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LIQUID CHROMATOGRAPHIC DETERMINATION OF FELYPRESSIN AFTER PRE-COLUMN DERIVATIZATION WITH FLUORESCAMINE

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SUMMARY

Felypressin, which is a nonapeptide with a vasopressoric effect, is present in pharmaceutical formulations used in odontological practice. At present, felypressin is determined by a pharmacopoeial bioassay (*United States Pharmacopeia* and *British Pharmacopoeia*) where the increase in blood pressure of rats is measured. As such a method is both tedious and expensive, the development of a faster, less expensive and more precise chemical assay method is very important.

A chemical method is described which includes the initial removal of interfering substances by extraction, pre-column derivatization of felypressin with fluorescamine, and fluorometric detection after liquid chromatography in a reversed-phase system. The relative standard deviation of the method is 1.2% and the detection limit is 0.3 ng of felypressin. The chromatographic system is selective towards closely related peptides as well as degradation products.

Results obtained with the chromatographic method are in close agreement with those obtained by bioassay for production batches of the pharmaceutical formulation as well as for degraded samples.

INTRODUCTION

Felypressin is a nonapeptide with a vasopressoric effect and is present in pharmaceutical formulations intended for use in odontological practice. These formulations contain the peptide at concentrations of *ca.* 0.5 μg/ml. Additional active components are the local anaesthetic drug prilocaine hydrochloride and the preservative methylparaben at concentrations of 30 and 1 mg/ml, respectively.

At present, felypressin is determined by a pharmacopoeial bioassay[1,2] where the increase in blood pressure of rats is observed. As this method is both tedious and expensive, the development of a fast, less expensive, more reliable and precise chemical assay method is considered very important.

High-performance liquid chromatography (HPLC) with UV detection at 210–220 nm has been used for the determination of felypressin-like nonapeptides in liquid pharmaceutical formulations and solid dosage forms[3,4]. However, for the

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analysis of our formulations, higher sensitivity and selectivity of the assay method are required. Derivatization of peptides with fluorescamine has been frequently used[5,6] and should offer the necessary sensitivity.

This article will present a liquid chromatographic method comprising pre-column derivatization with fluorescamine, chromatographic separation in a reversed-phase system and fluorometric detection. Factors affecting the derivatization (*e.g.* pH and reagent concentration), the influence of the composition of the eluent on the capacity ratio of the derivative as well as the stability and selectivity of the system are discussed. The precision of the method, including day-to-day variations, is 1.2% (R.S.D.), and down to *ca.* 0.3 ng (*ca.* 0.3 picomoles) can be detected. The results obtained by the bioassay and the chemical method are well correlated.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of the following components: LDC Constametric III pump (Laboratory Data Control, FL, U.S.A.), flow-rate 1.3 ml/min; Rheodyne 7120 injector (Rheodyne, CA, U.S.A.) equipped with a 50-μl loop; μBondapak C_{18} pre-packed column, 300 × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.); Brownlee MPLC guard column, 37 × 4.6 mm I.D., packed with Nucleosil C_{18} (Brownlee Labs., Santa Clara, CA, U.S.A.); Shimadzu fluorescence spectromonitor RF-530 (Shimadzu, Kyoto, Japan) with an excitation wavelength of 390 nm and an emission wavelength of 470 nm; W + W recorder (W + W electronic AG, Basel, Switzerland); HP 3390 integrator (Hewlett-Packard, PA, U.S.A.).

Chemicals

The eluent consisted of methanol and a phosphate buffer, pH 8.0 (ionic strength 0.1) in a proportion of about 55:45 (v/v), and it was degassed for 10 min in an ultrasonic bath before use. All chemicals used were of analytical grade and were obtained from E. Merck (Darmstadt, F.R.G.).

The borate buffer (pH 8.5) was prepared by mixing 50 ml of 0.2 *M* boric acid solution (containing 0.2 moles/l of potassium chloride) with 11.8 ml of 0.2 *M* sodium hydroxide and then diluting to 100.0 ml with water.

Fluorescamine was obtained from Roche Diagnostics (NJ, U.S.A.) and acetone from May and Baker (Dagenham, U.K.).

Felypressin (see Fig. 1) used for preparation of the standard solution was obtained as a concentrated solution containing 25 IU/ml (0.48 mg/ml). This stock solution was supplied by Sandoz (Basel, Switzerland) where it was assayed by the pharmacopoeial bioassay[1,2]. Further tests were HPLC, amino-acid analyses and nitrogen determination. The felypressin standard solution containing 5 μg/ml was prepared by dilution of the stock solution and assayed by the pharmacopoeial bioassay[1,2] using lypressin (WHO, international standard) as standard. The solution was stored

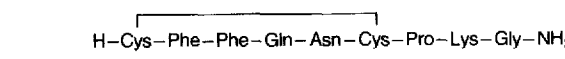

Fig. 1. Sequence of felypressin.

