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High-performance liquid chromatographic determination of bradykinin in saliva: a critical review and a new method

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Abstract

Because of difficulties or dubious results with previously published methodologies, a new semi-automated HPLC method with UV absorbance detection was developed and applied to the determination of bradykinin (BK) in human saliva. The new method consisted of an uncomplicated sample preparation involving the addition to saliva of an equal volume of 0.1 *M* orthophosphoric acid to stabilize BK, vortex-mixing, centrifugation, and separation, followed by chromatography of the supernatant phase on a C_8, 150×3.9-mm (I.D.) stainless steel column. The mobile phase was composed of 19% acetonitrile/0.1% trifluoroacetic acid at flow-rate of 0.4 ml/min. Using UV detection at 220 nm, the detection limit was 1 ng/ml for the BK standard, and 7 ng/ml for the assay of endogenous salivary BK. The orthophosphoric acid initially added to the saliva allowed BK to be stabilized from enzymic degradation at 20°C for 5 days and at 4°C for 60 days. Assignment made to the peak with the chromatographic properties of salivary BK was confirmed by HPLC–MS with an electrospray interface. This paper presents a new method that is reproducible, reliable and allows kinetic studies of salivary BK to be performed for clinical investigations.

Keywords: Bradykinin

1. Introduction

Bradykinin (BK), a highly active nonapeptide (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg), has many biological roles. Its pharmacological effects include cardiovascular and algogenic actions, the latter being associated with increased capillary permeability, production of edema, and the initiation of pain and associated reflexes [1]. BK is formed by the action of the enzyme kallikrein on high-molecular weight kininogen [2]. In plasma, BK has a half-life of only a few seconds [3]. In saliva, kallikrein has been identified [4–7] but the presence of endogenous salivary BK has not previously been confirmed with absolute methods.

Two methods using high-performance liquid chromatography (HPLC) have been previously described for analysis of BK in donated saliva. One method was based upon ultraviolet (UV) detection and *o*-phthalaldehyde (OPA) derivatized fluorescence detection [8,9]. Another method used benzoin as the derivatizing fluorophore to tag arginine-containing

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peptides [10–12]. Our aim was to use measured salivary BK as part of an ongoing clinical development program for pain diagnosis and treatment strategies. Accordingly, we used these published methods for salivary BK but found that neither could be replicated. Because we were concerned that the methodology and results obtained could be dubious, we developed and applied a new assay. Hence, the first part of this report provides a critical summary of our results with the previously published methods; the second part describes a new, simple method of sample preparation that offers a simple method for stabilizing salivary BK and a semi-automated method using HPLC–UV detection (HPLC–UV) for its measurement. In the process, we needed confirmation of our chromatographic assignment to salivary BK and made this by HPLC coupled to mass spectroscopy (HPLC–MS). Preliminary clinical data based upon application of the method are presented in this report: the results of a clinical kinetic study are to be presented separately.

1.1. Critical review of previously published methods

1.1.1. Omori et al. [8] (UV detection)

Omori et al. [8] used HPLC–UV to measure concentrations of BK in the saliva of healthy volunteer subjects and patients suffering from periodontal disease. The method required the use of two HPLC assays, with peak collection and evaporation, to concentrate the BK: it was thus very time consuming. Omori et al. [8] used an ultrafiltration membrane (10 000 MW cutoff, Immersible CX-10, Millipore) to separate BK from the enzymes responsible for its metabolism: they reported 90% recovery of BK standard in water that we confirmed. However, in our preliminary work, we found that when saliva was spiked with BK (50 ng), approximately 90% of the BK was held up in the filter (Acrodisc PF 0.2 μm, 25 mm diameter polysulfone low protein binding filter, Gelman Sciences, Ann Arbor, MI, USA). Omori et al. did not report the recovery of BK from saliva using filters, only from the standard in water, thus casting doubt as to the concentrations of salivary BK reported in patients from their clinical study. The difference in recovery may, in part, be due to the higher viscosity of saliva and/or salivary constituents. The original ultrafiltration membranes used by Omori et al. were no longer available for our study, but we assumed that the more recent replacement low protein binding filters would be at least comparable with the original membranes in not retaining the peptide of interest (BK). Due to the low recovery of BK found, we discontinued the use of filtration devices for the pretreatment of saliva.

