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Note

Effects of mobile phase and stationary phase on the quantitative determination of oxytocin

MAXIMILIAN ANDRE
Quality Assurance, Biochemie GmbH, A 6250 Kundl (Austria)
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Today the cyclic nonapeptide, oxytocin, a pituitary gland hormone, is produced by chemical synthesis. The concentration of liquid dosage forms is usually *ca.* 10 I.U./ml. With reference to the 4th Internal Standard for Oxytocin, this concentration is equal to 16.8 μg/ml oxytocin. Due to the low concentration, a precise quantitative determination is of predominant interest in quality control. The pharmacopoes requires biological assays, which usually lack precision, therefore time-consuming and repetitive test are necessary.

Since high-performance liquid chromatography (HPLC) has proved the most suitable method for the separation of peptides, various HPLC systems have been studied for the separation of oxytoxin samples[1–9]. Apart from the ion-exchange mode[6,7], the reversed-phase (RP) mode was most widely used, employing octyl- or octadecyl-bonded stationary phases.

Additionally, mobile phase effects on the separation of oxytocin from other nonapeptides or diastereoisomers have been investigated by several authors[4,5,7,9].

The quantitative determination of oxytocin by RP-HPLC was established as the method of choice in pharmaceutical quality control, since Krummen and Frei[1] have found a good correlation between biological assays and RP-HPLC.

The purpose of this study was to improve the precision of the quantitative determination and to investigate mobile phase effects on similar stationary phases from different manufacturers.

MATERIALS AND METHODS

HPLC equipment

The HPLC equipment consisted of a Beckman 110A pump and a Kratos 773 UV detector at 215 nm equipped with a 12-μl flow cell. The manuel injector was a Rheodyne Model 7125 with a 20-μl injection loop. The peaks were integrated by an HP 3357 Lab-Data-System.

HPLC columns and mobile phases

The HPLC columns used were slurry packed. The column dimensions were 150 × 3 mm I.D.

Nucleosil C_{18} 5 μm (Machery-Nagel, Düren, F.R.G.) or LiChrosorb RP-8, 5 μm (Merck, Darmstadt, F.R.G.) were used as stationary phases.

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The mobile phases were HPLC-grade acetonitrile–phosphate buffer (Tritrisol, Merck) at pH 7 or pH 3 (18:82) and acetonitrile–orthophosphoric acid (0.06 *M*) adjusted with triethylamine (Merck) to pH 3 (18:82). The flow-rate was 1 ml/min.

Standard and sample preparation

The 4th International Standard for Oxytocin (established in 1978) containing 12.5 I.U./ml dissolved in water was used as reference. The sample for testing the accuracy was an aqueous solution of oxytocin (10.5 I.U./ml) preserved by trichlorobutanol.

RESULTS AND DISCUSSION

Effects of pH and stationary phase on selectivity

The two stationary phases used were identical in particle diameter and alkyl-chain length but were from different manufacturers (LiChrosorb RP-8, 5 μm; Nucleosil RP-8, 5 μm). A marked difference between these materials was observed when the pH of the mobile phase was changed from 3 to 7. On LiChrosorb material, an approximately two-fold increase in the retention time of oxytocin was observed and

