Simplified Radioimmunoassay of Bradykinin in Human Plasma

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A greatly simplified radioimmunoassay for bradykinin in human plasma is described. Current techniques require multiple chromatographic steps or extraction procedures with analytical recoveries of bradykinin of often less than 60%. We present a method in which bradykinin is separated from components of higher relative molecular mass (including kininogens) in a single step, by use of a column of Sephadex G-25 medium (PD-10). The mean analytical recovery of tritiated bradykinin added to plasma is 85.5% (SD, 3.5%). The sensitivity of this radioimmunoassay is 25 pg per assay tube, equivalent to 125 ng per liter of plasma. Twenty to 30 blood samples may be completely processed and assayed within 6 h. As determined with this technique, concentrations of bradykinin in plasma from apparently normal subjects ranged from 2.5 to 5.2 µg/L (mean 4.2, SD 1.1 µg/L); these values are consistent with previously reported normal values.

Additional Keyphrases: reference intervals • peptide hormones

The nonapeptide, bradykinin (BK), 1 is generated in plasma from kininogen by plasma kallikrein, which is itself formed from prekallikrein by activated Hageman factor. BK is assuming steadily increasing importance in clinical medicine, and has been implicated in the pathophysiology of a wide variety of clinical disorders, including hereditary angioedema (1), dumping syndrome (2), Gram-negative septicaemia (3), carcinoid syndrome (4), and Bartter’s syndrome (5). Further delineation of its role in disease processes will require the availability of a rapid, accurate, and sensitive assay technique.

Initial attempts at bradykinin quantitation made use of bioassay techniques in which the contractile response due to an unknown sample was compared with that produced by a standard dose of BK on an isolated animal tissue such as guinea pig ileum (6). Talamo et al. (7) reported the first successful radioimmunoassay (RIA) in 1969; BK was separated from trichloroacetic acid-precipitated plasma on a column of ion-exchange resin (Amberlite IRC-50). This BK fraction was subsequently found to be contaminated with kininogen, which cross reacted with the BK antibody, thus necessitating an additional step, chromatography on QAE-Sephadex, to remove this kininogen (9). Mashford and Roberts (10) precipitated the blood sample with ethanol, followed by extraction with diethyl ether, and quantitated the BK in the aqueous phase by RIA. However, interfering substances in the sample make difficult the separation of free and antibody-bound BK on dextran-coated charcoal.

The major difficulties with current assay methods for plasma BK are their technical complexity and tedious length, such as multiple chromatographic steps or solvent extractions, the analytical recovery of BK diminishing with each step. We describe here a single-step separation technique for removing BK from plasma, for which recovery and reproducibility are excellent. This procedure can be adapted by any laboratory that can do RIA; 20 to 30 plasma samples may be completely processed and assayed within 6 h.

Materials and Methods

Apparatus

All centrifugations were done with a refrigerated centrifuge (Sorvall RC-3B; DuPont Instruments, New Town, CT 06470) equipped with an H-4000 swinging bucket rotor (DuPont). We counted tritium radioactivity with a liquid scintillation system (Model 6880, Analytic Mark III; Tracer, Des Plaines, IL 60018) and 125I radioactivity with an automatic gamma scintillation counter (Model 1185; Searle Analytic Inc., Des Plaines, IL 60018).

Reagents and Solutions

BK triacetate was obtained from Calbiochem, San Diego, CA 92112. 125I-labeled Tyr-BK (specific acty 279 Ci/g) was obtained from Nuclear International Corp., Waltham, MA 02154. [3H]BK triacetate (specific acty 50 kCi/mol) was from New England Nuclear, Boston, MA 02118. We used prepacked Sephadex G-25 (medium) columns (9.1 mL bed volume, 5-cm bed height, PD-10 column; Pharmacia, Piscataway, NJ 08854). The assay buffer contained, per liter, 100 mmol of tris(hydroxymethyl)methylamine, 140 mmol of NaCl, 1 mmol of disodium ethylenediaminetetraacetate (EDTA), 1 g of hexadimethrine bromide (Polybrene), and 1 g of bovine gamma globulin, Cohn Fraction II; this mixture was adjusted to a final pH of 7.5.