1.1.2. Omori et al. [9] (fluorescence detection with OPA)

OPA has been used for rapid (<1 min) pre-column and post-column derivatization of many biogenic amines including histamine, noradrenaline and serotonin [13], and amino acids [14,15]. Only one study by Omori et al. [9] has described OPA for derivatization of polypeptides. They used OPA in a post-column fluorescence detection method (340 nm excitation, 476 nm emission) for salivary BK, and reported that an approximate 10-fold increase in sensitivity was achieved over UV detection (0.16 ng BK with fluorescence detection [9], 2 ng BK with UV detection [8]). Omori et al. [9] used a post-column reaction at 50°C for 5 min, followed by cooling at 20°C for 2 min in mildly basic pH conditions (pH 9–9.5); however, the method was found to be problematic from a number of standpoints. First, sample pretreatment of saliva employed the same 10 000 MW cut-off filters. Second, we were unable to reproduce the formation of an OPA-BK derivative as reported by Omori et al. [9]. Third, Omori et al. [9] displayed two representative chromatograms in their article: one of the designated BK peak in a healthy subject, the other of the BK peak in a subject with periodontal disease which was 30-fold higher in peak height. However, in their results, they reported that only a 2- to 3-fold increase existed in BK from the periodontal disease group compared with the healthy group. Fourth, the published chromatograms surprisingly showed no peak broadening as would be expected from the 7-min reaction time between column and detector. The publication, a Short Communication, unfortunately did not contain sufficient information or data to rationalise the difficulties that we subsequently found in our attempt to reproduce this method.

1.1.3. Kai et al. [10–12] (fluorescence detection with benzoin)

Benzoin is used for pre-column derivatization in alkaline conditions, with 2-mercaptoethanol to stabilize the derivative. Excitation and emission maxima for the product were found to be 325 and 435 nm, respectively, and sensitivity for benzoin products have been found to be approximately 100-times higher than other fluorogenic products including fluorescamine, OPA and ninhydrin [11]. Benzoin has been investigated as a fluorogenic reagent for several arginine-containing peptides including tuftsin, substance P, angiotensin I, II and III [12], but its reaction with BK has not been reported. Considering that other arginine-containing peptides have been successfully tagged by benzoin, the method described by Kai et al. [10–12] was evaluated for its potential in forming a benzoin-BK product.

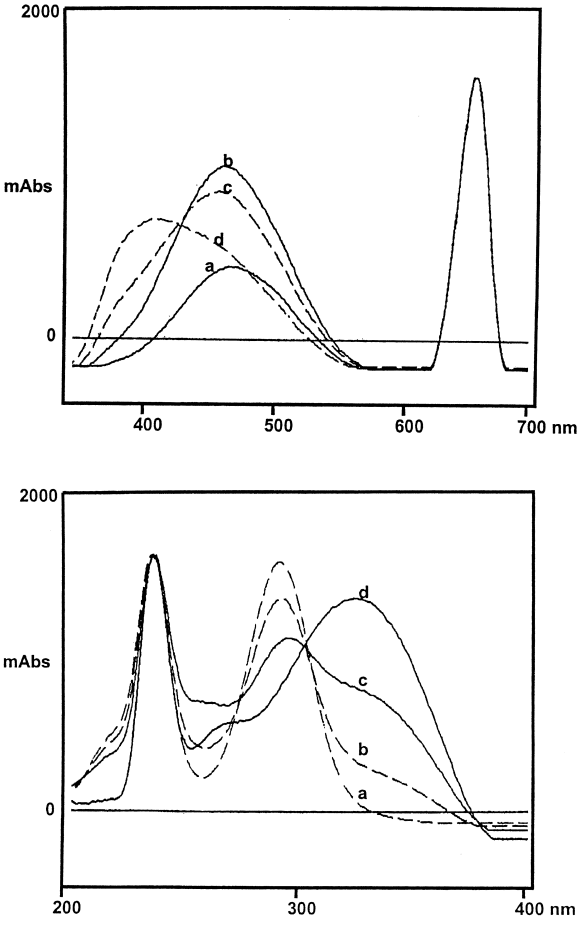

Fig. 1. Excitation and emission spectra of benzoin-mercaptoethanol in fluorescence detector flow cell (stopped flow) indicating the intrinsic instability of benzoin. (Lower) excitation scans (200–400 nm) of benzoin-mercaptoethanol: (a) immediately after injection into detector, (b) at 30 s, (c) at 90 s, and (d) at 180 s. The reagent was found to be unstable under excitation at various wavelengths including 325 nm. (Upper) Emission scans (350–700 nm) of benzoin-mercaptoethanol at excitation 325 nm shows the instability of emission spectra over time: (a) immediately after injection into detector, (b) at 30 s, (c) at 90 s, and (d) at 180 s. Emission at 435 nm shows instability.