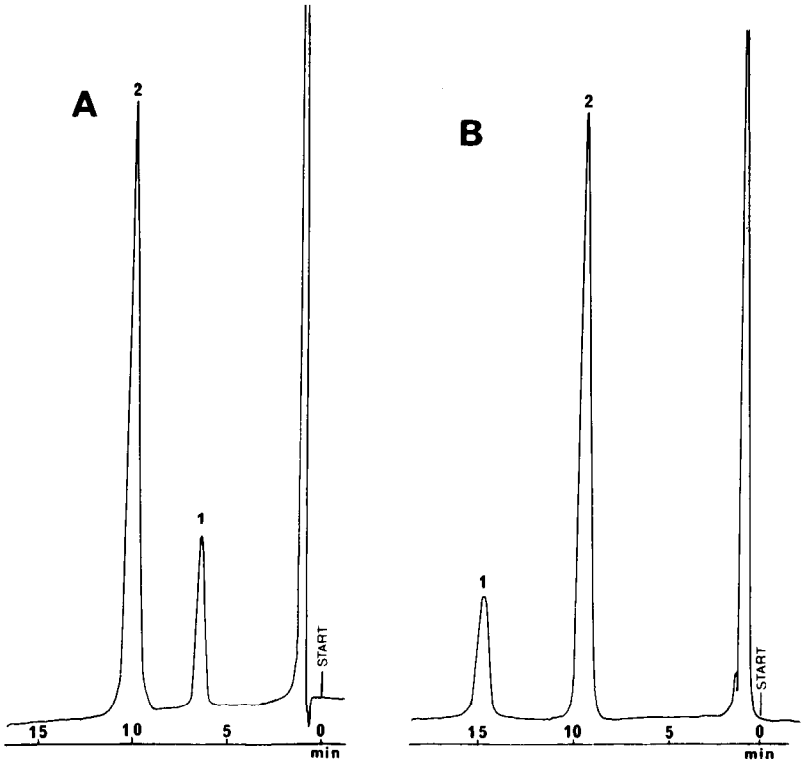

Fig. 1. Chromatogram of an oxytocin solution. Peaks: 1 = oxytocin; 2 = trichlorobutanol. Column: Nucleosil RP-8, 5 μm. Mobile phase A: acetonitrile–phosphate buffer: pH 3 (18:82); B: acetonitrile–phosphate buffer pH 7 (18:82); flow-rate, 1 ml/min; UV detection at 215 nm.

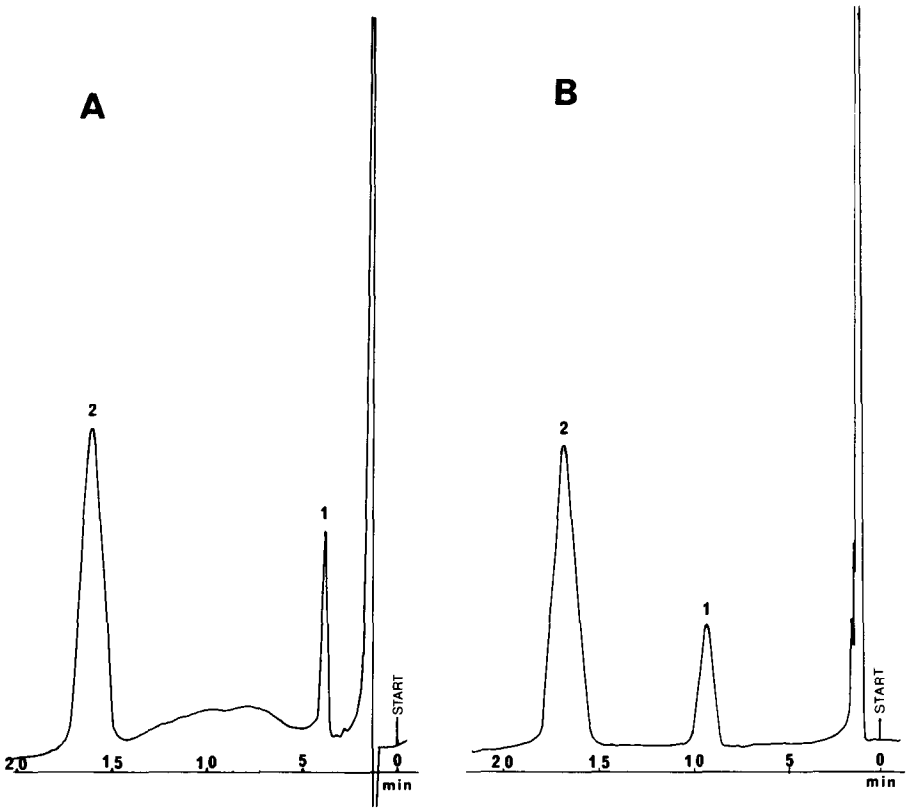

Fig. 2. Chromatogram of an oxytocin solution. Peaks: 1 = oxytocin; 2 = trichlorobutanol. Column: LiChrosorb RP-8, 5 μm. Mobile phase, A: acetonitrile–phosphate buffer pH 3 (18:82); B: acetonitrile–phosphate buffer pH 7 (18:82); flow-rate, 1 ml/min; UV detection at 215 nm.

oxytocin was eluted before trichlorobutanol. This is in agreement with the results reported by Krummen and Frei[1,3].