BK-depleted plasma. Four milliliters of pooled human plasma, determined by RIA to contain 5 ng of BK per milliliter, was loaded onto a PD-10 column. The column was then washed with assay buffer, and the initial 4 mL (void volume) of effluent was collected. This 4-mL sample (BK concentration, 500 ng/L by RIA) consisted of a slightly diluted plasma containing all those plasma components with relative molecular masses (M,) exceeding 5000.

BK antiserum. BK antibody was prepared with synthetic BK triacetate conjugated to ovalbumin by a modification of the method of Talamo et al. (11). The antigen was prepared by dissolving 40 mg of BK and 1.25 µg of [3H]BK (104 cpm) in 3.6 mL of 8 mol/L urea and 0.4 mL of 1 mol/L sodium phosphate buffer, pH 7.2, and cooling the mixture to 4 °C. To this we added 100 µL of tolune-2,4-diisocyanate, followed by 80 µg of ovalbumin dissolved in 8 mL of 50 mmol/L sodium borate. After the resulting solution was stirred at 45 °C for 2 h, it was dialyzed at 4 °C vs 2 L of 0.1 mol/L ammonium carbonate with three changes during 24 h, then against an equal volume of distilled water changed three times during 24 h. The final dialysate (i.e., nondialyzable material), determined by scintillation counting to contain 3.5 mol of BK per mole of ovalbumin, was then freeze-dried.

One milligram of this freeze-dried antigen preparation was then redissolved in 0.5 mL of isotonic saline, emulsified with an equal volume of Freund’s complete adjuvant, and injected intradermally, subcutaneously, and intramuscularly into white male New Zealand rabbits once monthly for three

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1 Nonstandard abbreviations: BK, bradykinin; RIA, radioimmunoassay; EDTA, ethylenediaminetetraacetate.

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months. Blood was then sampled from the animals, and they were given a booster injection of 1 mg of antigen. Ten days later the animals were again bled. Booster injections and bleeding were repeated at various intervals during five months. All rabbits developed antibodies in usable titer. Pooled antisera from all of them was used for RIA at a 5000-fold dilution, which was the dilution that bound 50% of $^{125}$I-labeled BK in the absence of unlabeled BK, as determined from standard dilution curves.

Preparation of plasma samples. Venous blood was sampled from 21 apparently normal volunteers (15 men and six women) into chilled polystyrene tubes containing 3.6 mg of Polylephene and 3.0 mg of disodium EDTA in a final volume of 0.1 mL. The blood was immediately centrifuged at 4 °C, and the plasma was transferred to another polystyrene tube surrounded by crushed ice. Sephadex PD-10 columns were equilibrated with 30 mL of assay buffer solution. A 2-mL plasma sample was then loaded onto the column, and the column was washed with 4 mL of assay buffer. The resulting void volume contained all plasma components of $M_r$ over 5000, including kininogens; the BK was retained. The BK was then eluted with a second 4-mL wash of assay buffer. This 4-mL BK fraction, clear and colorless, was collected in a plastic vial; duplicate 0.4-mL aliquots were then assayed.

RIA procedure. For RIA we used $12 \times 75$ mm polystyrene tubes, and kept all tubes and assay reagents at 4 °C throughout preparation. All standard solutions and dilutions were made with assay buffer immediately before use. BK standards, 20 µL, in concentrations from 0.025 to 10 ng/20 µL were pipetted in duplicate into assay tubes. Each unknown BK 0.4-mL fraction was similarly pipetted into assay tubes. We added 6000 cpm of $^{125}$I-labeled BK (10 pg) in 10 µL of buffer and 0.1 mL of diluted antibody to all tubes. A nonspecific binding tube was prepared by using 0.1 mL of buffer in place of diluted antibody. The total-count tube contained only 10 µL of $^{125}$I-labeled BK and buffer. Sufficient buffer was then added to each tube to provide a final volume of 0.5 mL, and the contents of the tubes were stirred on a vortex-type mixer and incubated for 4 h at 4 °C, by which time equilibrium was achieved. After incubation, bound and free BK were separated by precipitation with 0.5 mL of saturated ammonium sulfate solution, and the mixture was centrifuged at 3000 rpm with the H-4000 swinging-bucket rotor for 15 min. The precipitate was washed with 1 mL of half-saturated ammonium sulfate, and again centrifuged as above. The dried precipitates were counted in an automatic gamma scintillation counter sufficiently long to provide a counting error of 1%.

