High-Performance Liquid Chromatographic Analysis with Diode-Array Detection of Bradykinin, Neuropeptide K, and Substance P in Human Plasma

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Abstract

A method is described for the determination of bradykinin, neuropeptide K (NPK), and substance P in patients with atypical carcinoid syndrome. The developed method uses a combination of conventional and solid-phase extraction as well as high-performance liquid chromatographic techniques. A narrow-bore C_{18} column with ultraviolet detection is used (200 nm). The technique recovers bradykinin at a level of 98%, NPK at 96%, and substance P at 98% (when pure standards are dissolved) at concentration levels relevant to the atypical carcinoid syndrome. In biological samples, the recovery rate of bradykinin, NPK, and substance P drops to 88, 86, and 88% respectively. The overall analysis time is 150 min from receipt of samples. This method proves to be a valuable tool in the identification of neuropeptides and thus the diagnosis of atypical carcinoid syndrome, especially in puzzling cases with nonspecific symptoms.

Introduction

Bradykinin (COOH-Arg-Pro-Pro-Gly-Phe-Ser-Phe-Arg-NH_2), neuropeptide K (NPK) (COOH-Met-Leu-Gly-Val-Phe-Ser-Asp-Thr-Lys-His-Arg-Lys-Ser-Leu-Gln-Gly-His-Gly-TyrLeu-Ala-Lys-Leu-Leu-Ala-Val-Glu-Lys-Glu-Ile-Ser-Ser-Asp-Ala-Asp-NH_2), and substance P (SP) (COOH-Met-Leu-Gly-Phe-Gln-Gln-Pro-Lys-Pro-Arg-NH_2) are members of the tachykinin family of peptides (1). When produced in excess, all three cause a serious fall in blood pressure due to vasodilatation, edema of various organs (increased permeability of the blood vessels), stimulation of the immune system (direct effect on the lymphocytes), and inflammatory reactions and contraction of smooth muscles (2–4). When these symptoms appear, only specialized therapy with peptide analogues to block the peptide receptors in the tissues will be of any use (5). A correct diagnosis is thus essential to ensure a productive life.

It is possible to analyze bradykinin, NPK, and SP by radioimmuno assay (RIA) (6,7), but it is very time-consuming and expensive due to the required production of appropriate antiserum after cleanup of the peptides (usually by size-exclusion chromatography). Due to the chemical properties of the peptides, high-performance liquid chromatography (HPLC) offers an attractive alternative for analysis (8–13). An HPLC method was developed to analyze these substances in plasma. The main advantage using this method is that the shortened analysis time may be life-saving in certain cases (14).

A major problem in HPLC with ultraviolet (UV) detection is that the detector must operate at very low wavelengths (200–210 nm). This eliminates a large number of mobile phases (15). Various buffer systems were tried with acetonitrile as the organic modifier (15). Acetonitrile–water acidified with 0.1% trifluoroacetic acid (TFA) was found to be the best mobile phase for this specific analysis.

For determination in plasma, a method was developed using conventional and solid-phase extraction followed by reversed-phase HPLC separation with UV detection using a diode-array detector at 200 nm. A narrow-bore column was used due to the low concentrations and restricted volumes available in some cases. An anticoagulant and proteolytic degradation inhibitor cocktail were added to the Vacutube prior to sample collection.

Experimental

Materials

Acetone, TFA, acetonitrile, and acetic acid were all AR-grade and were obtained from Merck SA (Johannesburg, Gauteng, South Africa). Bradykinin, NPK, and SP were purchased from Sigma Chemicals (Johannesburg, Gauteng, South Africa). High-purity nitrogen and helium were purchased from Afrox SA (Johannesburg, Gauteng, South Africa). All glassware was obtained from Glass World South Africa (Johannesburg, Gauteng, South Africa). Diethyl ether was obtained from NT Laboratories (Midland, Gauteng, South Africa). The Seppak ODS and C_{18} minicolumns were purchased from Anatech South Africa (Johannesburg, Gauteng, South Africa). The water used was double glass-distilled and filtered through a 0.22-µm

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Millipore filter obtained from Anatech South Africa. The cocktail consisted of Heparin obtained from Merck South Africa, aprotonin from Bayer SA (Midland, Gauteng, South Africa), soyabean trypsin inhibitor from Sigma Chemicals, and polybrene and pepstatin from Sigma Chemicals. Kainic acid (2-carboxy-3-carboxymethyl-4-isopropenyl pyrrolidine) was purchased from Sigma Chemicals; its structure is shown in Figure 1.

