Quantitative Radioimmunohistochemical Method Using \([^{125}\text{I}]\)-Protein A to Measure the Content of Methionine Enkephalin in Discrete Rat Brain Areas

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We report a quantitative radioimmunohistochemical method, using \([^{125}\text{I}]\)-protein A in combination with a specific antibody to methionine enkephalin (Met-enk), for determination of the content of this peptide in discrete areas of rat brain. After paraformaldehyde fixation, rat brain sections were incubated with a Met-enk polyclonal antibody, followed by incubation with \([^{125}\text{I}]\)-protein A. After autoradiography with \(^3\text{H}\)-sensitive Ultrofilm, optical densities (OD) were quantified by computerized microdensitometry. The OD obtained were compared to a standard curve, constructed after determination by radioimmunoassay of the Met-enk content in corresponding brain areas from adjacent tissue sections. After comparing 15 different brain areas over a ninetyfold range of concentrations, we found a linear relationship between the content of Met-enk, as determined by radioimmunoassay, and the OD generated by autoradiography. The content of Met-enk in other discrete brain areas can be quantified by interpolation of the OD determined by autoradiography in the standard curve. The method allows, for the first time, precise quantification of peptide concentrations in discrete areas and nuclei from thin sections of rat brain. This technique has a more than 100-fold higher sensitivity than classical radioimmunoassays, with the additional advantage of neuroanatomical localization. It also has the potential for application to the quantification of many other antigens present in brain and other tissues. (J Histochem Cytochem 36:1379–1386, 1988)

KEY WORDS: Immunohistochemistry; Autoradiography; Computerized microdensitometry; Radioimmunoassay; Methods; Staphylococcal protein A; Neuropeptides; Enkephalin; Opioids; Brain/rats.

Introduction

Autoradiography has become a routine technique for visualization of neural constituents because of its higher sensitivity and better anatomical resolution than biochemical methods (20). The use of autoradiography has made it possible to identify and study the distribution, in restricted areas of the central nervous system, of macromolecules such as neurotransmitter and neuropeptide receptors (26,30,33,39,41) and enzymes responsible for neuropeptide synthesis or degradation (29,38). A major advantage of autoradiography is the possibility of quantitative assessment by comparison with appropriate standards (32,40). The use of iodinated ligands with autoradiography further increases its sensitivity and possibilities of quantification. Because of their higher specific activity and consequently reduced exposure times, iodinated ligands have made possible the use of autoradiography for kinetic studies in anatomically discrete areas (5,17).

Radioimmunohistochemical methods using radiolabeled antibodies to visualize the presence of the antigen or the antigen–antibody complexes in tissues are considered to be more sensitive than immunohistochemical methods (7,28). \([^{125}\text{I}]\)-Antibodies have been used for qualitative light or electron microscopy autoradiographic localization of putative neurotransmitters in the brain (24,25,27). The lack of adequate standards to correlate the generated optical densities (OD) with the actual content of antigen in the tissue is the major problem with the use of immunohistochemistry as a quantitative method. Accurate quantification of antigens in tissues still relies on biochemical methods, such as specific radioimmunoassays for measurement of the concentration of neurotransmitter peptides in the brain.

Protein A is a component of the staphylococcal cell wall which interacts strongly and specifically with the Fc fragment of IgG immunoglobulins of different species (11,16). \([^{125}\text{I}]\)-Protein A was used to demonstrate the presence of antibodies bound to cell membranes in vitro (1,9,21). Preliminary studies carried out in our laboratory

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Materials and Methods

Preparation of Animal Samples. Three male Sprague-Dawley rats (220-230 g) were anesthetized with sodium pentobarbital (45 mg/kg, IP). Heparin (4000 U/kg) was injected IV and the chest cavity was opened surgically. The descending aorta was clamped with a hemostat, and an 18-gauge needle connected to a perfusion apparatus was introduced into the ascending aorta through the left ventricle of the heart. Animals were perfused for 5 min with ice-cold saline, followed by a 30-min perfusion with 4% paraformaldehyde in 0.05 M PBS, pH 7.5. Perfusion rate was kept constant at 150 ml/hr. The brains were removed and soaked in 4% paraformaldehyde–PBS for 2 hr, followed by 24 hr in 0.32 M sucrose at 4°C. Brains were frozen in isopentane at −30°C and 20- or 100-μm frontal sections were cut on a cryostat at −16°C. The sections were cut from a region situated between the stereotaxic coordinates A7590 μm to A6650 μm (18). Tissue sections were thaw-mounted onto 0.05% chrome alum–0.5% gelatin–coated slides, and were kept frozen at −70°C until use.