During the course of confirming the optimal excitation and emission spectra for benzoin we noticed marked changes in absorbance of benzoin in the detector cell, thus raising questions over the validity of benzoin as a stable compound useful for tagging peptides, particularly in a postcolumn method where the fluorescent product moves slowly through the detector cell. An analysis of excitation and emission spectra of benzoin-mercaptoethanol showed the derivatization medium to be very unstable in the fluorescence detector cell. In particular, there was a marked time-dependent increase in intensity for both excitation and emission spectra indicating that flow-rate and/or residence time of the derivatization product in the detector flow cell may have been substantially affecting the peak size (area and height), and thus the apparent peptide concentrations (Fig. 1).

2. Methods: development using HPLC–UV and HPLC–MS

2.1. Experimental

2.1.1. Materials and reagents

BK, arginine-vasopressin, kallidin and substance P (purchased from Auspep, Parkville, VIC, Australia) were stored at −80°C until use. Stock solutions of BK, prepared by dissolving in Milli-Q water (1 mg/ml) and stored at 4°C away from light, were stable for 10 days. From the stock solution, working concentrations of BK at 100, 10 and 1 μg/ml, and 500, 250 and 100 ng/ml were prepared by serial dilution with Milli-Q water. HPLC grade reagents and their suppliers were: acetonitrile (BioLab Scientific, Melbourne, VIC, Australia), methanol (EM Science, Gibbstown, NJ, USA), glacial acetic acid

(Ajax Chemicals, Sydney, NSW, Australia), and trifluoroacetic acid (TFA) (Sigma, Sydney, NSW, Australia). Water was purified by a Milli-Q system (Millipore, Sydney, NSW, Australia). Nitrogen gas (5.0, BOC, Sydney, NSW, Australia) was of ultra-high purity.

2.2. Instrumentation

2.2.1. HPLC–UV

Analyses were performed on a Shimadzu HPLC system equipped with an autosampler and UV detector. The system was comprised of a LC 10AD model dual piston pump, CBM 10A controller and SPD 10A programmable UV detector, controlled by Shimadzu HPLC version 1.0 software and a 486 PC. Peak area and peak height calculations were performed using the same software. The chromatographic conditions are described in Section 2.4.1. For data analysis, calibration graphs of absorbance against amount of BK were subsequently fitted using least-squares linear regression. Peak homogeneity was determined on a Hewlett-Packard 1100 Series HPLC with diode array detection (DAD) using the same column and chromatographic conditions.

2.2.2. HPLC–MS

Mass spectrometric analyses were performed on a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer (MS) equipped with an electrospray interface and controlled by version 4.0 software running on an Alpha DEC-term workstation with a UNIX operating system. The solvent delivery system was an Applied Biosystems 140B dual 10-ml syringe system (Foster City, CA, USA) coupled to the MS by the electrospray interface. The autosampler consisted of a Gilson Dilutor 401 and a Gilson 232 Bio Sample Injector with a 20-μl loop (Villiers, Le Bel, France); 10-μl injections were used.

2.3. Sample preparation

2.3.1. HPLC–UV

Saliva (~1 g) was collected by having a subject spit into a 10-ml polyethylene centrifuge tube (Crown Scientific, Sydney, NSW, Australia); 0.5 ml saliva was then transferred into a second tube to which was added 0.5 ml 0.1 *M* orthophosphoric acid. The pH of the saliva mixture was measured, the tube capped, vortex-mixed for 30 s, then centrifuged at 1500 *g* for 5 min to separate particulate matter such as food debris.

2.3.2. HPLC–MS

Saliva (1 ml) was collected and 1 ml 1% aqueous acetic acid was added to stabilize BK from enzymic degradation, 10 ml Milli-Q water was added, followed by vortex-mixing, centrifugation and the decanting of the top layer from oral debris, as described in Section 2.3.1. To improve MS performance, the analysis of salivary BK required the prior removal of endogenous salts. These were removed from the supernatant by solid-phase extraction (SPE) using a C_{18} Extract-clean Highflow solid-phase extraction cartridge (Alltech, Deerfield, IL, USA) under vacuum, the cartridge having been preconditioned with 25 ml 50% acetonitrile/1% aqueous acetic acid. The elution of unwanted salts was carried out by loading the cartridge with 10 ml Milli-Q water and discarding the eluant. Elution of endogenous BK was subsequently effected with 5 ml 50% acetonitrile/1% aqueous acetic acid. Finally, the retained BK-containing eluant was concentrated by evaporation under nitrogen and reconstituted in 100 μl mobile phase A (see Section 2.4.3).

