Radioimmunoassay of Antibiotics and Chemotherapeutic Agents

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Therapeutic monitoring of drugs has become of major importance, and has stimulated development of new assay systems. Chemotherapeutic agents, many of which are toxic, have until recently been assayed by microbiological systems or other complex techniques that are impractical for a clinical laboratory. Radioimmunoassays have now been developed for some of these agents. We review the techniques for producing hapten protein conjugates and radiolabeling at specific sites on the drug molecule, and the use of these assays in clinical medicine and pharmacology.

The assay of drugs in biological fluids has attracted much attention in recent years, primarily for two reasons: first, for investigations into their absorption, distribution, metabolism, and excretion (pharmacokinetics) and second, for monitoring therapy (1). The therapeutic monitoring of drugs is justified by the finding that response to therapy is often better correlated with the concentration of the drug in the blood than with prescribed dosage (2). An additional indication for routine clinical assay for many toxic therapeutic agents is that as much as 8% of hospitalizations may be because of drug-induced illness (3). Werner et al. (4) have described concepts for the rational selection of drugs suitable for assay on a routine clinical basis. These included drugs that displayed the following characteristics: dangerously toxic, with poorly defined clinical end points; narrow therapeutic range; used in life-threatening disease; used chronically, widely used; and, finally, a technologically and pharmacologically valid assay must be available.

Many antibiotics fulfill the above criteria but are not measured routinely in the clinical laboratory. Interestingly, a microbiological assay for a chemotherapeutic agent was outlined in Fleming’s original paper on penicillin (5). This assay was later described by Florey and his colleagues (6) as being laborious and not having much clinical application. In the same paper, they also described what today is the standard microbiological assay system: radial diffusion, producing zones of inhibition. This method remains essentially unchanged, having the same disadvantages—i.e., imprecision, and lack of sensitivity and specificity—as described in the original paper. In a subsequent paper, Heatley (7) described in detail the microbiological assay of penicillin by this technique. The earliest chemical method for determination of a chemotherapeutic agent appeared in 1937 when Marshall et al. reported an assay for p-aminobenzenesulfonamide, based on a diazotization technique (8). This was later improved to become the basis of the present technique for determination of the sulfonamide drugs (9). Few antibiotics can be chemically assayed directly, most conventional methods requiring time-consuming solvent extraction procedures followed by spectrophotometry, or chromatography. Gas-liquid chromatography, which has sufficient sensitivity and specificity to monitor chemotherapy, is a heavy consumer of human and instrumental time and is not readily amenable to automation (1).

Radioimmunoassay techniques, originally used in drug assays for the detection of cardiac glycosides (11) but later extended to other drugs, offer an alternative approach for the assay of antibiotics. The procedure is suitable for semi-automation or full automation in clinical laboratories and can potentially be used for almost any antibiotic. Moreover, the assay is usually so specific that other naturally occurring compounds or drugs do not interfere. The theoretical sensitivity of radioimmunoassay is also equivalent to or greater than any conventional technique. However, development of a radioimmunoassay requires the production of a specific antibody and the development of a suitable radioactively labeled drug.

Antibody Production

Most chemotherapeutic agents have a molecular weight of less than 1000 and are therefore not immunogenic by themselves. Landsteiner (12) showed that small molecules could be coupled to a carrier protein as
a hapten and stimulate an immunologic response. Many proteins may be used as carriers (bovine serum albumin, hemocyanin, thyroglobulin, polylysine, etc.), but bovine serum albumin is probably used most frequently. The chemotherapeutic agents can be conjugated to a protein by coupling amine or carboxyl functional groups on the antibiotic molecule with either carboxyl or amine groups on the carrier protein (Figure 1). Antibiotics lacking the necessary functional groups for direct conjugation can be modified as shown in Figure 2 to form the appropriate derivatives. The resulting conjugate is purified by dialysis and chromatography and the molar ratio of drug to carrier protein determined spectrophotometrically or by incorporation of a radioactive tracer.

Fig. 1. Hapten–protein coupling reactions
A. Carbodiimide activation of carboxyl groups (58); B. Schiff’s base formation with glutaraldehyde (59); C. Formation of a mixed anhydride (60)

Fig. 2. Formation of a drug derivative suitable for conjugation to proteins
A. Esterification of a primary alcohol with succinic anhydride (67); B. Formation of an oxime from the reaction of a ketone group with carboxymethyl hydroxylamine (62); C. Introduction of a carboxyl group into a phenolic residue by using chloroacetate (63); D. Diazotization with p-aminobenzoic acid (64)
There have been numerous regimens proposed for the production of antisera, and the subject has been extensively reviewed \((13, 14)\). Although no method has been clearly established as superior, we have had success with the method of Hurn and Landon \((15)\), with use of New Zealand white rabbits and monthly intramuscular injections of conjugate emulsified in Freund adjuvant. After several booster injections, the resulting antiserum is evaluated for specificity and avidity.