The Nucleosil material showed nearly the same increase in retention of oxytocin but a change in selectivity when changing the pH. This is in contrast to the results reported earlier[1,3] using the same stationary phase. In both cases, the retention time of trichlorobutanol was virtually independent of pH.

At the same pH, a two-fold retention time was observed on Nucleosil RP-8 compared to LiChrosorb RP-8, indicating a considerable difference in the hydrophobicity between these stationary phases (see Figs. 1 and 2).

Precision and accuracy

Precision studies were accomplished in order to improve the chromatographic assay. Under the chromatographic conditions described in Fig. 2A, a relative standard deviation (R.S.D.) of 2.3% (n = 7) was obtained for a 10 I.U./ml solution. Additionally, after a repetition of *ca.* 100 injections, a gradual increase in retention time up to twice the initial retention time of oxytocin was observed, so resulting in

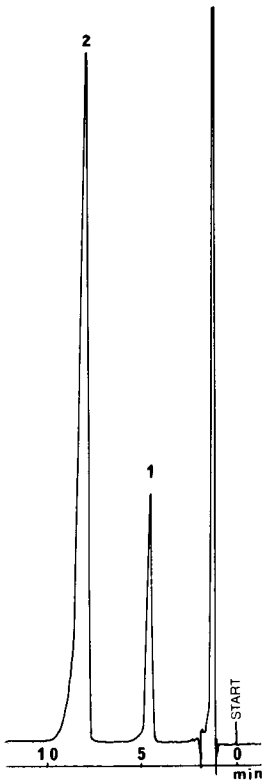

Fig. 3. Chromatogram of an oxytocin solution. Peaks: 1 = oxytocin; 2 = trichlorobutanol. Column: Nucleosil RP-8, 5 μm. Mobile phase: acetonitrile–phosphoric acid (0.06 *M*) adjusted to pH 3 with triethylamine (18:82), flow-rate, 1 ml/min; UV detection at 215 nm.

unsatisfactory reproducibility. This phenomenon may be caused by the irreversible adsorption of peptides on the stationary phase.

To prevent such adsorption, Rivier[10] has pointed out the advantage of trialkylammonium salts in a comprehensive study.

In a similar way, Biemond *et al.*[4] used tetramethylammoniumhydroxide as a powerful agent for silanol masking for the quantitative determination of oxytocin, But the studied oxytocin concentration was more than three hundred times higher than that used in this study.

By use of triethylammonium phosphate buffer and the chromatographic conditions described in Fig. 3, the optimum results for precision and peak shape were obtained with the systems studied. The R.S.D. (n = 8) for a 10 I.U./ml solution was 0.9%, corresponding to more than a three-fold increase in precision, which could probably be further improved by use of automatic injection systems[11]. Even after several hundred injections, no chromatographic instability appeared. This was caused by the effective silanol masking of the ammonium salts and the reproducible high recovery of the peptide. The accuracy of the method was tested with a 10.5 I.U./ml oxytocin solution, which was determined by two alternative biological assays.

The same content was obtained with the HPLC conditions described in Fig. 3, indicating that the selected k' value is high enough to obtain sufficient resolution for quantitative determination.

REFERENCES

1 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
2 B. Larsen, V. Viswanatha, S. Y. Chang and V. J. Hruby, *J. Chromatogr. Sci.*, 16 (1978) 207.
3 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27.
4 M. E. F. Biemond, W. A. Sipman and J. Olivié, *J. Liq. Chromatogr.*, 2 (9) (1979) 1407.
5 B. Larsen, B. L. Fox, M. F. Burbe and V. J. Hruby, *Int. J. Pept. Protein Res.*, 13 (1979) 12.
6 R. A. Pask-Hughes, R. E. Harslay and R. E. Gaines Das, *J. Biol. Stand.*, 11 (1983) 13.
7 R. A. Pask-Hughes, P. H. Corran and D. H. Calam, *J. Chromatogr.*, 214 (1981) 307.
8 K. Krummen, F. Maxl and F. Nachtmann, *Pharm. Technol.*, 9 (1979) 77.
9 M. Lebl, *J. Chromatogr.*, 264 (1983) 459.
10 J. E. Rivier, *J. Liq. Chromatogr.*, 1 (3) (1978) 343.
11 N. A. Parris, *Instrumental Liquid Chromatography* (*J. Chromatogr. Library*, Vol. 27), Elsevier, Amsterdam, 1984, pp. 87, 127.