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Note

Liquid chromatographic determination of felypressin using a column-switching technique and post-column derivatization

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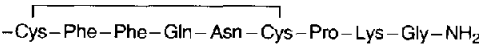
Felypressin (Fig. 1), which is a nonapeptide with a vasopressoric effect, is often present in odontological local anaesthetic formulations.

In the pharmacopoeias [1,2], felypressin is determined by bioassay, measuring the increase in blood pressure of rats. Felypressin-like nonapeptides have also been determined by high-performance liquid chromatography (HPLC) with UV detection at 210–220 nm [3,4]. A chromatographic method for the determination of oxytocine has also been described [5]. This method includes an on-line preconcentration and reversed-phase chromatography with post-column derivatization and fluorescence detection.

In a pharmaceutical formulation containing about 0.5 μmol/l (0.5 μg/ml) of felypressin and *ca.* 0.1 mol/l (30 mg/ml) of the local anaesthetic prilocaine hydrochloride (Fig. 2), felypressin has been determined after removal of prilocaine by extraction, precolumn derivatization with fluorescamine and fluorescence detection after reversed-phase chromatography [6]. As this method includes an extraction step, precolumn derivatization and manual injection, it is time consuming and not easily automated.

From studies of the retention behaviours of felypressin and prilocaine on different reversed-phase stationary phases, an automated method, including a column-switching technique and post-column derivatization, has been developed. In this method, felypressin is retained on a short column (extraction column), while other irrelevant components (*e.g.*, large amounts of prilocaine) are eluted. The final purification of felypressin is performed on a second column (separation column). Fluorescence detection after post-column derivatization with fluorescamine gives a detection limit of 0.6 ng (0.6 pmol).



Molecular weight: 1040

Fig. 1. Sequence of felypressin.

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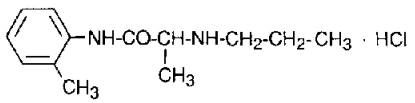

Molecular weight: 256.8

Fig. 2. Structure of prilocaine hydrochloride.

EXPERIMENTAL

Apparatus

The LC equipment consisted of an SP 8770 pump (Spectra-Physics, San Jose, U.S.A.) to deliver eluent I, an LDC/Milton Roy Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) to deliver eluent II, both at a flow-rate of 1.0 ml/min, and an LDC/Milton Roy 4711 pump (Laboratory Data Control) to deliver the post-column reagent, at a flow-rate of 0.25 ml/min. The samples and standards were injected with a Spectra-Physics SP 8780 XR autosampler and the vials were obtained from Chromacol (London, U.K.).

The eluate was mixed with the reagent in a 5 m × 0.5 mm I.D. knitted Teflon tube [7] and detected with a RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) with an excitation wavelength of 390 nm and an emission wavelength of 470 nm. A Shimadzu SPD-6A UV detector set at 210 nm was used in testing the different stationary phases and to monitor the effluent from the extraction column.

An automated switching valve (Waters Assoc. Milford, MA, U.S.A.), controlled by a Spectra-Physics SP 4270 integrator was used to perform the column-switching procedure. The fluorescence was recorded with a Nelson Analytical (Cupertino, CA, U.S.A.) Model 600 reintegration data system. UV absorbance was recorded on a Spectra-Physics SP 4270 integrator.

Stationary phases

The stationary phases and columns tested were a LiChrospher 60 RP-Select B (150 × 4.0 mm I.D.) stainless-steel column with 5-μm particles, a Superspher 60 RP-8e (50 × 4.0 mm I.D.) cartridge column with 4-μm particles, both from E. Merck (Darmstadt, F.R.G.), a Supelcosil LC-18 DB (150 × 4.6 mm I.D.) stainless-steel column with 5-μm particles (Supelco, Bellefonte, PA, U.S.A.), a Nucleosil C_{18} (125 × 4.0 mm I.D.) stainless-steel column with 3-μm particles (Macherey, Nagel & Co., Düren, F.R.G.) and a Zorbax-CN (150 × 4.6 mm I.D.) stainless-steel column with 5-μm particles (DuPont, Wilmington, DE, U.S.A.). The extraction column used in the method was a Superspher 60 RP-8 (25 × 4.0 mm I.D.) cartridge column with 4-μm particles (E. Merck).