The antibody recognition site (determinate group) on the hapten is generally the group most removed from the region of conjugation to the carrier protein. Therefore, the specificity of the immunoassay depends upon the site of drug–protein conjugation \((16)\). Thus the biologically active region of the drug or the moieties of the drug that differ from metabolic products should not be used for conjugation to the carrier protein. Radioimmunoassays that can differentiate between the cancer-treatment drug, methotrexate, and folic acid have been produced by conjugation through the carboxyl groups of the glutamic acid residue on the molecule (Figure 3), thus leaving the amine-substituted pteric acid portion exposed for antibody recognition \((17)\). Radioimmunoassays for aminoglycoside antibiotics also demonstrate the specificity of antisera produced by using drug–protein conjugates. Antibodies produced against gentamicin–bovine serum albumin conjugates do not cross react significantly with kanamycin, differing in the substitution of four functional groups, but cross react 65% with sisomicin, which differs only in the substitution of one functional group (Figure 4). An antibody produced against a tobramycin–bovine serum albumin conjugate cross reacts 12.2% with kanamycin but is relatively nonreactive with gentamicin or sisomicin (Table 1). In addition to specificity, the sensitivity of an immunoassay is determined by the avidity of the antisera.

The affinity constant of an antisera can be calculated by using a Scatchard plot \((18)\) or by an equilibrium technique \((19)\). Affinity constants so determined for an antisera produced with gentamicin–bovine serum albumin conjugates were \(8 \times 10^8\) and \(10.5 \times 10^9\) liter/mol, respectively \((20)\), values that compare favorably with affinity constants reported for polypeptide hormones \((14, 21)\). Zettner \((22)\) has proposed formulas, based on the affinity constant, for determining the optimum concentrations of antibody and labeled antigen to produce maximum sensitivity.

### Production of Radiolabeled Antibiotics

Because most drugs lack suitable residues for direct iodination, tritium or carbon-14 labeled antibiotics are used in many radioimmunoassays. The inherent technical difficulties involved with beta-emitting isotopes make gamma emitters, such as iodine-125, desirable for any radioimmunoassay \((23)\). Chemical manipulations similar to those used in the conjugation of antibiotics to carrier proteins may also be used to form iodinatable derivatives. Hunter \((24)\) has demonstrated this technique using \(^{125}\)I-labeled tyramine, tyrosine methyl ester, and histamine. Edwards et al. \((25)\) found that \(^{125}\)I-labeled histamine conjugated compounds in steroid immunoassays showed superior performance because of their increased affinity for antibodies when compared with \(^3\)H-labeled steroids. Bolton and Hunter \((26)\) used a preiodinated hydroxyphenylsuccinimide ester to label human growth hormone; the resulting compound had a high specific activity with minimal iodination damage. We have modified this procedure to label gentamicin \((20)\), sisomicin \((27)\), and tobramycin \((28)\), respectively (manuscript in preparation). This technique (Figure 5) has many advantages, which include not exposing the drug to oxidizing agents used in standard iodination techniques.

### Table 1. The Specificity of Antisera Produced Against Gentamicin or Tobramycin Bovine Serum Albumin Conjugates

<table>
<thead>
<tr>
<th>Drug</th>
<th>Anti-gentamycin/BSA</th>
<th>Anti-tobramycin/BSA</th>
</tr>
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<tbody>
<tr>
<td>Gentamicin</td>
<td>100</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Sisomicin</td>
<td>65</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>&lt; 1</td>
<td>100</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>&lt; 1</td>
<td>12.2</td>
</tr>
<tr>
<td>Amikacin</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
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</tbody>
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* Sisomicin also contains a double bond in the pyranose ring between the carbon atoms adjacent to \(R_1\) and \(R_2\).
though of low specific activity, was suitable for developing a radioimmunoassay (30). Meyers et al. (31), recently reported the production in high yield of an iodinated bleomycin of high specific activity, by the iodine monochloride technique. Although their compound was produced for use in in vivo scanning techniques, it should also be appropriate for use in radioimmunoassay.

Specific Assays

Penicillin. Dismukes et al. (32) described the specificity of antibodies raised against benzyl penicillin, oxacillin, and 6-amino-penicillinic acid conjugated with bovine gamma globulin. The beta lactam ring in these penicillins spontaneously cleaves to produce penicilloy1 derivatives, which then covalently bind to epsilon amino groups of lysine. The antibodies so produced were specific for the penicilloy1 derivatives of the native penicillin, but their activity against the native penicillins was greatly reduced. These results indicated that the method of conjugating haptons to protein carriers must be one that least disturbs the native configuration if radioimmunoassays are to be specific for an antibiotic and not its degradation products. Wal and Bories (33) produced an assay for the penicilloy1 derivatives in biological fluids by using preiodinated bovine serum albumin conjugated to penicillin groups. There was no cross reaction between the penicilloy1 groups and penicillin G in the radioimmunoassay. This assay was designed to detect penicilloy1 groups in dairy products and other biological fluids where they may be present in trace amounts. Such biologically inactive penicilloy1 derivatives can be formed in man or animals treated with penicillin and allergies have been reported following consumption of products from animals thus treated (34).