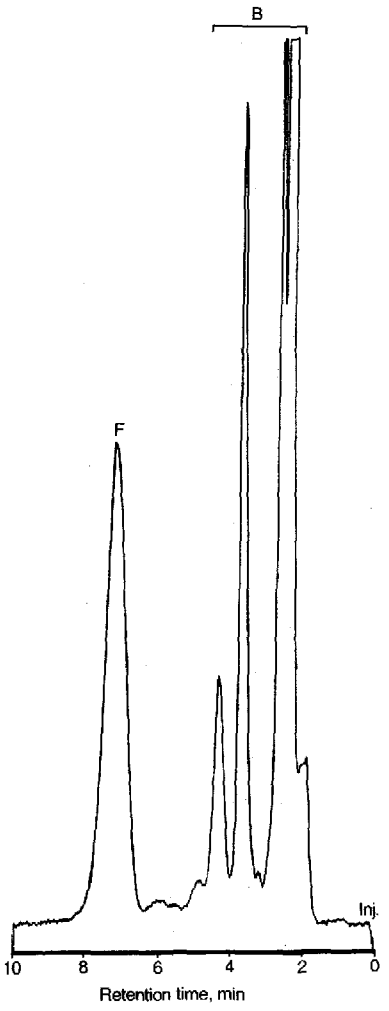

Fig. 2. Sample chromatogram. Separation column: μBondapak C_{18}, 300 × 3.9 mm. Eluent: 56% (v/v) of methanol in phosphate buffer, pH 8.0 (μ = 0.1). Flow-rate: 1.3 ml/min. Peaks: F = felypressin derivative; B = blank peaks as well as peaks originating from other sample components.

in closed glass ampules at +4°C and used for further dilutions as described in the Method section, below.

Method

For the assay 1.50 ml of sample solution were placed in a 10-ml glass test-tube and 3.0 ml of dichloromethane (water-saturated) and 75 μl of 2 *M* sodium hydroxide were added. The tube was immediately stoppered and mechanically shaken for about 10 min. An aliquot (500 μl) of the supernatant was transferred to another 10-ml glass test-tube and 500 μl of borate buffer (pH 8.5) were added. Fluorescamine solution (500 μl of a 0.05% (w/v) solution in acetone; freshly prepared and protected from light) was added while vortex-mixing for *ca.* 30 sec. A 50-μl aliquot of the solution was injected on the column and peak height was used for the quantitation of fely-

pressin in the sample. The standard curve was prepared from 500 μl of known concentrations of felypressin (0.3–0.8 μg/ml) obtained by dilution of the standard solution with 0.9% (w/v) sodium chloride. The derivatization was performed as described for the sample.

A typical sample chromatogram is shown in Fig. 2.

RESULTS AND DISCUSSION

UV detection

Initially UV detection at 210 nm was tried with an eluent containing 27% (v/v) of acetonitrile in phosphate buffer, pH 6.6. Before injection on the column, the sample was extracted with dichloromethane to reduce disturbances in the chromatogram originating from the large amounts of prilocaine and methylparaben originally present in the sample. A pH value below 6.5 at the extraction step resulted in a large disturbing peak while at pH 8 this disturbance was negligible, but degradation of felypressin occurred in the extraction step. Consequently, for a successful analysis, the pH had to be balanced very carefully without dilution of the sample to any appreciable degree. To obtain the peak height necessary for quantitation, 100 μl or more of the sample had to be injected.

Fluorescence detection

In order to avoid the drawbacks of UV detection, a fluorescence method based on pre-column derivatization with fluorescamine was developed. The studies of the parameters affecting the derivatization were performed using the apparatus and chemicals described under Experimental.