Sample collection and extraction

Blood samples were collected in vacuum tubes containing the anticoagulant and proteolytic degradation inhibitor cocktail. The blood samples were immediately centrifuged at 1800 *g* for 15 min. To 5 mL of plasma, 1 mL of 1 mol/L acetic acid containing kainic acid monohydrate as the internal standard was added (200 pmol/L). This was mixed well. Acetone (5 mL) acidified with acetic acid (2 mL/1000 mL) was added for the removal of protein. The sample was then centrifuged at 30,000 *g* for 15 min. The acetone was removed by bubbling helium or nitrogen through the sample. Diethyl ether (5 mL) was added for lipid extraction. This step was repeated four times. The diethyl ether was then discarded, and the sample was loaded on a Seppak ODS minicolumn previously activated with methanol (2 mL) and washed with distilled water (5 mL). This was necessary for the removal of salts and sugars. This step was repeated a second time. Acetonitrile acidified with TFA was used as an eluant. The sample was evaporated to dryness by bubbling helium or nitrogen through the sample. The sample can be stored in this form for long periods at –40°C. The sample was redissolved in acetonitrile containing 0.1% TFA. The standards were prepared in the same way containing 0, 50, 100, 150, 200, 500, 1000, and 2000 pmol/L each of bradykinin, NPK, and SP. Then 1 µL was injected into the HPLC.

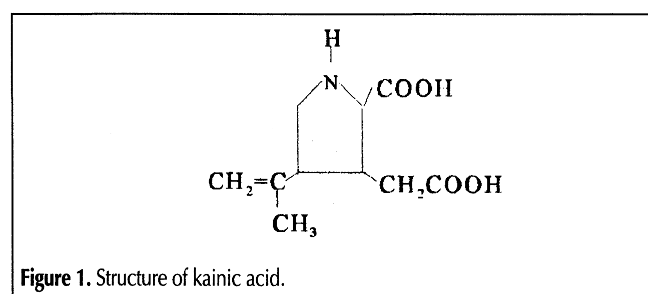

Figure 1. Structure of kainic acid.

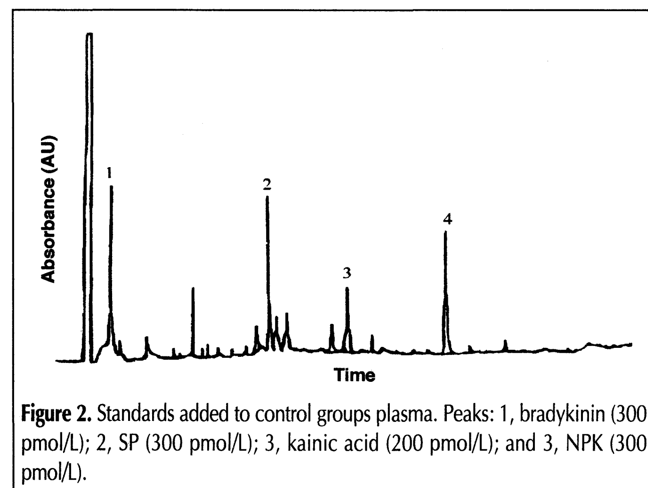

Figure 2. Standards added to control groups plasma. Peaks: 1, bradykinin (300 pmol/L); 2, SP (300 pmol/L); 3, kainic acid (200 pmol/L); and 3, NPK (300 pmol/L).

HPLC analysis

Analyses were performed with a Varian 9010 solvent delivery system and a Rheodyne 7125 injector with a 1-µL loop connected to a Nova Pak C_{18} narrow-bore column (4-µm particle size) supplied by Microsep SA (Johannesburg, Gauteng, South Africa) (2.1 × 150 mm). According to the literature, C_{18} is a very suitable column packing for protein and thus also peptide analysis (1–16). A sentry guard column (Nova Pak C_{18} Microsep SA) was used to protect the narrow-bore column. The bradykinin, NPK, and SP were detected at 200 nm with a Varian 9065 Polychrom diode-array detector modified with a 2-µL flow cell. From the UV spectra of all three compounds, the choice of 200 nm was made because all three showed good absorbance at 200 nm. SMM South Africa supplied all the Varian products except the flow cell. Elution of the peptides was done using a 90-min linear gradient of 20% acetonitrile in water containing 0.1% TFA to 60% acetonitrile in water containing 0.1% TFA. Acetonitrile was used due to its low absorbance at 200 nm. The whole system was computer-controlled by the Varian LC Star workstation. Quantitation was accomplished by comparing the peak areas of external standards with those of the sample. Figure 2 shows the chromatogram of standards added to a blank plasma sample.