Isoniazid. Schwenk et al. (35) recently reported an assay for this anti-tuberculosis agent. Antibodies were raised against an isoniazid–bovine serum albumin conjugate, and the drug was radioactively labeled with tritium. The sensitivity of this assay was 50 μg/liter and cross reactivity was 2% with acetylsalicylic acid, 1% for isonicotic acid, 48% with isonicotinamide, and less than 1% with p-aminosalicylic acid. Isoniazid is metabolized by acetylation, and patients can be divided into two genetically distinct groups based on their metabolic rates (36). The authors recommended that the radioimmunoassay for isoniazid be used to detect fast and slow metabolizers of the drug and thus enable optimization of chemotherapy.

Aminoglycoside antibiotics. The aminoglycoside antibiotics are active against infections with Gram negative bacilli but are potentially ototoxic and nephrotoxic (37). Thus, it is important to monitor aminoglycoside chemotherapy, particularly in patients with impaired renal or cardiac function. The first aminoglycoside radioimmunoassay was developed by Lewis et al. (38) for gentamicin. They prepared antibodies by using a carbodiimide activated conjugation of gentamicin to a variety of carrier proteins. A sensitivity of 2 ng was achieved in the radioimmunoassay with a tri-
onium-labeled antibiotic. There was little or no cross reactivity with other aminoglycoside antibiotics: neomycin, kanomycin, and streptomycin. Mahon et al. (39) improved the sensitivity of this assay by chromatographic purification of the tritium-labeled drug. An iodinated gentamicin radioimmunoassay was developed in our laboratory (20) by use of the pre-iodinated hydroxyphenyl succinimide ester technique, which further improved the assay sensitivity to 80 pg. Comparison of results for this assay with those for a microbiological assay gave a correlation coefficient of 0.87. The antisera did not cross react with kanomycin, tobramycin, or streptomycin, but displayed 65% cross reactivity with sisomicin. This cross reactivity facilitated the development of a sisomicin radioimmunoassay (27) with a sensitivity of 140 pg. Comparison of the sisomicin radioimmunoassay with a microbial assay yielded a correlation coefficient of 0.85. Radioimmunoassays for other aminoglycosides have been reported: Lewis et al. (40) produced an assay for amikacin with a sensitivity of 5 ng; our laboratory has also developed one for tobramycin with a sensitivity of 280 pg (manuscript in preparation).

In addition to microbiological and radioimmunoassays, there are also radioenzymatic assays for aminoglycosides (41, 42). These assays are based on the following principle: An enzyme mediated by Escherichia coli "R" factor adenylates aminoglycosides, utilizing ATP in the reaction. The ATP in the assay is labeled with 14C and serves as the source of the adenylic group. Quantitation is achieved by measuring the amount of 14C incorporated onto the aminoglycoside. A comparison of the radioimmunoassay of 3H-labeled gentamicin with the radioenzymatic assay by Minshew et al. (43) resulted in a correlation coefficient of 0.9 and a linear-regression line with a slope of 0.99. This indicated that both methods yield comparable results, which are not statistically significantly different.

Cancer chemotherapeutic agents. These drugs are highly toxic by virtue of their pharmacological action. Radioimmunoassays have been developed for a few of the agents and used to investigate their pharmacokinetics and metabolism in patients. The capacity to monitor chemotherapeutic drugs routinely may provide a basis for monitoring therapy and avoiding or minimizing toxicity.

Methotrexate. Methotrexate, a folate antagonist that binds to folate reductase (EC 1.5.1.3), was first assayed by radioimmunoassay with use of an antibody raised in rabbits immunized with a carboxyimide activated methotrexate/methylated bovine serum albumin conjugate and a tritium-labeled drug (17). The antibody prepared in this manner cross reacted less than 0.1% with folate. Levine and Powers (44) also reported a radioimmunoassay for methotrexate in which hemocyanin was used as the carrier protein for antibody production. This assay could detect 80 pg of the drug and was applicable for determining methotrexate concentrations in patients receiving folic acid "rescue" therapy (45). The antisera used in this radioimmunoassay cross reacted five orders of magnitude less with folate, tetrahydrofolate, or folic acid, which lack either the 4-amino or 10-methyl group of methotrexate (Figure 3). The authors suggested this indicated an immunodominance of either one or both of these groups (44). Raso and Schrieber (46) also developed a radioimmunoassay for methotrexate and investigated the characteristics of their antisera (similarly developed using methotrexate/methylated bovine serum albumin conjugates). They found that compounds resembling methotrexate lacking the 10-methyl substitution but having the 4-amino substitution (aminopterin) showed 10% cross-reactivity whereas compounds lacking the 4-amino substitution displayed less than 0.01% cross reactivity. These data suggested that the 4-amino substitution in methotrexate was immunodominant (46). Arons et al. (47) used the binding of methotrexate to dihydrofolate reductase (EC 1.5.1.4) instead of an antibody as the basis of a radioassay for methotrexate. Their sensitivity was 500 pg, and they reported no interference in the assay by physiological concentrations of folate and other analogs.