Influence of fluorescamine concentration. Reagent solutions containing 0.01–0.2% (w/v) of fluorescamine in acetone were tested. The reagent:peptide molar ratios were 690:1–13,800:1 in those experiments. The resulting peak height of the felypressin derivative obtained from an original sample as well as a standard solution was plotted against reagent concentration (see Fig. 3). For the sample, a 0.03% (w/v) fluores-

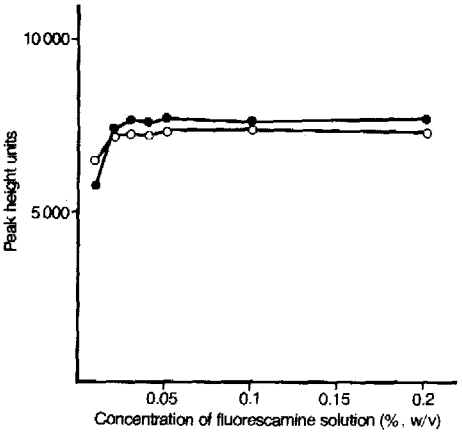

Fig. 3. Influence of fluorescamine concentration on the yield of the fluorescent derivate of felypressin. For chromatographic conditions, see Fig. 2. (●) Sample solution containing 0.53 μg/ml of felypressin; (○) felypressin standard solution containing 0.50 μg/ml of felypressin.

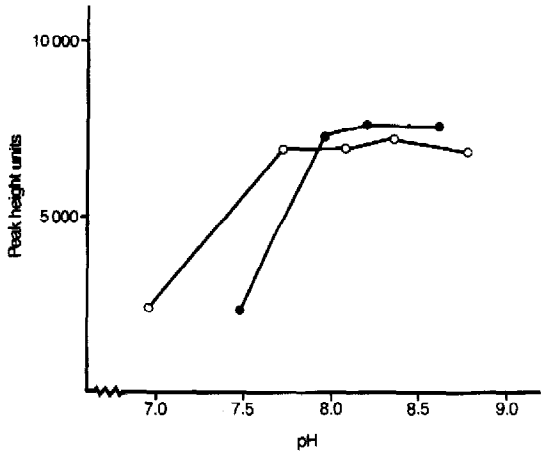

Fig. 4. Influence of pH of the derivatization medium on the yield of the fluorescent derivative of felypressin. The pH before addition of fluorescamine reagent is given. For chromatographic conditions, see Fig. 2. (●) Sample solution containing 0.53 μg/ml of felypressin; (○) felypressin standard solution containing 0.50 μg/ml of felypressin.

camine solution was sufficient for maximum fluorescence while a somewhat lower concentration (0.02%, w/v) was required for pure felypressin standard solutions. At concentrations below 0.02% (w/v) an additional peak appeared in the chromatogram (*cf.* under pH studies, below). In the assay method, a 0.05% (w/v) solution of fluorescamine was used.

Influence of pH. The pH of the derivatization step was varied by addition of borate buffers of pH 7.7–8.9 to sample and standard solutions. The resulting pH was measured before addition of the fluorescamine reagent. The peak height of the felypressin derivative was measured and plotted against pH in Fig. 4. Maximum fluorescence was obtained at pH above 8.1 which was obtained by addition of a borate buffer of pH 8.5, as described in the assay method, above. A subsequent lowering of the pH after derivatization at pH above 8.1 had no influence on the fluorescence intensity.

Derivatization below pH 8 resulted in an additional fluorescent derivative giving a peak with a higher capacity ratio than that of the original derivative (peaks 2

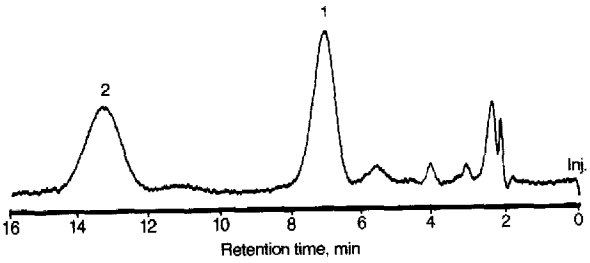

Fig. 5. Chromatogram obtained by derivatization at pH below 8. A phosphate buffer of pH 6.0 (μ = 0.1) was added to the derivatization medium instead of borate buffer pH 8.5. For chromatographic conditions, see Fig. 2. Peaks: 1 = original derivative obtained by derivatization according to the method; 2 = derivative obtained by derivatization at pH below 8.

and 1, respectively, in Fig. 5). The size of the additional peak increased with decreasing pH. The formation of this additional derivative was time-dependent and at pH 5–6 the relative amount (calculated from the peak areas by internal normalization) increased from 60 to 90% within 15 min. At an even lower pH both derivatives were formed but the peaks were very small (*cf.* ref. 7). Both derivatives also appeared when using reagent concentrations below 0.02% (w/v) of fluorescamine and also when using a degraded fluorescamine reagent. The derivatization reaction seems to go via the derivative giving peak 1 to the derivative giving peak 2. The reaction has a high initial rate and seems to be irreversible.

