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Immunofluorescence Detection of Drugs in Postmortem Tissues: A New Technique with Potential for Assessment of Drug Influence in Cause of Death

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ABSTRACT: This report describes a new technique, immunofluorescence, for the detection and possible characterization of drug content in postmortem tissues. By using antisera generated against a drug-protein conjugate, the stabilization of tissue-sequestered drug is accomplished by incubation of fresh frozen sections of tissue with dilute solutions of rabbit anti-drug antibodies. Secondary incubation with a fluorescence-labeled anti-rabbit immunoglobulin labels these points of sequestration. Tissue sections so stained are examined by fluorescence microscopy. In studies with rats given graded doses of morphine sulfate, there were discernible differences in tissue binding of morphine in brain sections from animals treated "therapeutically," fatally, and chronically. Extension of these studies to human autopsy material is anticipated and potential problems are discussed. This technique offers the forensic toxicologist the potential for evaluating the drug content of tissues in situ.

KEY WORDS: toxicology, spectroscopic analysis, postmortem examinations, immunofluorescence

The target organ attacked by today's drugs has to be the human brain. The toxicologist encounters these drugs each working day, and certainly most would agree that if a drug were to produce an acute lethal effect, it most likely would do so by acting on the brain. Effects on other organ systems would perhaps be less significant or be a direct result of the initial action on the brain.

When concentrations of central nervous system (CNS) depressants in the brain are evaluated, the differences between the concentrations of therapeutically applied agents and of agents in gross lethal intoxication of a nontolerant individual are reasonable and consistent with the expectations associated with the differences in dosage of the agent.

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A large majority of CNS agents, however, possesses properties that may complicate an evaluation of demonstrated brain concentrations. Tolerance and dependence are outstanding examples of such properties. Often the interpretation of brain findings without a detailed history of chronic drug use is exceptionally difficult. Attempts to provide solutions to this problem are exemplified by the investigations of metabolite ratios and gross brain distributions [*1*].

The aim of this report is to acquaint the toxicologist with another possible approach devoid of complex analytical techniques such as gas chromatography/mass spectrometry (GC/MS) and perhaps returning to the microscope as an analytical tool. Immunofluorescence techniques are as yet only beginning to be explored. Fluorescent immunological techniques have been used clinically for the demonstration of bacterial involvement in disease processes and for the assessment of autoimmune processes both premortem and postmortem. The use of immunological agents to sequester and identify tissue-bound exogenous materials is not widely reported. We have previously reported our observations concerning morphine disposition [*2*]. We wish here to update this technique and report its possible application to the assessment of the influence of drugs in the causation of death.

Materials and Methods

Immunological Reagents

Morphine antiserum was obtained from Technam Laboratories, Park Forest South, Ill. This antiserum was the same as that used in the hemagglutinin inhibition approach to immunoassay. Anti-rabbit immunoglobulin, rhodamine-conjugated antiserum was obtained from Miles Research Laboratories.

Histological Reagents

Thionine stain was used to evaluate anatomical orientation during tissue section preparation. Tissue sections were embedded in O.T.C. Compound (Scientific Products, Edison, N.J.), frozen, and sectioned. After immunological staining, the sections were mounted in a semipermanent fluorescein isothiocyanate (FITC) mounting medium (BioDX, Hackensack, N.J.).

Microscopic Equipment

A Leitz Orthoplan incident light fluorescence microscope was used for the evaluation of prepared sections with rhodamine excitation/emission fluorescence parameters. Photomicrography was done with Ektachrome 160 transparency film, processed for ASA 400.

Procedures

Preparation of Tissue Sections—Brain tissue was grossly sectioned into blocks. Particularly relevant areas of the brain were chosen for sampling purposes (that is, medullary nuclei, thalamic nuclei, hypothalamic nuclei, and so forth). The tissue blocks were then flash-frozen in liquid nitrogen or precooled hexane. The frozen blocks were fixed into brass chucks with O.T.C. Compound and 5- to 10-μm-thick sections were prepared. The sections were thaw-mounted onto gelatin-coated glass slides and stored frozen until stained. Sections for anatomical orientation were stained with thionine.