Adriamycin. This anthracycline antibiotic, isolated from Streptomyces pencilis is used in treating several different types of neoplastic disorders (48, 49). At therapeutic concentrations, this drug can sometimes produce toxic side effects, of which cardiotoxicity is the most serious (57). Van Vunakis et al. (51) described a radioimmunoassay for adriamycin and the related compound, daunomycin (Figure 7), with a sensitivity of about 1 μg/liter of serum. Antisera were produced by using carbodiimide- or glutaraldehyde-activated conjugates of adriamycin to human serum albumin or hemocyanin. These conjugates were injected into rabbits, monkeys, and a goat. The goat immunized with a glutaraldehyde-activated conjugate of adriamycin to human serum albumin produced an antibody that gave the most sensitive assay. The other antibodies that were produced varied in specificity with the species of animal and type of conjugate. The radioactively labeled drug used in this assay was prepared by carbodiimide conjugation of p-hydroxyphenylacetic acid to adriamycin. The resultant adriamycin derivative was iodinated by the lactoperoxidase method. This conjugation technique produced an amide derivative at the same site on the

![Fig. 7. Structure of adriamycin (R = COCH₂OH) and daunomycin (R = COCH₃)]
adriamycin molecule as used in carbodiimide conjugation of the drug with the carrier protein. The goat antisera recognized this bond with the carrier protein, as indicated by its increased affinity for N-acetyladriamycin compared with adriamycin.

Langone et al. (52) used a combination of radioimmunoassay and high-pressure liquid chromatography to identify and measure adriamycin and its metabolites in biological fluids. This procedure demonstrated the advantage of combining the sensitivity of radioimmunoassay with the separation achieved by liquid chromatography for identifying variations in drug metabolism among individual patients.

**Bleomycin.** This antineoplastic antibiotic, isolated from a mutant strain of *Streptomyces verticillus*, has been effective against a variety of neoplasms, including squamous cell carcinoma, lymphoma, and testicular carcinoma (53). The most common toxic effect of the drug is a minor cutaneous reaction. However, pulmonary toxicity occurs in about 10% of the patients, resulting in pulmonary fibrosis in about 1% (54).

Investigations into the toxicity and pharmacokinetics of this drug have been hampered by lack of a precise and sensitive assay (55). The published microbiological assays (56, 57) lack accuracy and sensitivity at bleomycin concentrations below 1 mg/liter of serum. We have developed a radioimmunoassay for this antibiotic with antisera produced in rabbits by using carbodiimide-activated conjugation of bleomycin to bovine serum albumin, and iodinated bleomycin prepared as described above (30). The assay was sensitive to 250 pg (2.5 μg/liter) and did not cross react with other antineoplastic agents. Comparison with a microbial assay for bleomycin gave a correlation coefficient of 0.978. Studies on patients receiving bleomycin by continuous infusion indicate that the assay can be used to monitor steady-state concentrations and estimate pharmacokinetic variables in patients receiving multiple drug therapy (Broughton, Strong, and Holoey; manuscript in preparation).

Therapeutic monitoring of drug concentrations in blood will be requested more frequently by physicians to optimize therapy and avoid toxicity (2). The clinical laboratory is the logical department to perform these assays, using methods that are rapid, precise, sensitive, specific and suitable for automation. Previously, assays for antibiotics have involved microbiological techniques or complicated procedures requiring highly sophisticated instrumentation and personnel. Radioimmunoassays, however, are eminently suitable for routine use. Therefore, the continued development of reliable radioimmunoassays for antibiotics and other chemotherapeutic agents will allow clinical laboratory medicine more fully to participate in the delivery of health care by providing a quantitative basis for rational modification of chemotherapy.

We appreciate the supply of bleomycin from Bristol Laboratories, Syracuse, New York, and the aminoglycosides from Dr. Gerald P. Bodey, Sr., M. D. Anderson Hospital, Houston, Texas. Dr. Bodey also performed the microbiological assays of these drugs. Dr. S. T. Crooke of Bristol Laboratories performed the microbiological assays of bleomycin.

**References**