Chemicals

The eluents used were prepared from acetonitrile and phosphate buffers of different pH (ionic strength 0.05 or 0.1), all degassed with helium for at least 10 min before use.

Acetonitrile was of chromatographic purity grade and all other chemicals were

TABLE I

STEPS IN THE CHROMATOGRAPHIC PROCEDURE

Step[a]	Time (min)	Eluent No.[b]	Column	Dimensions (length × I.D.) (mm)
1	0–14	I	Superspher 60 RP-8	25 × 4
2	14–23	II	Superspher 60 RP-8 + Superspher 60 RP-8e	25 × 4 50 × 4
3	23–30	I	Superspher 60 RP-8	25 × 4

[a] 1 = removal of prilocaine; 2 = chromatography of felypressin; 3 = re-equilibration of the extraction column (see Fig. 3).

[b] I = acetonitrile–phosphate buffer (pH 6.0) (12:88); II = acetonitrile–phosphate buffer (pH 6.0) (20:80).

of analytical-reagent grade and were obtained from E. Merck.

Fluorescamine was obtained from Fluka (Buchs, Switzerland) and was dissolved in acetonitrile at a concentration of 0.30 g/l. This reagent also contained 0.1% (v/v) of Brij-35 (Technicon, Tarrytown, NY, U.S.A.).

Felypressin was obtained as a concentrated solution containing 25 IU/ml (0.48 g/l), supplied by Sandoz (Basle, Switzerland). In the described method, a felypressin stock standard solution [6] containing 5 mg/l was used. Prilocaine hydrochloride was obtained from Astra (Södertälje, Sweden). When testing the different stationary phases, a solution containing 36 mg/l of felypressin and 9.6 mg/l of prilocaine hydrochloride was prepared in deionized water. Citanest Octapressin injection solution was obtained from Astra.

Method

The assay of felypressin in the pharmaceutical formulation (Citanest Octapressin) was performed by the injection of undiluted sample into the chromatographic system. The different steps in the chromatographic procedure are described in Table I and a schematic diagram is given in Fig. 3.

The volumes of samples and standards injected were 20 μl and the flow-rates for both eluents were 1.0 ml/min. Detection was effected by post-column derivatization (flow-rate 0.25 ml/min) with fluorescamine and fluorescence detection (λ_{ex} = 390 nm, λ_{em} = 470 nm). For quantification, peak areas were compared with a calibration graph [0.3–0.7 mg/l of felypressin dissolved in 0.9% (w/v) sodium chloride solution].

RESULTS AND DISCUSSION

Choice of chromatographic system

On five different stationary phases (two C_{18}, two C_8 and one CN), the capacity factors (k') of felypressin and prilocaine were determined with variation of the pH between 3 and 7, using an eluent containing 20% (v/v) of acetonitrile. For the octadecyl- and octyl-bonded phases, the increase in k' for prilocaine was more pronounced than that for felypressin between pH 6 and 7, *i.e.*, the separation factor (α) increases. In this pH range felypressin was eluted first. On the nitrile phase, the retention order