Experimental Procedure. Each rat brain was sectioned in three groups of four 20-μm sections and one 100-μm section. The 20-μm sections were divided into two consecutive sections cut anteriorly and two cut posteriorly to the 100-μm section (Figure 1). The three groups of sections corresponded, respectively, to the coordinates A7630 ± 101, A7098 ± 59, and A6690 ± 20 μm (19). The thinner sections were used for radioimmunohistochemical visualization of Met-enk by autoradiography. Sections used for radioimmunohistochemistry were divided into two sets: one for total binding and other for nonspecific binding. Each set contained one section anterior and one posterior to the 100-μm thick section (Figure 1). After autoradiography, the sections were stained with hematoxylin–eosin for anatomical localization of the area studied. The 100-μm sections were used for radioimmunoassay (RIA) of Met-enk in the tissue.

Radioimmunohistochemical Procedure. Autoradiography was performed using 20-μm sections. The sections were treated as follows: (a) rehydrated for 5 min in 0.05 M phosphate buffer containing 0.9% NaCl (PBS); (b) incubated for 2 hr with immune sera, diluted in PBS–0.2% Triton X-100; (c) washed twice in PBS (5 min each); (d) incubated for 30 min with 500,000 dpm of [125I]-protein A, diluted in PBS–0.2% Triton X-100; (e) washed three times in PBS (5 min each); (f) dried under a stream of cool air; and (g) exposed to [3H]-Ultraprof (KLB, Bromma, Sweden) for 14 days. All reactions were carried out at 23–25°C in a humidified incubation chamber (Accurate Chemical, Westbury, NY). Rabbit Met-enk antiserum (lot #519/8603016; Immuno Nuclear Corp, Stillwater, MN) was used at a 1:500 dilution, according to the manufacturer's suggestion. Preliminary observations indicated that the background increased markedly at lower dilutions of the primary antibody in autoradiograph generated with [125I]-protein A. Met-enk antiserum pre-incubated overnight with 100 M Met-enk was used as control for the specificity of the reaction (nonspecific binding). [125I]-Protein A (85.3 μCi/μg) was purchased from New England Nuclear (Boston, MA).

Sets of [125I]-standards were prepared as previously described (17). Known amounts of increasing concentrations of [125I]-were thoroughly mixed with rat brain tissue aliquots previously ground to a paste and degassed by repeated mixing under vacuum. The aliquots were placed as blocks of tissue on micrometre specimen holders and frozen on dry ice. Tissue sections 20 μm thick were cut in a cryostat at −14°C and thaw-mounted onto gelatin-coated glass slides. Parallel sets of standards obtained from consecutive sections were used for determination of protein concentrations (23) and radioactivity. Tissue sections and complete sets of [125I]-standards were placed in cassettes and exposed to [3H]-Ultraprof for 14 days. The films were developed at 4°C for 4 min with undiluted D19 Kodak developer and OD were quantified by computerized microdensitometry in both [125I]-standards and specific areas of tissue sections from each film (17). The OD of the tissue sections were related to the concentration of radioactivity present by comparison with standard curves generated by processing sets of standards with each film.

Each set of standards contained concentrations of radioactivity/μg of protein varying from about 10–1000 dpm. There was a linear relationship between the ln of the optical densities and the ln of the concentration of radioactivity at any exposure of [3H]-Ultraprof to the standards. The molar quantities of ligand bound/mg of tissue were determined by interpolating the OD in the straight line obtained from the ln–ln standard curve.