Lypressin, being a closely related peptide containing lysine in the same position as felypressin, *i.e.* close to the C-terminal amide, behaved analogously. However, oxytocin, being a nonapeptide without lysine in the molecule, did not exhibit the same behaviour, *i.e.* no additional fluorescent derivative was formed either at a low pH or when degraded fluorescamine was used. Substance P, a peptide without a disulphide bridge, containing 11 amino acids and with lysine located close to the primary amino terminus, was also studied. This peptide behaved like oxytocin.

It is reasonable to presume that, at pH above 8.1, fluorescamine reacts with the primary amino terminus as well as the ε-amino group in lysine. At lower pH values, the fluorescamine molecule bound to lysine may be involved in an additional reaction, *e.g.* with the C-terminus giving rise to a peak with longer retention than the first peak.

Stability of fluorescence. The fluorescence of the felypressin derivative has a limited stability and a *ca.* 10% reduction in peak area of the derivative was observed at room temperature after 2 h.

Separation system

Column. In addition to the pre-packed μBondapak C_{18} (300 × 3.9 mm I.D., 10-μm particles) column which was used in the method described, shorter columns of the same material as well as Bio-Sil ODS-5S (Bio-Rad Labs., Richmond, CA, U.S.A.) and μBondapak Phenyl (Waters Assoc.) columns were investigated. All the columns gave symmetrical peaks with tailing factors of *ca.* 1.1. The capacity ratios (k' values) on the Bio-Sil column were somewhat higher than those on the μBondapak C_{18} columns. The μBondapak Phenyl material gave the highest k' values, very symmetrical peaks but somewhat lower efficiency than the C_{18}-materials.

The μBondapak C_{18} column used in the method exhibited good selectivity towards fluorescamine derivatives of closely related peptides such as lypressin and oxytocin. This is illustrated in Fig. 6. The stability of the column was good. A slight tendency to decreased k' values during the lifetime of the column (several months) was observed. This effect could, however, be counteracted by slight modification in the composition of the eluent.

Eluent. A methanol–phosphate buffer mixture gave good results with the fluorescamine derivative well resolved from the blank as well as from peaks which appear early in the chromatogram and which originate from prilocaine. Furthermore, this eluent gave good selectivity as discussed above in connection with the separation column.

The capacity ratio is very sensitive to small changes in methanol concentration. This is illustrated in Fig. 7. A 1% decrease in the concentration of methanol (*e.g.*

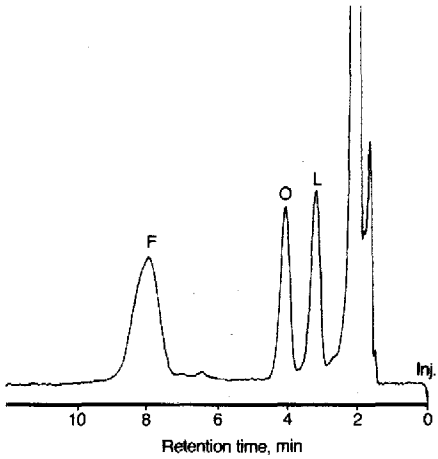

Fig. 6. Separation of closely related peptides. For chromatographic conditions, see Fig. 2. Peaks: F = felypressin; O = oxytocin; L = lypressin.

from 56 to 55%) results in a 25% increase in the k' value. The pH of the eluent has a minor influence on the retention, but there is a tendency to increased retention with decreased pH.

Recent findings indicate that it would be possible to use an eluent of lower pH than that used in the above method. For instance, by using a buffer of pH 6.5 instead of pH 8.0, the fluorescence intensity of the fluorescamine derivative formed at pH above 8.1 is not affected, as discussed in connection with the derivatization, but the column lifetime would probably be increased.

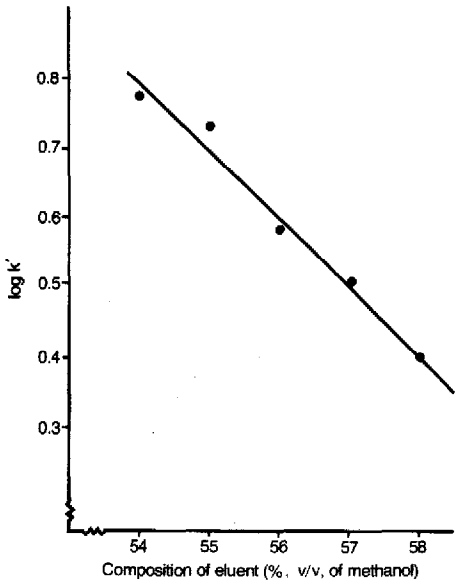

Fig. 7. Influence of eluent composition on the capacity ratio of the felypressin derivative.