2.4. Chromatographic methods

2.4.1. HPLC–UV and effect of pH on UV absorbance

The mobile phase was composed of 19% acetonitrile/0.1% TFA and was pumped isocratically at a flow-rate of 0.4 ml/min. The column was a Waters C_8, 7 μm particle size, 100 Å pore size, 150×3.9 mm (I.D.) stainless steel, column (Waters, Sydney, Australia), used at room temperature. The analytical absorbance wavelength of the UV detector was set at 220 nm.

The UV absorbance characteristics of peptides can be influenced by the surrounding medium. In particular, ion-pairing of H^+ with basic terminal amino acids occurs at low pH and this conveys two advantages for reversed-phase chromatography: increased sensitivity, and greater separation of similar peptides [16]. As BK has two basic terminal amino

acid (arginine) residues, an analysis was carried out over a pH range (pH 2–7) to determine the extent to which sensitivity might be improved for BK under UV detection. Aliquots of Milli-Q water were adjusted with 0.1 *M* H_3PO_4 and 0.1 *M* NaOH to give samples at each of pH 2, 3, 4, 5, 6, 7, and spiked with BK at 50 ng per injection; BK in Milli-Q water was used as a control.

Peak homogeneity, at DAD detector wavelengths of 210, 220 and 280 nm, was ascertained with the threshold for peak purity being set at 900 and 1 mAU, and five spectra being analysed across the native salivary BK peak.

2.4.2. Direct infusion MS

Direct infusion electrospray mass spectra utilized a Harvard Apparatus Syringe Infusion Pump 22 (South Natick, MA, USA) operating at a flow-rate of 5 μl/min directed into the electrospray interface. The *m*/*z* scan range for the direct infusion was 200–600 with a scan rate of 3 s.

2.4.3. HPLC–MS

Gradient chromatography was carried out such that mobile phase B increased 0–100% over 60 min: mobile phase A (13% methanol/1% aqueous acetic acid), mobile phase B (80% methanol/1% aqueous acetic acid). The flow-rate was 0.2 ml/min with a 20:1 split, thus admitting 10 μl/min into the electrospray interface. An Alltima C_{18}, 5 μm particle size, 100 Å pore size, 150×2.1 mm (I.D.), stainless steel column (Deerfield, IL, USA) with an Alpha Resources Opti-guard C_{18} guard column, 20–40 μm particle size, 15×1 mm (I.D.) (Sydney, NSW, Australia) were used at room temperature. The pressure of the sheath gas (nitrogen) was 414 kPa (=60 p.s.i.). The electrospray ionization interface was operated in positive ion mode with a probe voltage of +4.5 kV. Full scan mode employed a mass to charge ratio (*m*/*z*) range from 400 to 700 with a scan rate of 2 s.

2.5. BK stability study

Using an ex vivo method, six volunteer subjects (three males, three females, age range 23–40 years) each donated 0.5 ml saliva to which 0.5 ml 0.1 *M* orthophosphoric acid was added; each sample was then vortex-mixed, centrifuged and the pH recorded as described in Section 2.3.1. Each sample was then divided into two aliquots, each aliquot spiked with BK to give either 50 or 500 ng BK per HPLC injection. Each aliquot was then subdivided into two further aliquots for storage at 20 or 4°C, and stability assessed over 5 and 60 days, respectively. BK concentrations were measured using the method as described in Section 2.4.1.

2.6. Salivary BK pilot study in healthy volunteer subjects

To assess whether the developed method had the sensitivity required for the clinical application we carried out a pilot study on five healthy volunteer subjects by collecting saliva at two time points (10:00 and 15:00 h) for three consecutive days and assaying the samples for BK as described.

3. Results

3.1. HPLC–UV method: optimization of pH conditions and analyte identity

H^+ ion pairing-pH optimization gave approximately 5-fold greater peak area for BK at pH between pH 2 and 5 compared to BK in Milli-Q water, with pH 3 being the optimal pH to assay salivary BK ($P<0.05$, ANOVA, followed by Tukey post hoc comparison).