Calculations

The calculations for standard curve and unknown samples are expressed as percent inhibition of binding, defined as:

\[
\text{% inhibition} = \frac{(B_0 - B)}{B_0} \times 100
\]

where $B_0 =$ mean cpm in the standard tube containing no bradykinin minus mean cpm in the nonspecific binding tube and $B =$ mean cpm in standard or sample tube minus mean cpm in the nonspecific binding tube. A standard curve is plotted on logit-log paper as percent inhibition of binding vs concentration of standard, and the concentrations of the unknown samples are determined from the curve. These concentrations are then multiplied by the factor 5.8 to derive the actual bradykinin concentration per milliliter of plasma. This factor derives from the use in the final assay of a 0.4-mL aliquot of the 4-mL elution volume obtained after loading 2 mL of plasma onto the column, and correcting for 85% recovery through the PD-10 column.

BK recovery and accuracy determinations. Recovery of BK from the PD-10 column was determined by adding Blue Dextran, cytochrome c, and $[^3H]$BK (8000–10 000 cpm) to 2-mL plasma samples and processing as previously described, but collecting 1-mL fractions. The ratio of the counts detected in the BK fractions to the total activity added represents the overall recovery. The Blue Dextran and cytochrome c in the samples mark the void volume and indicate contamination with larger plasma components that may interfere with BK assay. We estimated the accuracy of this assay procedure by adding various concentrations of unlabeled BK (350 to 6000 pg) to 2-mL volumes of both BK-depleted plasma and distilled water, which we then processed and assayed as for unknown samples. The measured BK values were expressed as a percentage of the true (added) concentrations, and these percentages were used as an index to the accuracy of the procedure.

Results

Bradykinin separation. The antigenic site of BK is located at the carboxy terminus. The antibody cross reacts with the BK precursor, kininogen (7); thus BK must be separated from kininogens before assay, by column chromatography on Sephadex G-25. Figure 1 shows a typical elution pattern. All plasma components with $M_r$ greater than 5000, including kininogens with both high ($M_r$ approximately 120 000) and low ($M_r$, approximately 80 000) relative molecular masses (8), are eluted with the void volume, as indicated by the cytochrome c ($M_r = 23000$) elution pattern. The elution pattern of Blue Dextran ($M_r > 300000$), virtually superimposable with that of cytochrome c, is not shown. Analytical recovery of $[^3H]$BK added to the 2-mL plasma sample before its application to the column ranged from 82 to 87% (mean 85.5, SD 3.5%, n = 42) in the 4-mL BK fraction. Because the column chromatography represents the only purification step and the BK fraction is assayed directly, this represents the total recovery of plasma BK.

RIA. Antibody dilution curves indicate that the rabbit antibody at a 5000-fold dilution bound 50% of the total $^{125}$I-labeled BK added per tube. The avidity of this antibody, as calculated from a modified Scatchard plot, is $3.23 \times 10^5$ L/mol. Figure 2 shows a typical standard curve obtained for BK (25–10 000 pg), and demonstrates the sensitivity of this RIA down to 25 pg of BK per assay tube, or 125 pg per milliliter of plasma. The percent inhibition corresponding to 25 pg of BK differed significantly ($p \leq 0.05$) from the percent inhibition corresponding to 0 pg of BK in every standard curve. Blank values, obtained by using distilled water, were indistinguishable from the 0-pg tubes. This sensitivity could be increased to a lower limit of 25 ng/L by utilizing the entire elu-
tion volume rather than a 0.4-mL aliquot of it, but this elution volume would have to first be concentrated. We did not do this, because the greater sensitivity is not required for routine plasma BK quantitation, and it would prolong the procedure unnecessarily.

Nonspecific binding of the $^{125}$I-labeled BK was determined to be 4.5% (SD 0.27%). The coefficient of variation at each point was less than 6%. The intra-assay coefficient of variation (≤6.68%) was obtained by assaying 14 aliquots from each of three plasma control samples, and the inter-assay coefficient of variation (≤7.63%) by assaying the same plasma control samples on 10 different days (Table 1). Accuracy was assessed by adding increasing amounts of BK to both 2-mL portions of water and BK-depleted plasma samples, and assaying for total BK (Tables 2 and 3, respectively).