Staining Procedure—The staining procedure using immunological reagents was a modification of the technique of Coons and Kaplan [*3*] (Table 1, Fig. 1). The tissue sections were warmed to room temperature and then overlaid with an appropriate primary

TABLE 1—*Procedure for the immunologic staining of tissue sections.*

1. Prepare frozen sections.
2. Incubate with a primary antibody solution in a humid chamber, then wash with buffer for 30 min.
3. Incubate with fluorescence-labeled secondary antibody in a humid chamber, then wash with buffer for 30 min.
4. Cover the sections with FITC mounting medium and a coverslip; evaluate by fluorescence microscopy.

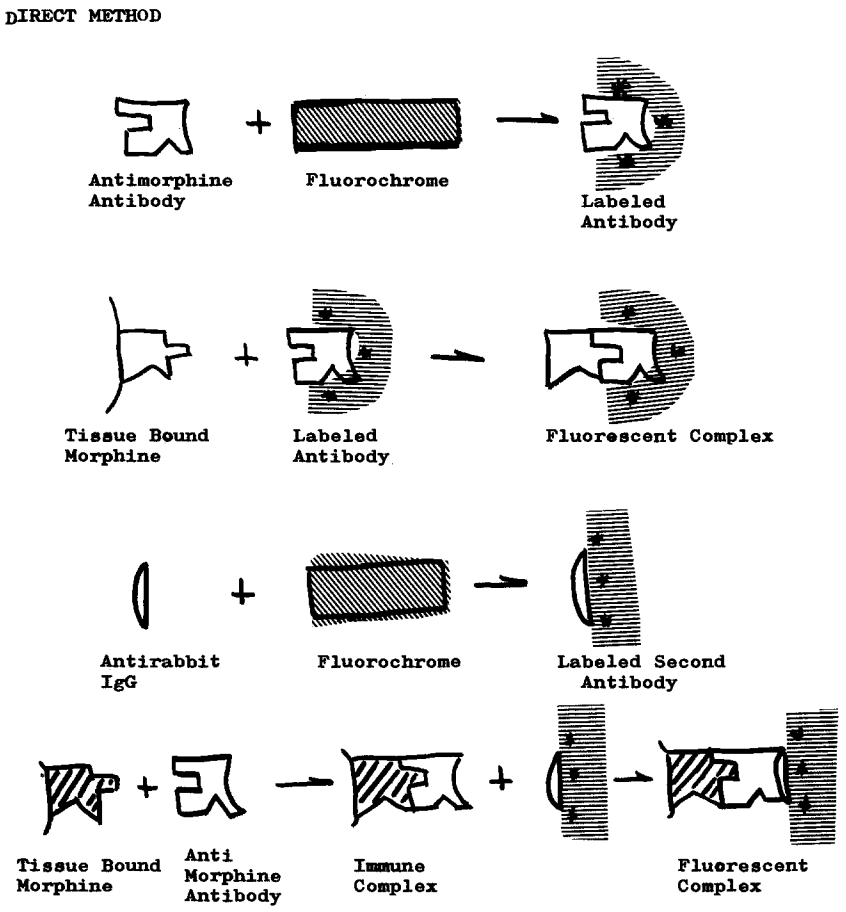

FIG. 1—*Diagram of indirect staining procedure for immunofluorescence.*

antiserum, about 30 μl in a 1:20 dilution (1 mg/ml protein), for 30 min in a humidified chamber. For the work reported here, morphine antisera were used.

After this primary incubation the sections were washed in phosphate buffer and then overlaid with the secondary antiserum conjugated with an appropriate fluorophore, in this case rhodamine. After incubation for 30 min and a second washing to remove non-interacting reagents, the sections were overlaid with FITC mounting medium and covered with a glass slip. The sections were then ready for fluorescence microscopic evaluation.

Controls—Control sections ruled out the possibility of nonspecific staining by either of the immunological reagents. Generally, a section stained with only the secondary antisera,

an unstained section, and a section that had reacted with complexed primary antibody were sufficient to prove that nonspecific staining had not occurred.

Microscopy—A Leitz Orthoplan incident illuminating fluorescence microscope was used to evaluate the stained sections. Some sections were viewed with transilluminating fluorescence microscopes (Olympus and Nikon). "Epiillumination" (incident light excitation) proved to be a more sensitive approach to the evaluation of fluorescence staining, especially at higher magnifications.