Calibration

One aliquot (1 µL) of each calibration solution of bradykinin, NPK, and SP was injected, and the peak areas were determined.

Table I. Recovery

	Bradykinin				Substance P				Neuropeptide K			
Concentration (pmol/L)	Pure (pmol/L)	%	Plasma (pmol/L)	%	Pure (pmol/L)	%	Plasma (pmol/L)	%	Pure (pmol/L)	%	Plasma (pmol/L)	%
50	13	25	7	14	42	80	36	72	17	34	12	24
100	68	68	56	56	97	97	86	86	81	81	67	67
150	155	103	146	97	148	99	132	88	145	97	129	86
300	296	99	264	88	294	98	264	88	289	96	259	86
500	492	98	439	88	489	98	443	89	481	96	436	87
1000	979	98	885	89	982	98	884	88	959	96	861	86
2000	1964	98	1764	88	1968	98	1769	88	1922	96	1720	86

Results and Discussion

Recovery

The accuracy was assessed for each substance by measuring the recovery of known quantities of the substances added to the base (plasma previously tested negatively for all three substances).

Different amounts of the peptides were added to 5 mL (exactly measured with a certified pipette) of plasma. Each was submitted to the same sample preparation steps already described. The final concentration percentages of each peptide were calculated. Each sample was successively injected five times into the HPLC system. The mean percentage ratio of the experimentally measured concentration to the calculated concentration was determined for each peptide (five replicates) (Table I). The concentration range was 50–2000 pmol/L. The poor recoveries for bradykinin at 50 and 100 pmol/L concentrations can be attributed to the fact that these concentrations are close to or lower than the limits of detection (LOD) (57.0 pmol/L) and quantitation (LOQ) (190.0 pmol/L) of bradykinin. For SP, the recoveries at the low concentrations of 50 and 100 pmol/L were good because these concentrations were higher than the LOD (10.2 pmol/L) and LOQ (33.9 pmol/L) of SP. For NPK, the recoveries were intermediate between those for bradykinin and SP, as was expected because the LOD (40.8 pmol/L) and LOQ (136.0 pmol/L) of NPK was intermediate between the values of bradykinin and SP.

The three different compounds had the following mean recovery values in plasma: 88% for bradykinin, 86% for NPK, and 88% for SP. The results summarized in Table I show that a satisfactory degree of accuracy (86–88%) was achieved for all the compounds at concentration levels greater than the corresponding LOQs. An accuracy above 80% was acceptable for proteins (14). When the pure synthetic polypeptides were dissolved and analyzed, the following recovery rates were achieved: 98% for bradykinin, 96% for NPK, and 98% for SP. The loss of accuracy (± 10%) in biological samples reflected a matrix effect because quantitative recovery was achieved with the pure synthetic peptides. The absorbance of bradykinin was much better than the absorbances of NPK and SP (Table II). The reason for this probably lies in the structure of the peptides. Both bradykinin and NPK have two benzene rings per unit in their chemical structure, but the peptide chain in the case of NPK is substantially longer than bradykinin, and it is thus possible that folding may have taken place and masked the chromophores. SP only has one benzene ring per unit, but the peptide chain is very short.

Linearity of response

The linearity of response was confirmed for each peptide by injection of aliquots containing known differing amounts of each pure synthetic peptide. The standards contained 0, 50, 100, 150, 300, 500, 1000, and 2000 pmol/L each of bradykinin, SP, and NPK.

Table II. Absorbance

Peptide	Concentration (pmol/L)	Absorbance (AU) at 200 nm	Area
Bradykinin	2000	0.4334	69687637
SP	2000	0.0389	6118782
NPK	2000	0.0300	4721104

Table III. Statistical Evaluation of the HPLC Determination of Bradykinin, SP, and NPK