Each concentration was assayed in quadruplicate and the results were averaged. Those samples run in BK-depleted plasma were corrected for the BK present in the plasma, as indicated in Table 3. The percentages of measured to true BK concentrations ranged from 98 to 103.6 (mean 101.6, SD 3.1) for the water samples, and 102 to 109 (mean 106.3, SD 3.0) for the BK-depleted plasma. The excellent agreement between the measured and true BK concentrations indicates the absence of systematic error from the assay.

**Plasma BK.** Plasma BK concentrations in 21 normal subjects ranged from 2.5 to 5.2 µg/L (mean 4.2, SD 1.1 µg/L), values similar to those obtained by Talamo et al. (7) and Wintraub et al. (9).

**Discussion**

The plasma kallikrein–kinin system is regulated by the presence of the proenzyme, prekallikrein, which, when activated by Hageman factor, releases BK from kininogens. The system is modulated by plasma kallikrein inhibitors and kininases. Therefore the initial problem in any BK assay is to prepare the plasma sample in such a way as to prevent BK destruction by kininases or prekallikrein activation, resulting in artifactually low or high BK values. Polybrene has been used to inhibit Hageman factor activation of prekallikrein, and EDTA to inhibit kininase activity, and these are used for the same purposes in this procedure (7).

The initial RIA for BK of Talamo et al. was reportedly sensitive to 1.25 µg/L of plasma (7). After trichloroacetic precipitation of plasma proteins, BK was adsorbed onto an Amberlite IRC-50 column and subsequently eluted with an equivalence mixture of acetic acid and water and flash-evaporated before assay. Of the added $^{14}$C]BK, 60% was recovered before assay. With this methodology, plasma BK concentrations were reported as less than 3 µg/L. The fraction containing BK was subsequently determined to be contaminated with kininogen, which cross reacted with the BK antibody (9), thus producing falsely high BK values. To eliminate the kininogen, it was necessary to apply the re-neutralized trichloroacetic acid supernate to a QAE-Sephadex column, which retained the kininogen. The BK-containing eluent was then desalted by Amberlite IRC-50 chromatography. The additional step reduced BK recovery even further and prolonged the overall procedure. Mashford and Roberts (10) collected blood in ethanol to precipitate proteins; after centrifugation and evaporation, the supernate was acidified and extracted with diethyl ether. The aqueous phase was examined by RIA. $^{14}$C]BK recovery ranged from 56 to 117% in six subjects; concentrations in plasma were reported to be 70 (SD 30) ng/L of blood. This procedure was further complicated by the presence of interfering substances, probably lipids, in the assay sample, which complicated the separation of free from antibody-bound BK.

The primary and most important improvement in our procedure is the separation of kininogens from BK by use of Sephadex G-25, a molecular sieve that allows the unimpeded passage of proteins of high relative molecular mass (Mr >5000)

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**Table 1. Intra- and Inter-assay Coefficients of Variation**

<table>
<thead>
<tr>
<th>Plasma sample</th>
<th>Mean (SD) BK concn, µg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.70 (0.18)</td>
<td>6.67</td>
</tr>
<tr>
<td>2</td>
<td>4.50 (0.27)</td>
<td>6.00</td>
</tr>
<tr>
<td>3</td>
<td>3.61 (0.23)</td>
<td>6.37</td>
</tr>
<tr>
<td><strong>Inter-assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.81 (0.20)</td>
<td>7.12</td>
</tr>
<tr>
<td>2</td>
<td>4.70 (0.32)</td>
<td>6.81</td>
</tr>
<tr>
<td>3</td>
<td>3.54 (0.27)</td>
<td>7.63</td>
</tr>
</tbody>
</table>

Three pooled plasma samples were prepared containing various BK concentrations; inter-assay measurements were done over 20 days (n = 14 for all determinations).