The retention time (t_R) of the BK standard was 22.5 min (Fig. 2). The sample of saliva showed the native BK peak to be eluting at 22.5 min with good separation from other native peaks (Fig. 3); two other endogenous peaks at $t_R=16.9$ and 27.5 min acted as readily identifiable internal salivary markers concerning any slight day-to-day changes in t_R of BK. The method was selective for BK in saliva in that there was no interference from other standard peptides with a terminal arginine residue including arginine-vasopressin ($t_R=7.3$ min), kallidin (lysine-BK) ($t_R=15.3$ min), and substance P ($t_R=36.0$ min). However, there was a lack of spectral selectivity for BK at 220 nm with arginine-vasopressin, kallidin and substance P all demonstrating similar absorbance spectra over the range 200–260 nm. Peak homo-

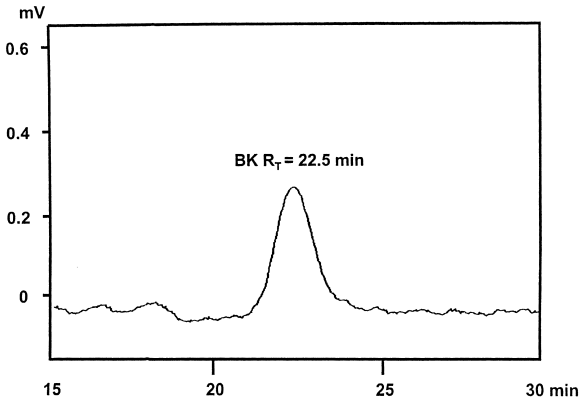

Fig. 2. Assay of the BK standard peptide using HPLC–UV shows a retention time (t_R) of 22.5 min.

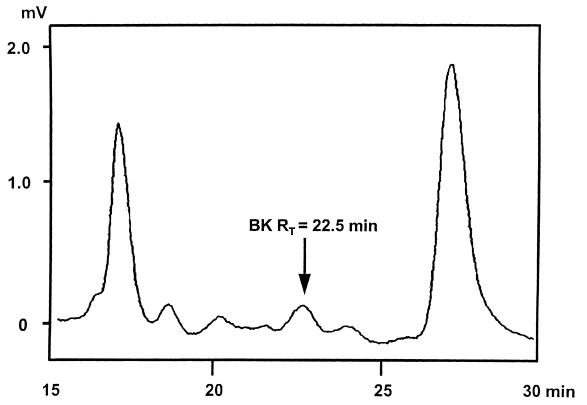

Fig. 3. Assay of saliva using HPLC–UV showing a native peak (arrowed) eluting at a retention time (t_R) of 22.5 min.

geneity analyses, determined by DAD, showed that the purity of the BK peak exceeded the specified threshold, confirming that there were no co-eluting substances.

3.2. HPLC–UV method: linearity, recovery, accuracy and precision

The assay was linear from 1 to 20 ng of BK as determined by least-squares linear regression [equation, mean (±SD): $y=3608(\pm 154)x$, $n=6$; $r^2=0.995$]. The coefficient of variation of the assay was found to be 4.7% at 1 ng/ml, 4.6% at 5 ng/ml, and 2% at 20 ng/ml. The limit of detection of BK was 1 ng on column: this was established by injection of saliva samples to which BK was added in decreasing concentration until a signal-to-noise ratio (S/N) of 3:1 was reached. The limit of quantitation of BK in saliva samples was 7 ng/ml: this was based on the sample preparation described in Section 2.3.1 using 0.5 ml 0.1 *M* orthophosphoric acid added to 0.5 ml spiked saliva, with a maximum HPLC injector volume of 50 μl. The recovery of BK for the sample extraction procedure using the 50 ng and 500 ng BK-spiked samples ($n=6$) was 97.6±4.3 and 99.9±5.5%, respectively. The accuracy of the method, determined at three concentrations with three replicates per concentration, was 85–97% (Table 1). The intra-day precision, determined at three concentrations (three replicates per concentration), was 2.2–4.3% at 25 ng, 8.9–12.6% at 50 ng, and 2% at 500 ng. The inter-day precision was −2.3–1.4% at 25 ng, −0.1–4.9% at 50 ng, and 4–6.7% at 500 ng. The residual variability was between 4.2 and 6.3% for intra-day precision, and 0.3–3.7% for inter-day precision (ANOVA, with repeated measurements).

To determine the ruggedness of the method for peak area at 50 and 500 ng of BK (three replicates per concentration) and for t_R of BK, a second Waters column of the same specification (Lot number T43501) was compared to the column used through-

Table 1
Accuracy (mean and relative SD) for the analysis of bradykinin in spiked saliva samples

BK concentration of sample	BK standard in 0.1% TFA (mean peak area (RSD) per HPLC injection)	Saliva spiked with BK standard in 0.1%TFA (mean peak area (RSD) per HPLC injection)	Accuracy (%)	n[a]
50	12206 (93)	10505 (544)	86	3
250	23528 (463)	22815 (872)	97	3
500	49625 (370)	42209 (233)	85	3

[a] *n*, number of replicates.

out the study (Lot number T41231). The t_R on the second column was 20.8 min. There was no significant difference for peak area between the columns at 50 ng (Student's *t*-test, $P=0.07$; T43501 = 105±3.1% of T41231) but significant difference in peak area was found at 500 ng (Student's *t*-test, $P=0.03$; T43501 = 102±0.7% of T41231).