Parameter	Bradykinin	Substance P	Neuropeptide K
Correlation coefficient (r)	0.9997	1.0000	0.9998
r^2	0.9993	0.9999	0.9997
Slope (b)	35233	3065	2379
Intercept (a)	–563139	–8268	–26927
Regression line	$y = 35233x - 563139$	$y = 3065x - 8268$	$y = 2379x - 26927$
Random error (Sy/x)	669183	10386	32353
Slope error (Sb)	370	5.74	17.88
95% confidence limit of slope	35233 ± 905	3065 ± 14	2779 ± 44
95% confidence interval of slope	$34328 < b < 36137$	$3051 < b < 3079$	$2335 < b < 2422$
Intercept error (Sa)	303156	4705	14657
95% confidence limit of intercept	–563139 ± 741796	–8268 ± 11512	–26927 ± 35864
95% confidence interval of intercept	$-1304935 < a < 178657$	$-19781 < a < 3244$	$62791 < a < 8936$
LOD (pmol/L)	57.0	10.2	40.8
LOQ (pmol/L)	190.0	33.9	136
Experimental retention time (min)	13.62	48.45	88.12
Calculated retention time (min)	12.65	44.81	85.77

Table IV. Precision

Amount added (pmol/L)	Bradykinin		Substance P		Neuropeptide K	
	Average recovery	RSD (%)	Average recovery	RSD (%)	Average recovery	RSD (%)
50	9.4	51.00	53.40	18.2	8.6	26.000
100	55.00	27.00	87.00	3.30	67.20	22.700
150	144.80	1.30	133.60	1.40	128.00	3.780
300	265.20	0.05	264.60	0.09	260.40	0.500
500	441.00	0.04	449.00	0.05	436.80	0.020
1000	885.40	0.03	884.80	0.02	863.60	0.020
2000	1764.40	0.02	1768.60	0.02	1725.00	0.007

The correlation coefficient, standard error, slope, intercept, and corresponding confidence limits (p = 0.5) for each peptide were determined by regression and ANOVA analysis. The results are shown in Table III There was a linear correlation between the concentrations and the peak areas, as is shown by the correlation coeffiecients of 0.9997, 0.9999, and 0.9998 for bradykinin, SP, and NPK, respectively.

Precision

The precision of the assays was determined by carrying out replicate determinations for 0, 50, 100, 150, 300, 500, 1000, and 2000 pmol/L of peptides in plasma medium. Five aliquots were analyzed for each amount of each substance according to the sample preparation procedure already described. The average recovery and the relative standard deviation (RSD [%]) were calculated for all three compounds (Table IV).

The poor RSDs for bradykinin at 50 and 100 pmol/L concentration can be attributed once again to the fact that these concentrations were close to or lower than the LOD (57.0 pmol/L) and LOQ (190.0 pmol/L) of bradykinin.

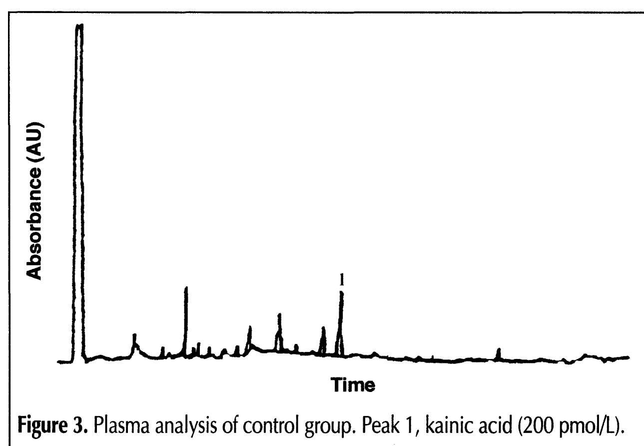

Figure 3. Plasma analysis of control group. Peak 1, kainic acid (200 pmol/L).

For substance P, the RSD at the low concentration of 50 pmol/L was acceptable because this concentration was higher than the LOD (10.2 pmol/L) and LOQ (33.9 pmol/L) of substance P.

For NPK, the RSDs were intermediate between those for bradykinin and SP for the same reasons discussed in the *Recovery* section.

Reproducibility

Each substance was injected five times successively into the HPLC system. The average area and RSD were calculated (Table V).

The reproducibilities were excellent, as manifested by the low RSD values (except for bradykinin and NPK for concentrations 50 and 100 pmol/L, respectively). The reason for this was that these concentrations were close to or lower than the corresponding LODs and LOQs of bradykinin and NPK.

Elution order of the peptides

According to Sasagawa et al. (16), the retention times of peptides depend on the interaction of the free silanol groups of the packing materials and the basic groups of the peptides and are predictable under certain experimental conditions. The following formulas can be used to calculate retention times.

$$T_{Ri} = A\ S\ D_j n_{ij} + B \qquad \text{Eq 1}$$

or

$$T_{Ri} = A\ (\ln[1 + \Sigma D_j n_{ij}]) + C \qquad \text{Eq 2}$$

where T_{Ri} is the retention time of peptide i; D_j is the retention constant of amino acid residue j; n_{ij} is the number of residue j in peptide i; and A, B, and C are constants. From this data, it seemed that the elution order would be as it appears on the chromatograms (Table III).