Initial extraction step

To allow determination of the very small amounts of felypressin (0.5 μg/ml) present, the sample should be diluted as little as possible. However, the sample also contains large amounts of prilocaine hydrochloride (30 mg/ml) and methylparaben (1 mg/ml) which should be reduced in order to minimize over-loading effects as well as degradation of the column. Prilocaine, being a secondary amine, also reacts with fluorescamine, thus interfering with the reaction between felypressin and fluorescamine. In the initial extraction step, about 87% of the prilocaine and more than 95% of the methylparaben are removed. In this step, the initial pH is 8.4 but decreases rapidly to *ca.* 6 at equilibrium. A higher pH would remove more of the prilocaine but would lead to degradation of felypressin.

Adsorption

Adsorption of felypressin to glass and plastic surfaces is a problem when working with very diluted solutions. To overcome this, all dilutions of the standard solution were made with a 0.9% (w/v) sodium chloride solution.

Sensitivity and precision of the method

The detection limit is 0.3 ng of felypressin, indicating that concentrations down to 20 ng/ml can be detected when analyzed by the method.

Its accuracy was tested on three simulated samples containing 400–600 ng/ml of felypressin which were analyzed in duplicate. The mean recovery was 100.8%.

The linearity of the standard graph was tested by preparing standard solutions of five felypressin concentrations ranging from 37 to 156% of the expected concen-

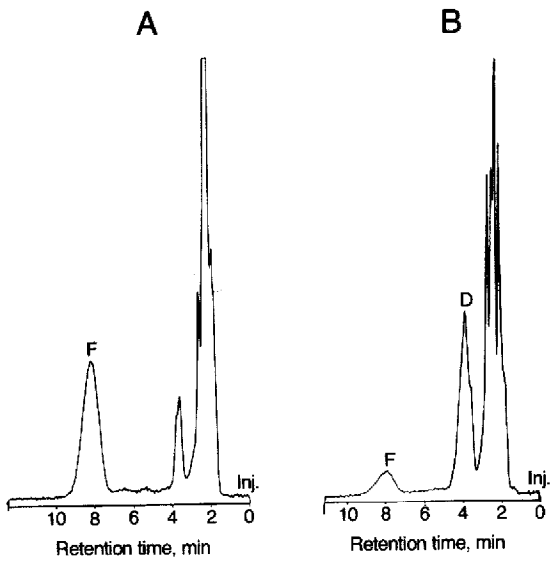

Fig. 8. Stability-indicating properties of the chromatographic system. For chromatographic conditions, see Fig. 2. (A) Chromatogram from a freshly prepared solution of felypressin of pH 7. (B) Chromatogram from the same solution stored for 3 days at room temperature. Peaks: F = felypressin derivative; D = derivative of degradation product.

TABLE I

COMPARISON BETWEEN CHEMICAL AND BIOLOGICAL ASSAYS ON DEGRADED SAMPLES

Sample treatment	*μg/ml of felypressin*	
	Chem. method	*Bioassay*
Untreated	0.55	0.56
2 days at 70°C	0.46	0.46
pH 7.5, 6 h at 70°C	0.38	0.39

tration in a normal sample solution. The standard graph had a correlation coefficient of 0.9995.

The reproducibility (at intervals of some days) of the method was determined by duplicate analyses of a sample on four occasions. The relative standard deviation was 1.2%.

Stability-indicating properties

For analyses of storage samples, the selectivity of the separation system must be sufficiently high to separate possible degradation products from intact felypressin. Fig. 8 gives the chromatograms obtained before and after storage of a felypressin standard solution, pH 7, for three days at room temperature. An extra peak appeared before the dramatically decreased peak of the felypressin derivative.

Comparison with bioassay

Comparisons were made between the chromatographic method and the official pharmacopoeial bioassay[1,2] on degraded samples as well as on production batches. A sample solution (pH 4) which had been stored at 70°C for two days, and one with pH increased to 7.5 and stored for 6 h at 70°C, were analysed by both methods. The results which are presented in Table I are in very close agreement.

In addition, more than 30 production batches were analysed with both methods. The data were evaluated statistically by the *t*-test for matched pairs. There were no significant differences between the results of the two methods at the 5% level, but a tendency to slightly higher values (less than 1%) with the chemical method was observed.

CONCLUSION

The chemical method based on liquid chromatography has good precision and selectivity which makes it suitable for quality control of production samples as well as for stability testing of samples containing felypressin.

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