3.3. Stability

The addition of an equal volume of 0.1 *M* orthophosphoric acid to saliva resulted in the mixture having a pH in the range of pH 2–4.5. At pH 2, saliva spiked with BK at two concentrations (50 and 500 ng per injection) was stable for 5 days at 20°C and for 60 days at 4°C (Table 2). Kruskal–Wallis one-way ANOVA on ranks indicated that there were no significant differences between the peak values obtained at the different time points (n = saliva samples from five subjects with five replicates at 50 ng and six replicates at 500 ng per time period). The high recovery rates (>100%) in a number of samples may be explained by adjacent endogenous small peaks being integrated with the much larger BK peaks in the spiked samples, or by slow synthesis of BK from degradation of a parent compound in the samples occurring at low pH. Further analysis was not carried out to identify the source of any candidate peaks. The stability gained by the addition of the orthophosphoric acid to saliva allowed the endogenous BK concentrations to be measured with negligible loss, precluded the need for enzyme inhibitors in subsequent clinical studies, and allowed automated HPLC assay.

Table 2
Stability of bradykinin spiked into saliva with different storage conditions

BK concentration of spiked sample (per HPLC injection) and storage condition	Recovery (%)	RSD[a] (%)	*n*
50 ng at 20°C			
1 day	97.3	4.9	5
2 days	98.8	5.9	5
3 days	99.8	3.3	5
5 days	103.6	3.4	5
50 ng at 4°C			
10 days	109.2	17.6	5
20 days	112.4	11.2	5
30 days	108	10.7	5
60 days	106.6	13.2	5
500 ng at 20°C			6
1 day	106.2	2.4	6
2 days	103	2.3	6
3 days	105.1	3.1	6
5 days	107.5	8.4	6
500 ng at 4°C			
10 days	107	3.6	6
20 days	108.7	6.4	6
30 days	110.2	3.1	6
60 days	107.1	4.2	6

[a] RSD (%), relative standard deviation; *n*, number of replicates.

3.4. MS and HPLC–MS

Direct infusion of BK standard showed it to have a base peak at a mass to charge (m/z) ratio of 531. The m/z ratio observed was for the double-charged molecular ion of BK, $[M+2H]^{2+}$. The t_R of BK standard was 27.2 min. Blank injections made after the peptide standard showed no carry over to the next injection. Employing the described HPLC–MS conditions, a native peak in saliva was detected with a m/z of 531 and with a t_R of 27.4 min (Fig. 4). This is similar to the t_R of the BK standard of 27.2 min and the minor variability observed can be attributed to the long assay time used (60 min). The HPLC–MS method thus provided confirmation that saliva contained endogenous BK.

3.5. Pilot study of salivary BK in healthy volunteer subjects

Twenty-three out of 30 samples (from four of the five healthy volunteer subjects) had measurable concentrations of endogenous BK (Table 3).

4. Discussion

We required a method for the determination of BK in saliva for use in a clinical research program that involves studies of pain and pain relieving drugs. The pilot study on five healthy volunteer subjects indicates that the developed method had the sensitivity required for the clinical application. Briefly, from our ongoing use of the method, we report that