LOD

The LODs for all three compounds were calculated according to the following formula:

$$y_{LOD} = a + 3S_y/x \qquad \text{Eq 3}$$

where $y_{LOD} = bx_{LOD} + a$, a is the intercept, S_y/x is the standard error, and b is the slope (data from regression and ANOVA analysis, Table III). The LODs of the three compounds are summarized in Table III.

LOQ

The LOQ for all three compounds was calculated according to the following formulas summarized in Table III (data from regression and ANOVA analysis, Table III).

$$y_{LOQ} = a + 10S_y/x \qquad \text{Eq 4}$$

$$y_{LOQ} = bx_{LOQ} + a \qquad \text{Eq 5}$$

The LOQs of the three compounds are summarized in Table III.

Table V. Reproducibility

Amount added (pmol/L)	Bradykinin Average area	Bradykinin RSD (%)	Substance P Average area	Substance P RSD (%)	Neuropeptide K Average area	Neuropeptide K RSD (%)
50	302126	26.50	125465	10.10	55528	26.00
100	2012261	15.60	290931	3.10	159056	14.70
150	5226767	0.30	458897	0.20	354084	3.10
300	1045348	0.20	917828	0.07	708348	0.10
500	1742291	0.05	1529747	0.03	1180093	0.10
1000	3484383	0.03	3059401	0.10	2360574	0.20
2000	6968766	0.20	61118782	0.02	4721097	0.09

Table VI. Range of Peptides in Three Patients

Patient	Bradykinin (pmol/L)	SP (pmol/L)	NPK (pmol/L)
Control group	< LOD	< LOD	< LOD
1	< LOD	1160	368
2	460	288	1635
3	< LOD	< LOD	> 2000

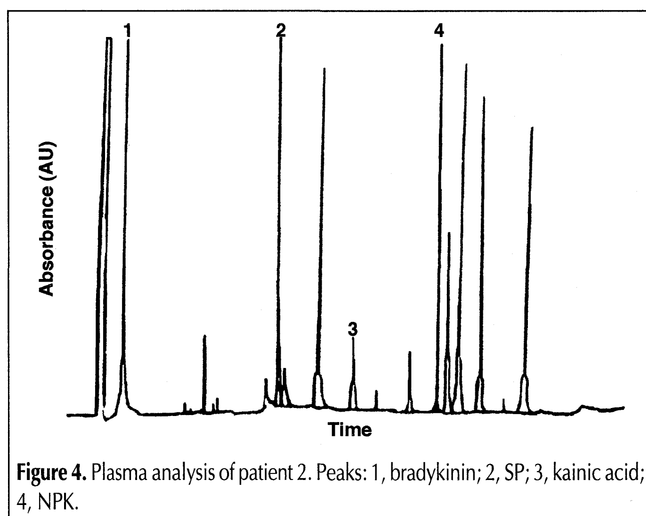

Figure 4. Plasma analysis of patient 2. Peaks: 1, bradykinin; 2, SP; 3, kainic acid; 4, NPK.

Case studies

The plasma of three patients with clinical diagnosis of atypical carcinoid syndrome were analyzed for all three substances and compared to samples of 15 individuals without any symptoms. (Figure 3). As compared with concentrations of bradykinin, NPK, and SP in the control group, all three patients had elevated concentrations of NPK (Table VI). One patient had an elevated concentration of bradykinin. Two patients had elevated concentrations of SP (Table VI). The chromatogram of patient 2 is shown in Figure 4. Patient 2 had severe episodes of flushing and edema. It must be noted that this patient had elevated levels of all three substances. The sample was obtained during such an episode. The flushing lasted for at least 45 min, and the edema for much longer (hours). All three patients had fluctuations in blood pressure ranging from 135/90 to 60/40. Patient 2 had the most severe fluctuations. The control group had values for all three compounds below the compound's LOQ.

Conclusion

The use of conventional extraction methods combined with solid-phase extraction, separation by HPLC, and UV detection was demonstrated to be a useful alternative to RIA detection in cases of atypical carcinoid syndrome. It must be noted that this method is not suitable for analysis of the three compounds below the LOQs.

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