The semi-log plot of the OD read from the film vs the exposure time and amount of radioactivity present in the standards generated the so-called characteristic curve of the film. The slope of the curve gives a measure of

![Figure 1](https://example.com/f1.png)

Figure 1. Graphic representation, corresponding to the coordinate A7190 μm of König and Klippel's rat brain atlas (19), indicating the experimental procedure used to obtain samples for generation of internal standard curves for quantitation of Met-enk in paraformaldehyde-fixed rat brains. The 20-μm sections were used for radioimmunohistochemical determination of the content of Met-enk in discrete brain regions by computerized microdensitometry of autoradiographs generated using [125I]-protein A. Sections A and D were incubated with rabbit Met-enk antiserum, pre-absorbed with 100 M Met-enk (nonspecific binding), before [125I]-protein A. Sections B and E were incubated only with Met-enk antiserum (total binding). Outlined areas in the figure represent the regions corresponding to areas dissected from the 100-μm sections, in which the peptide content was determined by radioimmunoassay.
the photographic contrast. At OD of 0.2 units or less, the changes in the slope as a function of exposure time are very small and discrimination between different values is difficult. At OD greater than 1.6 units, the film rapidly saturates and no useful data can be obtained. A linear relationship exists between OD and dpm/µg of protein \( \times \) time of exposure for values between 0.3-1.6 OD units. In this case, the contrast is high between different values, making it easy to discriminate between small differences in concentrations of binding sites for a particular structure. With this information, the exposure time that produces optimal contrast can be estimated for a particular tissue section. It is therefore desirable in preliminary experiments to expose the labeled tissue sections for various lengths of time to determine the adequate conditions for optimal contrast (OD between 0.6-1.4 units for each particular structure).

The amount of \([^{125}I]\)-protein A (MW 42,000) specifically bound to the tissue was calculated as pg/mg protein using values corresponding to the difference between total and nonspecific binding. The results were corrected for the \([^{125}I]\)-decay and the protein content of the standards (23).

Radioimmunoassay Procedure. Square or rectangular cubes (0.132-0.375 mm\(^3\)) were dissected from the 100-µm sections under stereomicroscopy with the use of a sharpened dissection instrument. The concentration of Met-enk in homogenates of discrete areas of the rat brain was determined by RIA after extraction from the tissue. Tissue Met-enk was extracted in 35 µl of 0.1 N HCl (5,15) by sonicating for 10 scc, using a micro-ultrasonic cell disruptor (Kontes; Vineland, NJ). Homogenates were centrifuged at 12,000 \( \times \) g for 2 min at 4\(^{\circ}\)C in a model 235C microfuge (Fisher Scientific; Springfield, NJ) and 15-µl aliquots were taken for Met-enk quantification using a RIA kit (ImmuNuclear). Met-enk values were corrected for the protein content measured using the method of Lowry et al. (23), modified as follows: (a) 20 µl of 1 N NaOH containing 0.2% Triton X-100 were added; (c) mixed and allowed to stand at room temperature for at least 10 min; (d) 25 µl of Folin reagent (Fisher) diluted 1:1 with distilled water; and (e) mixed immediately and allowed to stand at room temperature for 30 min and read at 750 nm using a Ultrospect II spectrophotometer (LKB; Cambridge, England).

### Table 1. Correlation between values derived from radioimmunohistochemical and radioimmunoassay measurements of content of Met-enk immunoreactivity in different areas of rat brain

<table>
<thead>
<tr>
<th>Region</th>
<th>Radioimmunohistochemistry (Bound ([^{125}I])-protein A) pg/mg protein ± SEM</th>
<th>Radioimmunoassay (Met-enk) ng/mg protein ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>2.0 ± 0.8(^{a}) (12)</td>
<td>1.2 ± 0.4(^{a})</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>14.8 ± 2.4(^{a}) (12)</td>
<td>6.0 ± 0.8(^{a})</td>
</tr>
<tr>
<td>Dorsal caudate putamen</td>
<td>22.0 ± 7.2(^{a}) (12)</td>
<td>6.9 ± 1.2(^{a})</td>
</tr>
<tr>
<td>Lateral caudate putamen</td>
<td>174.4 ± 44.4(^{a}) (3)</td>
<td>90.9 ± 12.3(^{a})</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>5.6 ± 1.6(^{a}) (9)</td>
<td>4.0 ± 1.3(^{a})</td>
</tr>
<tr>
<td>Septum</td>
<td>42.0 ± 6.0(^{a}) (12)</td>
<td>9.5 ± 1.3(^{a})</td>
</tr>
</tbody>
</table>

\( r = 0.979^{c} \)

\( r = 0.994^{c} \)

\( r = 0.969^{c} \)

\(^{a}\) Results correspond to combined data from the three groups of sections described in Methods. Each value corresponds to the average of paired determinations (number in parenthesis) from areas situated between the coordinates A7630 and A6690 µm of the König and Klippel rat brain atlas (19).