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**Table 2. Analytical Recovery in the Bradykinin Assay**

<table>
<thead>
<tr>
<th>Added to Plasma</th>
<th>Measured, mean (SD) BK, pg</th>
<th>% recovery</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>342.8 (28.6)</td>
<td>98</td>
<td>8.34</td>
</tr>
<tr>
<td>625</td>
<td>647.5 (30.7)</td>
<td>103.6</td>
<td>4.74</td>
</tr>
<tr>
<td>1250</td>
<td>1287.5 (52.8)</td>
<td>103</td>
<td>4.09</td>
</tr>
<tr>
<td>2500</td>
<td>2566.2 (65.7)</td>
<td>102.6</td>
<td>2.58</td>
</tr>
</tbody>
</table>

Known quantities of unlabeled BK were added to 2-mL volumes of distilled water and assayed as unknown samples; the measured values expressed as a percentage of the true (added) value is an index of the accuracy of the assay. Each sample was run in quadruplicate.

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**Table 3. Analytical Recovery in the Bradykinin Assay**

<table>
<thead>
<tr>
<th>Added to Plasma</th>
<th>Measured, mean (SD) BK, pg</th>
<th>% recovery</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>1090 (117)</td>
<td>109</td>
<td>10.73</td>
</tr>
<tr>
<td>2000</td>
<td>2150 (203)</td>
<td>107.5</td>
<td>9.44</td>
</tr>
<tr>
<td>4000</td>
<td>4270 (290)</td>
<td>106.8</td>
<td>6.79</td>
</tr>
<tr>
<td>6000</td>
<td>6125 (410)</td>
<td>102.1</td>
<td>6.69</td>
</tr>
</tbody>
</table>

Known quantities of unlabeled BK were added to 2-mL volumes of BK-depleted plasma and assayed as unknown samples; the measured values were corrected for the concentration of BK in the plasma before addition of BK. Each sample was run in quadruplicate.

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Fig. 2. Typical standard curve obtained for BK; the % inhibition is linear over BK concentrations from 25 pg to 10 000 pg.
absence and lipids. Both high- and low-molecular-mass kininogens, as marked by the presence of Blue Dextran and cytochrome c, are completely eluted in the initial 4-mL void volume; 82–87% of [3H]BK is then recovered in the subsequent 4-mL buffer wash. The BK fraction is crystal clear, indicating the absence of any substantial quantities of lipid. The speed of the separation of BK from kininogen is remarkable: 15–20 plasma samples can be processed within 15 min and the PD-10 columns can be reused after buffer washes.

BK-antibody not only cross reacts with kininogen but also with Lys-BK and Met-Lys-BK (7). Because these are rapidly converted to BK when present in plasma (12), there is no need to attempt to separate them or to assay them separately.

This procedure allows BK concentrations of 125 ng/L to be detected, starting with 2-mL plasma samples. Addition of a concentration step before RIA would lower the detection limit to 25 ng/L, but this additional sensitivity is not needed for routine plasma BK assay and needlessly lengths the procedure.

Our analytical recovery of [3H]BK (mean 85.5%, SD 3.5%) is considerably higher than the 60% reported by Talamo et al. (7) before modification of the technique to further separate BK and kininogen, and it is considerably more reproducible than the 56–117% range found by Mashford and Roberts (10). Furthermore, this BK is free of both kininogen and lipids, which may interfere with subsequent RIA procedures. When we added known concentrations of BK to water or BK-depleted plasma, measured values ranged from 98 to 109% of the corresponding known concentrations. These studies suggest that the accuracy of this method is satisfactory, and that there is no systematic error in the procedure. The intra- and inter-assay precision is adequate. The coefficient of variation for 14 samples run on the same day was 6.66%; it was 7.62% for the same samples run on 20 different days.

The values for plasma BK we found approximate those reported in venous blood of normal subjects by several other authors (5, 7, 9, 13–16). The much lower values reported by Mashford and Roberts (10)—70 ± 30 ng/L of blood—are difficult to explain.

A continuing problem in the RIA of BK is its instability in dilute solution. BK dilutions should be freshly prepared from a concentrated stock solution (stored at −70 °C) just before each RIA. Because such multiple dilutions may lead to an occasional pipetting error, reference plasma samples must be assayed with each set.

We conclude that our BK assay is suitable for both investigational and routine clinical use. The principle feature of this assay is that although it is both simple and rapid, it is still precise and sensitive. With this technique the controlling factors governing BK generation in both normal and pathological states can be studied. Such studies are currently in progress in our laboratory.

We thank Ms. Carolyn Umstott for her excellent technical assistance.

References