it is presumed that it is caused by the normal scatter of the biological assay. Calculation of the linear regression and the 95% confidence limits resulted in a slope of 0.78 ± 0.41, an intercept of 1.06 ± 1.96 and a correlation coefficient of 0.9621. The confidence limits for slope and intercept include the theoretical values of 1.00 and of zero, respectively. There is no statistically significant difference between the two assay methods. However, HPLC seems to be more precise in analysing thermally degraded samples and, as a consequence, HPLC would be the preferred technique for stability testing of ornipressin.

The data in the Tables II and III also show the laboratory-to-laboratory variation of the two methods. Standard deviations for HPLC are up to 2.75%, those for the bioassay are up to approximately 9%. HPLC is more reproducible and easier to transfer to other laboratories.

With respect to method correlation it should be noted that only samples of highly purified ornipressin were used for the study. If there were further compounds with a vasopressor effect present in the test solution, *e.g.* active by-products from the synthesis, the biological assay would reflect the overall activity of the sample without distinguishing between the activity of ornipressin and the activity of the by-products and in this case a correlation between HPLC and biological assay would not longer be given.

TABLE IV

RESULTS OF ORNIPRESSIN BULK SOLUTIONS AND ORNIPRESSIN INJECTIONS ANALYSED BY BIOASSAY AND BY HPLC

Results given as a percentage of the declared activity.

Sample	Bioassay result (%)		HPLC result (%)
	Laboratory 1	Laboratory 2	
Bulk solution (150 I.U./ml)			
Batch 12	107.1	91.7	93.3
Batch 13	105.4	105.1	106.2
Batch 14	108.2	99.1	105.0
Injections (5 I.U./ml)			
Batch 114	100.3	105.4	105.1
Batch 115	–	104.1	105.1
Batch 116	100.1	–	104.4
Batch 117	–	99.8	101.0
Batch 118	103.3	100.3	101.4
Batch 119	100.9	102.1	101.1
Batch 120	104.2	–	105.4
Batch 121	–	101.9	106.8
Batch 122	–	101.0	102.2
Batch 123	–	103.7	102.6

Experience with HPLC in the routine quality control of ornipressin

Experience was gained with the use of HPLC in the routine analysis of ornipressin in bulk solution and in the pharmaceutical preparation. A number of batches were analysed by both methods, with the internal reference standard being used for calibration. The results are given in Table IV. The data from both methods are in good agreement, which confirms that both methods can equally be applied for the quantitation of ornipressin in bulk solutions and in pharmaceutical preparations.

CONCLUSIONS

The present study shows that HPLC can successfully be used for the quantitation of ornipressin in bulk material and in pharmaceutical preparations. HPLC is a quick and inexpensive method which can easily be applied on automated analysis. It shows good selectivity and the results obtained are identical to those of the biological assay within the normal variation of the methods. From this it can be concluded that reliable potency estimations can be performed by HPLC in future and that the blood pressure test in rats, which has been applied so far, is no longer required.

Based upon these findings Sandoz Pharma plans to reduce the animal tests for ornipressin and to use HPLC instead. In detail, the following quality control concept is foreseen: ornipressin bulk material will be analysed by HPLC and by bioassay. HPLC is used for the precise potency determination, while the biological assay serves as an identification test which assures that the product exhibits the required vasopressor effect. The ornipressin formulations will be analysed by HPLC only, not by the biological assay. Biological identification of the final product is no longer seen as necessary as the drug potency has already been proven on the bulk material.

The proposed concept allows a reduction in animal assays for ornipressin by 90%. This is a significant contribution in minimizing animal experiments in the pharmaceutical industry and it is expected that the health authorities will agree to this new control concept.

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