\(^{b}\) Data from Hong et al. (14).

\(^{c}\) Significant at \( p < 0.01 \), Spearman rank correlation test (37).
Figure 2. Autoradiographs obtained by use of $[^{3}H]$-protein A as radioimmunohistochemical method to visualize Met-enk antisera binding to specific regions of the paraformaldehyde-fixed rat brain. (A) Hematoxylin–eosin-stained sections indicating the anatomical structures visualized in the autoradiographs, corresponding to coordinates between A7190–A6670 µm of König and Klippel's rat brain atlas (19). (B) Met-enk immunoreactive fibers evidenced in the caudate putamen (Cd), globus pallidus (GP), lateral septal area (LS), preoptic area (POA), and stria terminalis (ST). (C) Consecutive control sections incubated with immune serum pre-absorbed overnight with $10^{-4}$ M Met-enk.
Discussion

We report a quantitative radioimmunohistochemical method for determination of the Met-enk content in specific areas within thin sections of rat brain. Radioimmunohistochemistry was performed by incubation of thin brain sections with a specific polyclonal antibody, followed by incubation with $[^{125}\text{I}]$-protein A. Quantitative autoradiography was carried out by comparison with $[^{125}\text{I}]$-standards (17). A standard curve can be generated by comparison of the actual peptide concentrations determined by RIA in several discrete brain areas to the autoradiographic values obtained in homologous areas from adjacent sections. Quantification of peptide concentrations in other specific brain areas is possible by interpolation of the autoradiographic values obtained in these areas in the standard curve.

Met-enk distribution has been extensively studied in rat brain with classical immunohistochemical methods (15,37). The actual peptide concentrations can be reliably estimated by RIA in discrete brain areas dissected from sections of paraformaldehyde-fixed tissue (6). These characteristics made Met-enk an excellent candidate for validation of the new quantitative radioimmunohistochemical technique.

$[^{125}\text{I}]$-labeled secondary antibodies have been used for qualitative radioimmunohistochemistry (22,25,27). However, protein A, either as a fluorescein isothiocyanate or as a peroxidase conjugate, has been successfully used to detect tissue antigens by histofluorescence (1,2,10), and preliminary observations in our laboratory indicated that peroxidase-conjugated protein A can advantageously substitute the corresponding secondary antibodies in immunohistochemical procedures to localize neuropeptides or related enzymes in rat tissues (3). These observations prompted us to use $[^{125}\text{I}]$-protein A in conjunction with a specific polyclonal antibody to...
generate autoradiographic images of Met-enk distribution in rat brain.

The anatomical distribution of Met-enk as reported here by autoradiography agrees with that previously reported using classic immunohistochemical methods (4,34,36). The autoradiographs generated after incubation of tissue sections with \[^{125}\text{I}\]-protein A are very clear and the background is very low. The effectiveness of protein A as a reagent is probably due to its low nonspecific binding to tissues (1,2,10) and to its high affinity for IgG immunoglobulins of several mammalian species (13,18).

The OD obtained in the different brain areas studied could be precisely quantified as pg of \[^{125}\text{I}\]-protein A bound/mg protein, by comparison with sets of \[^{125}\text{I}\]-standards, using computerized microdensitometry (12,17,40). However, to quantify the actual concentration of peptide in specific brain areas it was necessary to construct a standard curve, which we did by determining the relationship of the observed autoradiographic units in several specific brain areas to the actual concentrations of peptide in homologous brain areas as measured by RIA. Such a correlation was possible, in the case of Met-enk, because the peptide could be reliably extracted from and quantified in small areas of paraformaldehyde-fixed brain (6). To minimize variations resulting from the heterogeneity of peptide concentrations, we compared the RIA values obtained in a thick (100 µm) section of the brain with the mean autoradiographic values of areas identical in size obtained from thinner (20 µm) adjacent sections both frontal and rostral to this section. We compared autoradiographic and RIA values from several brain areas, representing a ninefold variation in peptide concentration, and obtained a linear relationship. There was a good correlation between the values obtained here by radioimmunoassay and by autoradiography with those of the literature, obtained by radioimmunoassay in similar areas of microwaved rat brain (14). These observations indicated that interpolation in the standard curve of autoradiography values from specific brain areas, as described here, will result in accurate quantitative determinations of Met-enk concentrations.