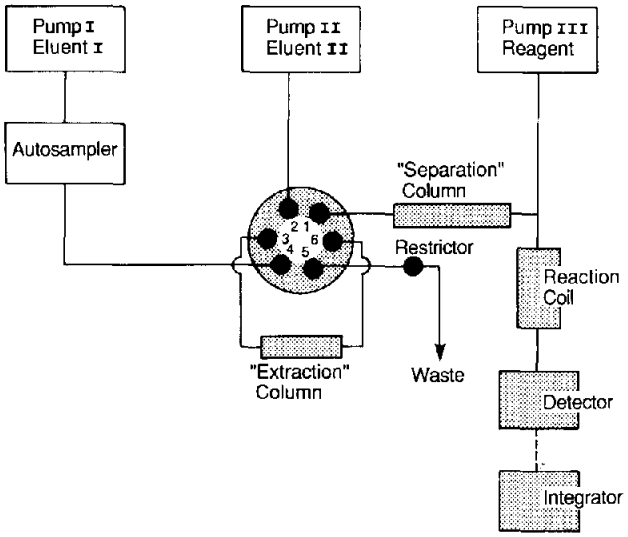

Fig. 3. Schematic diagram of the chromatographic system.

was changed and k' was less affected by pH. As the results were both unpredictable and irreproducible, no further experiments were done on this phase.

When varying the concentration of acetonitrile in the eluent, Superspher 60 RP-8e was selected as it gave the highest α value and good peak symmetry. Felypressin was more sensitive than prilocaine to variations in acetonitrile concentration and the retention order changed at a concentration about 18% (v/v). The effect of acetonitrile concentration is shown in Fig. 4. The large differences in the k' values of prilocaine and felypressin at concentrations below 14% (v/v) were utilized to remove prilocaine by the column-switching technique. Thus, felypressin can be extensively retained on a short extraction column, while *e.g.*, large amounts of prilocaine can be removed as waste (Fig. 5).

By a subsequent increase in the acetonitrile concentration, felypressin can be eluted and chromatographed on a second column. When using the chromatographic procedure described in Table I, clean chromatograms and symmetrical peaks are obtained for both samples and standards (Fig. 6).

Detection

Detection was performed by post-column derivatization with fluorescamine and fluorescence detection. As this reagent reacts only with primary amines, a high selectivity is achieved and a detection limit of 0.6 pmol (signal-to-noise ratio = 3) is obtained. The reaction took place in a 5 m × 0.5 mm I.D. knitted Teflon tube [7]. To avoid the formation of bubbles when mixing the reagent and eluent, Brij-35 was included in the reagent [5].

Usually a pH above 8 is used for the reaction of fluorescamine with peptides. On increasing the reaction pH from 6 to 8, no differences were observed. This favours the use of pH 6 (eluent pH), as a higher pH requires an additional pump and leads to dilution of the sample.

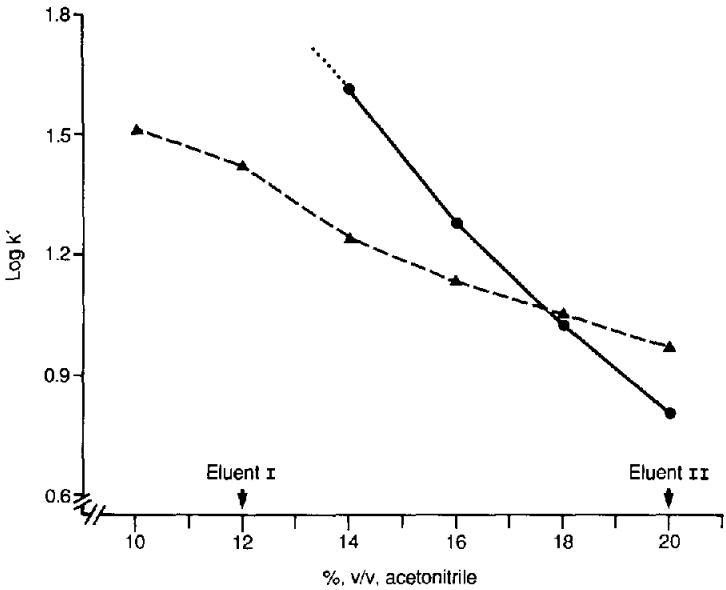

Fig. 4. Influence of eluent composition on the capacity factors of (●) felypressin and (▲) prilocaine. Eluent, acetonitrile in phosphate buffer (pH 6.0); ionic strength, 0.05; column, Superspher 60 RP-8e (50 × 4 mm I.D.), 4-μm particles.