Results

Modeling studies were performed with Sprague-Dawley rats given graded doses of morphine sulfate subcutaneously [*2*]. At designated times the animals were killed and their brains were excised, flash-frozen, and processed by immunofluorescence staining for morphine content. Frontal blocks were also evaluated for morphine content by a GC/MS procedure.

Typical immunofluorescence photomicrographs are presented in Figs. 2 through 4. A distinct neuronal dispositional character is evident and may vary according to dose and specific site evaluated. Comparison between the gross disposition in rat brain of morphine as determined by immunofluorescence and that determined by an autoradiographic technique [*1,4*] revealed a distinct localization of morphine in cellular gray areas that were not easily identified by chemical techniques (Fig. 5). Autoradiographic techniques tend to closely approximate the results obtained by wet chemical methods [*1,4*].

Discussion

The method described represents a logical extension of the concepts of radioimmunoassay and enzyme-multiplied immunoassay to the analysis of a micro sample of brain tissue for drug substances. Results from the modeling studies using rats dosed with morphine indicate that the technique can be successfully applied to the microdispositional study of at least this agent. Sher et al [*5*] have also evaluated this technique and compared their results to findings provided by chemical analysis. An excellent correlation was

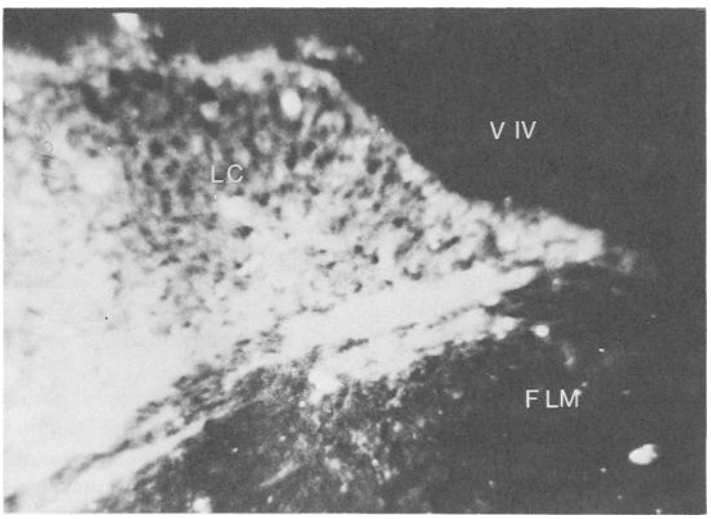

FIG. 2—*The light areas in the rat medulla indicate a morphine-positive result after staining*; LC = *locus cerulus*, V IV = *fourth ventricle, and* FLM = *fasciculus longitudinalis medialis.*

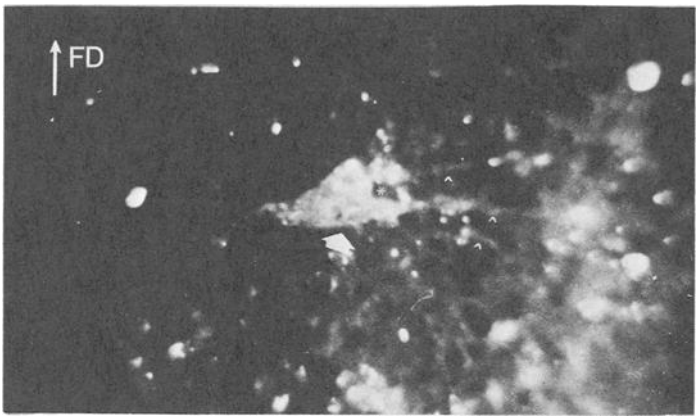

FIG. 3—*Motor neuron in rat cervical spinal cord shows a positive result after staining. The large arrow in the center points to the neuron and the smaller arrows indicate associated neural fibers*; FD = *funiculus dorsalis and* * = *nucleus.*