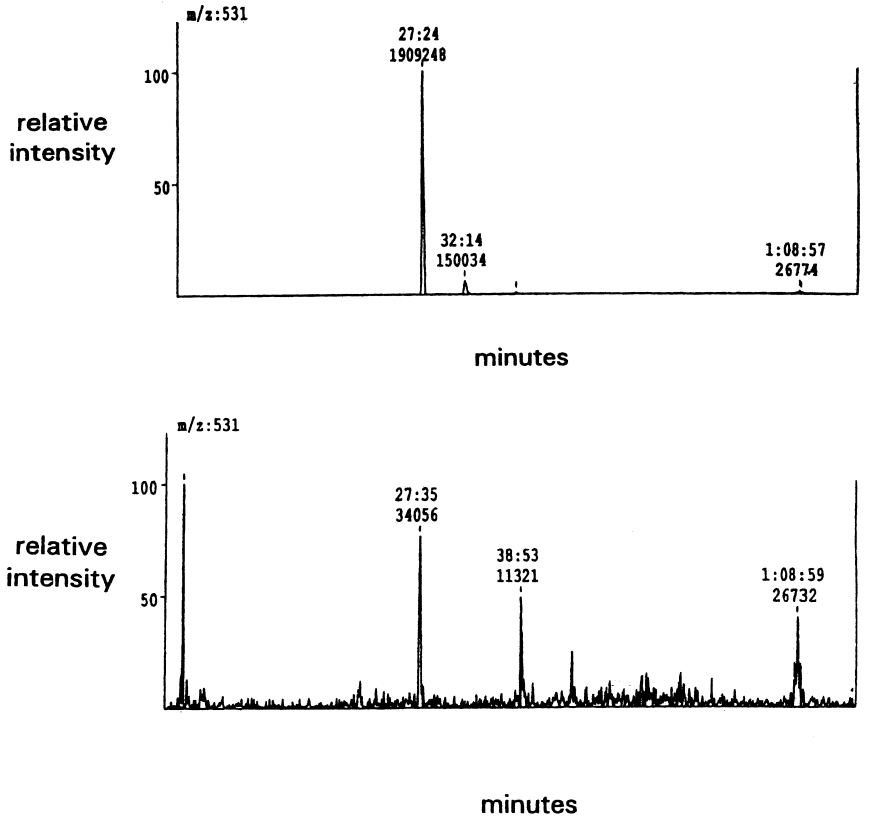

Fig. 4. (Upper) Assay of the BK standard using HPLC–MS in full scan mode (m/z 400–700) shows a retention time at 27.24 min. (Lower) Assay of saliva using HPLC–MS in full scan mode (m/z 400–700) shows a native peak eluting at a similar time (27.35 min) that has an identical m/z as the BK standard.

patients with pain have higher mean salivary BK concentrations (493±SD 358 ng/ml) than the healthy volunteer subjects (Vickers ER, PhD Thesis, University of Sydney, 1999) and we are currently conducting clinical pharmacokinetic studies of salivary BK for which this developed method is suitable. Such data will be reported separately. From a clinical perspective, there are considerable advantages in collecting saliva as an alternative to blood. These include: (i) excellent subject compliance, (ii) ease of obtaining repeated samples, (iii) pain-free technique, and (iv) avoidance of the risk of needle-stick injury.

Although the new methodology described for the salivary BK assay is relatively simple and this may suggest that its development was straightforward, it was, however, a complex project for several reasons.

First, our initial aim was to replicate and apply previously published methodology to a clinical study. However, as discussed above, the previous methods [8–12] were problematic. Moreover, a major difficulty is caused by the extremely rapid degradation of BK. Thus, the method needed to provide for stabilization of BK to allow automated HPLC analysis of many samples.

Second, there is a paucity of information applicable to preparing saliva in a form suitable for HPLC and HPLC–MS analysis. Given the difficulties with application of the previously published methods, it was considered important that chromatographic assignment of the BK peak be confirmed with an independent method (MS analysis). The HPLC–MS equipment required the removal of salts prior to analysis: saliva has high salt concentrations that act as the primary buffering mechanism to prevent tooth

Table 3
Variations of salivary bradykinin concentrations in healthy volunteer subjects

Subject	Day	Time	BK concentration (ng/ml)
Male	1	10:00 h	<7
35 years		15:00 h	<7
	2	10:00 h	<7
		15:00 h	<7
	3	10:00 h	<7
		15:00 h	<7
Female	1	10:00 h	23
40 years		15:00 h	19
	2	10:00 h	21
		15:00 h	<7
	3	10:00 h	46
		15:00 h	36
Female	1	10:00 h	92
38 years		15:00 h	104
	2	10:00 h	49
		15:00 h	61
	3	10:00 h	27
		15:00 h	12
Female	1	10:00 h	39
29 years		15:00 h	16
	2	10:00 h	16
		15:00 h	33
	3	10:00 h	11
		15:00 h	18
Male	1	10:00 h	9
43 years		15:00 h	21
	2	10:00 h	47
		15:00 h	132
	3	10:00 h	32
		15:00 h	33

