Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 4, pp. 529–532, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

Short Communication

The determination of bradykinin in human saliva by high-performance liquid chromatography with UV detection*

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Keywords: *Bradykinin; saliva; liquid chromatography.*

Introduction

The kinins, a group of biologically active polypeptides, are generally considered as important chemical mediators in the inflammatory process [1–3]. However, information is lacking for the role of these mediators in periodontal diseases, due to the lack of a suitable analytical method of determination. Several methods that have been reported for the determination of kinins in saliva and gingival fluid were based on a biological assay using guinea pig ileum or rat uterus [4]. However, the biological reactions involved were not specific to kinins so that the values obtained from these methods were not accurate.

The present work was attempted in order to establish a method for the analysis of bradykinin (BK) in human saliva without employing a biological reaction. We have developed an HPLC method which does not require any complicated anatomical technique nor any biological reaction but which was able to specifically quantify BK.

Experimental

Sample preparation

Whole saliva from periodontally healthy patients was collected in an ice-cold polyethylene tube, and immediately filtered using an ultrafiltration membrane (Immersible CX-10, Millipore Co., Bedford, MA, USA). This method of avoiding degradation and/or synthesis of the BK in the saliva is preferred to the addition of an

*Presented in part at the 61st General Session of the International Association for Dental Research, Australia, August 1984.

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inhibitor for kallikrein or kininase. A sample (2 ml) of the filtrate was then introduced into a SEP-PAK C_{18} cartridge (Waters Assoc., Milford, MA, USA). After washing this column with 2 ml of 60% (v/v) methanol and 2 ml of 80% (v/v) methanol, the kinins were eluted with 2 ml of methanol containing 1% (v/v) acetic acid. This fraction was dried under vacuum at 40°C and the residue dissolved in 100 μl of the mobile phase used for the HPLC system I, described below.

Materials

The following chemicals were used: BK (Sigma, St Louis, MO, USA), tetramethylammonium phosphate (PIK-A and Low UV PIC-A) (Waters Assoc.), triethylamine (Nakarai Chemicals, Tokyo, Japan), acetic acid and HPLC grade methanol Wako Chemicals, Tokyo, Japan). Other chemicals were of analytical grade and purchased from commercial sources. Water for HPLC was prepared with a MILLI-Q system (Millipore Co.).

Apparatus

The chromatograph was a modular isocratic system consisting of a Waters 6000A pump, U6K injector (50 μl loop), 440 absorbance detector and a 300 × 4 mm i.d. column packed with C_{18} μ Bondapak (Waters Associates, Milford, MA, USA).

HPLC analysis

The following analytical systems were used:

(1) The HPLC system I for the preliminary isolation of the BK from human saliva; methanol–aqueous triethylamine (0.5% w/v) and PIC-A (1% w/v) adjusted to pH 6.0 with acetic acid (35;65, % v/v). The flow-rate was 1.0 ml min^{-1} and the detector was operated at 254 nm.

(2) The HPLC system II for the determination of BK; methanol–aqueous Low UV PIC-A (1% v/v) (1;1, % v/v). The flow-rate was 1.5 ml min^{-1} and the detector operated at 214 nm.

Procedure

The preliminary isolation of BK from human saliva was achieved using HPLC system I by chromatographing 50 μl of the saliva extract (as described under sample preparation) that had been spiked with 2 μl authentic BK. This calibrated the separation so that the time for collecting the BP peak was known. A second 50 μl saliva extract (not spiked) was then injected and the BK collected (Fig. 1). The eluate fraction was evaporated to dryness under vacuum at 40°C and dissolved in 100 μl of the mobile phase required for HPLC system II, and assayed (Fig. 2).

Results and Discussion

Preliminary isolation and recovery

Since the enzymatic system for kinins, kallikrein and kininase occurs in saliva [5,6], the BK in the saliva must be isolated as quickly as possible at 0°C, to suppress any enzymatic reactions. As the ultrafiltration membrane used in this stage passed only small-sized molecules below 10,000 daltons, enzyme activity was considered to be suppressed. The SEP-PAK Silica cartridge was found to be unsuitable, providing a low recovery of BK from saliva. However, the SEP-PAK C_{18} cartridge was found to be satisfactory.

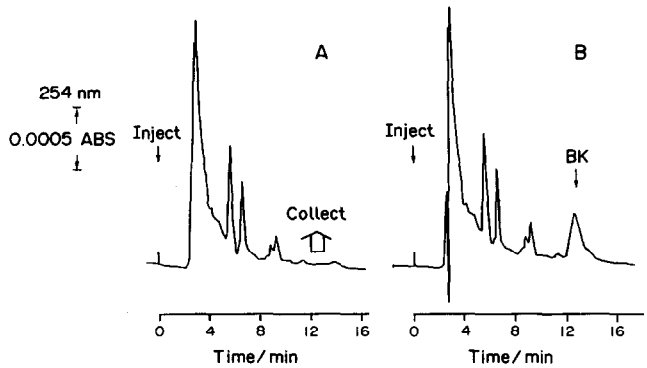

Figure 1
Chromatograms with HPLC system I: pretreated saliva (A) and pretreated saliva spiked with 2 μg of BK (B).

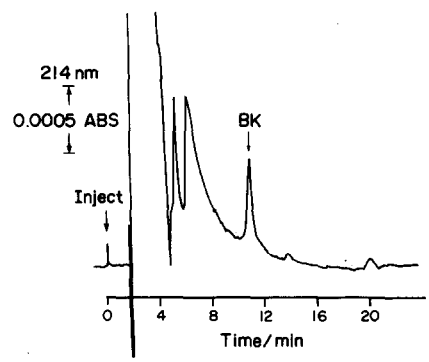

Figure 2
Chromatogram with HPLC system II. The sample was prepared according to the method described in text.

Recovery of BK added to a blank solution at a concentration of 5 ng ml^{-1} followed by ultrafiltration and SEP-PAK C_{18} treatment [7] was about 90% and the total recovery was about 85%.

Chromatographic studies

Reversed-phase HPLC conditions were used for the isolation and quantitation procedures. Several types of stationary phase were compared for peak shapes given by BK. It was found that the 'silanol effect' was not desirable to obtain a good shape peak, and the 'capped' material μ Bondapak C_{18} was chosen. The silanol effect was further suppressed by adding triethylamine to the mobile phase. For HPLC system I, however, it was found that this reduced sensitivity to BK at 214 nm, so that 254 nm was preferred. In HPLC system II, Low UV PIC-A was used instead of PIC-A and triethylamine was omitted. This permitted 214 nm to be used with improved sensitivity.

Using authentic samples containing BK, kallidin and Met–Lys–bradykinin, the peak in Fig. 2 was considered to be formed only by the BK.

Standard curve and application to saliva

A standard curve for the authentic BK solution was linear over the range 0–50 ng and passed through or close to the origin. The reproducibility at 5 ng ml^{-1} ($n = 5$) was 5.19 ±

0.30 (mean ±S.D.). The lower limit of detection was about 2 ng per injection. The concentration of the BK in filtrated saliva from clinical healthy subjects was from 0.9 to 7.3 ng ml^{-1} ($n = 5$).

Conclusion

An HPLC method has been developed in which the BK in human saliva can be separated and quantified. Since this method required no complicated techniques or radioactive compounds, it is considered to be suitable for routine assays. We use this method in periodontology studies and the results will be published in the near future.

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[*Received for review 6 March 1985; revised manuscript received 2 July 1985*]