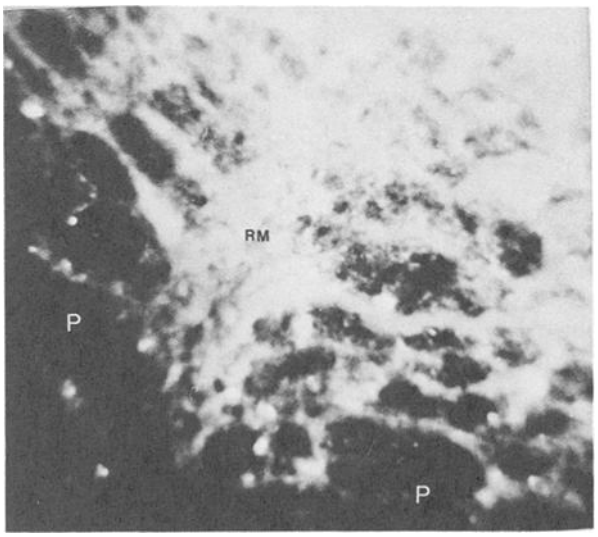

FIG. 4—*The respiratory control center of the medulla from a rat lethally overdosed with morphine sulfate shows a positive result after staining*; RM = *raphe magnus and* P = *tractus corticospinalis.*

observed (Table 2). Their studies included staining for morphine, methadone, and barbiturates in both human and rat brain tissue.

Interestingly, the dispositional characteristics of these agents appeared quite different from each other when the staining was evaluated.

Comparison of the immunofluorescence results with autoradiographic results previously obtained by others provided an indication of the comparability of the immunofluorescence approach with the more traditional analytical techniques. The autoradiographically identified regional disposition of morphine [*4*] produced results comparable to those found by radioimmunoassay [*1*]. Immunofluorescence appears to amplify yet specifically visualize tissue-drug interactions, eliminating the interference of free interstitial drug. By eliminating this "background," distinctions between the nontolerant individual and the

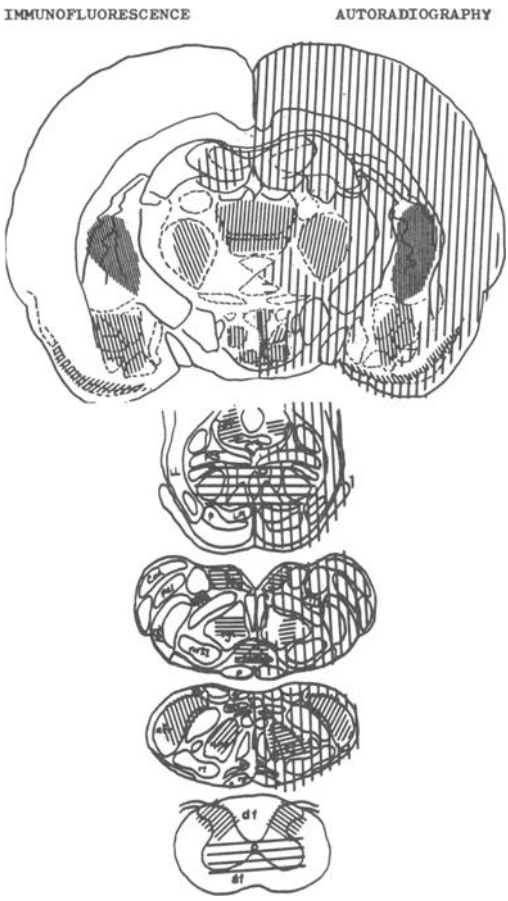

FIG. 5—*Comparison of results of morphine distribution as determined by immunofluorescence and autoradiography* [4].

TABLE 2—*Correlation of immunofluorescence findings with analytical toxicologic findings in human brain after a drug overdose* [5].

Drug	Immunofluorescence-Positive	Toxicology-Positive
Methadone	17	18
Morphine	2	2
Propoxyphene	4	5

chronic drug abuser may be more easily made. Moreover, the specific demonstration of the toxic agent in certain centers of the brain such as the respiratory center or the vagal center may provide convincing evidence for a direct toxic effect causing a death.

It must be admitted that this new approach presents certain problems to the forensic toxicologist. The toxicologist may have to become more familiar with brain neuroanatomy and will have to become adept at the preparation of frozen sections and the handling of

a fluorescence microscope. These are techniques we are certain that toxicologists are able to handle.

The fundamental problem that may be encountered in attempts to use this technique may be the state of the tissue specimen as it is received by the forensic toxicologist. Important aspects of tissue preparation should include rapid freezing in liquid nitrogen and storage in a sealed container at −20°C. While this may appear to be problematical, it should be indicated that similar procedures have been effectively mastered in assays such as receptor evaluation in breast masses. Specimens that have lost their cellular architectural integrity would yield as little information by this technique as they would by any other histological method. In spite of these problems, it is thought that the benefits to be gained from evaluations of this type are evident.

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