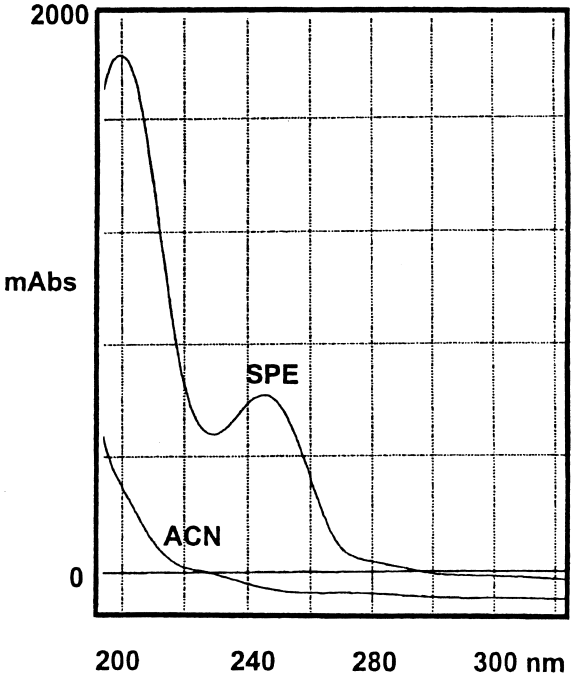

Fig. 5. UV scans of acetonitrile before preconditioning the SPE cartridge (ACN) and after 5 ml acetonitrile passing through the cartridge (SPE). The UV scans clearly show material in the acetonitrile after prewashing: this subsequently reduced MS performance. The UV scan of acetonitrile returned to its original spectrum after 25 ml of preconditioning.

enamel dissolution. SPE was considered the most suitable method to remove the salts. However, following the manufacturer's recommendations of preconditioning the SPE cartridge with 3–5 ml methanol, there was insufficient MS sensitivity to establish the identification of salivary native BK peak. It was not until 25 ml of acetonitrile were used to precondition the SPE cartridges that there was adequate MS sensitivity. The technique that we employed compared UV scans of acetonitrile before preconditioning the cartridge to acetonitrile eluent collected after preconditioning (Fig. 5). These scans clearly showed that there was extraneous material eluting from the SPE cartridge and this markedly reduced the sensitivity of the MS analysis. Satisfactory MS performance was possible only after washing the SPE with 30 ml acetonitrile. We found that comparing UV scans provided a simple and effective method to determine the correct preconditioning volume of acetonitrile necessary to rid the SPE cartridges of extraneous material and which we now use as a standard procedure.

Third, the separation of BK from other salivary constituents required extensive testing of the stationary and mobile phases. C_{18} and cation exchange (weak and strong for the terminal BK arginine residues) columns could not provide separation of BK from other salivary constituents, whereas only the C_8 column accomplished the separation. Moreover, the t_R of BK was found to be quite susceptible to relatively minor changes (0.5–1%) in the acetonitrile concentration that moved the BK peak into other

interfering endogenous salivary peaks. However, once BK was resolved from the adjacent peaks (on the C_8 column), the presence of the adjacent endogenous substances acted as internal surrogates that were stable at low pH.

The majority of published HPLC methodologies using saliva as an analyte matrix have concentrated on assaying drugs. However, there are few experimental data available on methodology for endogenous analytes that may be subject to enzymic degradation. Our concern about the potentially rapid degradation of salivary BK, based on the knowledge of its plasma kinetics, required a rapid, but simple, method for its stabilization after donation of saliva. In particular, we preferred to find an alternative to the addition of organic enzyme inhibitors. The addition of an equal or greater volume of orthophosphoric acid or 0.1% TFA to the sample achieved the stabilization necessary for clinical research purposes.

A principal alternative for measuring BK is immunoassay and plasma BK concentrations have been measured using this technique [17]. A potential disadvantage with immunoassay is that the antisera to BK can also bind to kininogen, the precursor of BK, that is present in much greater concentration than BK [17]. Although ethanol has been used to precipitate kininogen to overcome this problem, this consequently limits the use of immunoassay in prospective kinetic studies investigating both BK and kininogen. Furthermore, we considered that the inclusion of ethanol may also precipitate any (added) enzyme inhibitors for stabilising salivary BK. Hence, our chromatographic assay for BK offers an alternative to immunoassay for specific research applications, or can be used to concentrate BK for subsequent immunoassay techniques should kits become commercially available.

In summary, a HPLC–UV method that is reliable and suitable for semi-automation has been developed for the measurement of salivary BK concentrations. The method described is also applicable to similar endogenous analytes in saliva by adjusting the acetonitrile concentration in the mobile phase. The sample preparation is simple and offers excellent stability of salivary BK. The developed HPLC–UV method has found application in our clinical development program examining BK concentrations and the pharmacokinetics of BK in healthy volunteer subjects and specific patient groups.

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