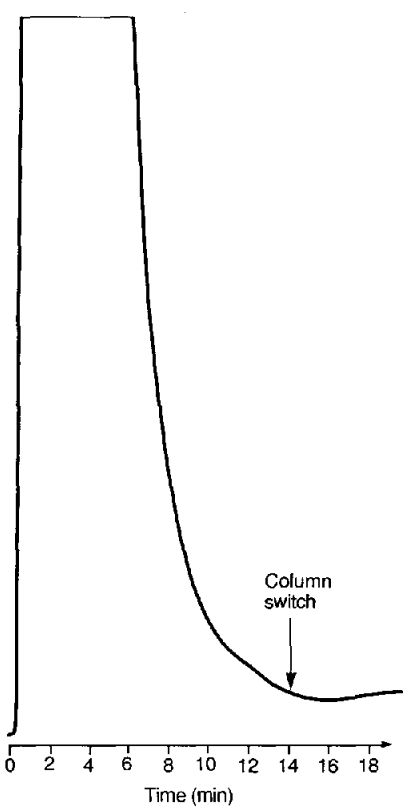

Fig. 5. UV trace (210 nm) from the extraction column. Eluent No. I (see Table I).

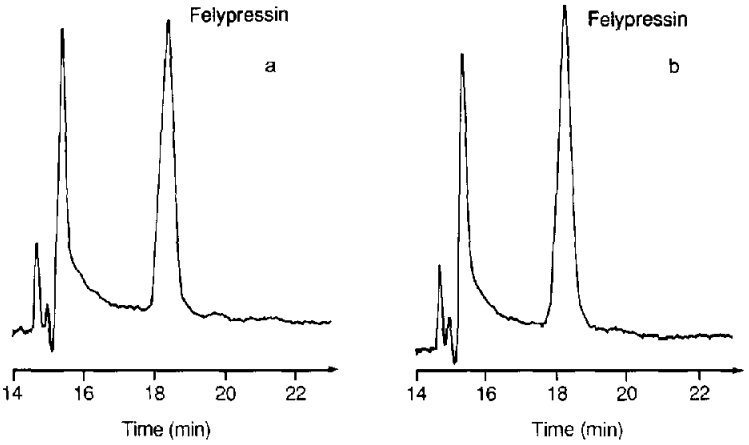

Fig. 6. Chromatograms after column switching of (a) standard and (b) sample solutions. About 10 ng of felypressin were injected.

Validation data

A calibration graph from five standard solutions, corresponding in concentration to 20–150% of the labelled amount of felypressin in a sample, was constructed. The correlation coefficient, r, was found to be 0.9998.

Six simulated samples were prepared and assayed according to the method. The mean recovery was found to be 101.8%.

The reproducibility (including day-to-day variations) of the method was 1.7% and 1.3% (relative standard deviation) for sample and standard, respectively. This precision was obtained only if special precautions to avoid adsorption to glass and plastic surfaces had been taken. Rinsing all glass and plastic ware with 1 mol/l acetic acid and deionized water and diluting all standards with 0.9% (w/v) sodium chloride solution minimize adsorption.

The selectivity of the method was tested on two closely related peptides, lypressin and ornipressin. Neither of them was detected, hence the method can be considered to be selective for felypressin. The proposed method is stability indicating, as degradation products are separated from intact felypressin.

As seen in Fig. 6, the peak symmetry and peak width from sample and standard were identical. After *ca.* 100 injections of a sample, the peak width was increased. Consequently, the extraction column should then be replaced.

CONCLUSION

An automated system which reduces sample handling to a minimum has been developed for the determination of felypressin in a pharmaceutical formulation. The running time with the proposed method is 30 min, including re-equilibration of the extraction column. Although this is a fairly long time, pre-extraction and precolumn derivatization procedures are eliminated [6].

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