As described here, the radioimmunohistochemical method offers advantages over standard techniques in terms of performance, sensitivity, and precise quantification. In addition, the anatomical resolution of this technique is good. The method allows quantitative determination of Met-enk concentrations in very discrete brain areas. The maximum resolution of the currently available microdensitometer systems, when used with \[^{3}\text{H}\]-sensitive Ultrascan, is approximately 0.01 mm². Therefore, OD can be accurately determined in very small brain areas from thin sections. In our studies, for reasons of convenience we utilized 20-µm sections, but thinner sections could be prepared as well. In contrast, we found that when peptide concentrations were low the currently available RIA require the dissection of areas about 1.0-1.5 mm² from brain sections at least 100 µm thick. This being the case, we can estimate the sensitivity of the present radioimmunohistochemical method to be on the order of 100-500-fold higher than available RIA methods. For areas with low peptide concentrations, the intensity of the signal can be increased by extending the exposure time.

The use of \[^{125}\text{I}\]-protein A as described here represents an additional advantage, since it carries the potential for application to quantitative determination of a wide variety of tissue antigens. Protein A interacts with the Fc fragment of the IgG molecules of different species (31). This property allows the use of similar techniques with many different primary antibodies for determination of many different tissue antigens. In addition, it avoids the need for different species-specific markers, necessary when using labeled antibodies.

However, quantitative radioimmunohistochemical methods, although attractive, demand a careful validation. The use of fixed tissue is essential for adequate immunohistochemical detection of cell antigens, such as neuropeptides. Internal calibration standards such as those used here can be used only when the tissue antigen is extracted quantitatively. Such a property must be determined for each antigen in question. In some cases, autoradiographic values could be successfully compared to an external standard curve generated by RIA determinations in similar areas from unfixed tissues. Alternatively, semiquantitative methods could be developed involving the use of standard curves, constructed after autoradiographic determination of OD generated by different concentrations of authentic antigens coupled to an inerti matrix, as demonstrated for immunofluorescence techniques (8,35).

We propose the following procedure to set up a quantitative radioimmunohistochemical method: (a) select one region that has a high and homogeneous concentration of the antigen to be studied; (b) using a fixed concentration of \[^{125}\text{I}\]-protein A, generate autoradiographs for different dilutions of the primary antibody, non-absorbed or pre-absorbed with the antigen, and select the dilution that yields the highest signal-to-background ratio; (c) fixing the selected dilution of the primary antibody, generate autoradiographs for different concentrations of \[^{125}\text{I}\]-protein A, and select the concentration that yields the highest signal-to-background ratio; (d) determine the correct exposure time to \[^{3}\text{H}\]-sensitive Ultrascan that generates autoradiographs with OD in the linear range (0.3-1.6); (e) select regions that have different concentrations of antigen and cut consecutive thin and thick sections for radioimmunohistochemical and biochemical measurements of the antigen, respectively; (f) use the correlation between radioimmunohistochemical and biochemical data as internal standard curve to determine the concentration of the antigen in other areas of the tissue; and (g) process one set of internal standard sections simultaneously with experimental sections, to provide an accurate comparison with the biochemical determinations.

Quantitative radioimmunohistochemistry has advantages over biochemical techniques because of its higher sensitivity, its anatomical resolution, and the possibility of permanent recording of the autoradiographs. Furthermore, autoradiographic methods allow enhancement of signal intensity by extending the exposure time of the film. Radioimmunohistochemical methods have advantages over classic immunohistochemical techniques. In addition to having similar or higher sensitivities that allow precise quantification of antigens in very small areas, there is the possibility of permanent recording of entire sections by autoradiography. The use of \[^{125}\text{I}\]-protein A could represent an additional advantage over methods using \[^{125}\text{I}\]-specific antibodies, since it allows the use of the same radiolabeled probe for determination of different tissue antigens. Finally, the use of serial thin sections may allow quantification of several antigens within a discrete region, permitting correlation studies between different transmitters or neuropeptides, with the additional advantage of possible concomitant co-localization of an-
tigens by the combination of radioimmunohistochemical methods and classic immunohistochemical techniques.

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Literature Cited

