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I. General Survey

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Review

DRUG IMMUNOASSAYS*

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Because of their specificity, sensitivity, rapidity and convenience, drug-specific immunoassays are now widely used both in clinical and investigative laboratories. Drug immunoassays are of clinical value in the determination of appropriate dosage schedules with certain drugs and in the documentation of recent ingestion of other drugs, particularly certain drugs of abuse. Immunoassays have already been of investigative value in studies of the bioavailability, absorption, metabolic degradation and excretion of several drugs.

This review deals with the synthesis and characterization of drug–protein conjugates, immunization with drug–protein conjugates, the detection and immunological characterization of drug-specific antibodies, the use of drug-specific antibodies in the development of immunoassays, general applications of drug immunoassays and, finally, individual descriptions of currently available drug immunoassay methods.

1. Introduction

The past decade has been accompanied by significant advances in the capacity to measure drug concentrations, at the picomolar level, in tissues and biological fluids (Vesell and Passananti, 1971). These advances have resulted, in large measure, from the refinement of quantitative spectrofluorometric (Udenfriend, 1962; Trevor et al., 1971), gas chromatographic (Gudzinowicz, 1967; Hammar et al., 1969; Wilkinson, 1971; Hawks, 1974) and mass spectrometric (Hammar et al., 1969; Jenden and Cho, 1973; Horning et al., 1973; Hawks, 1974) methods for the sensitive and specific determination of drug concentrations in specimens obtained from man and experimental animals. Most recently, drug-specific antibodies have been used with increasing frequency in the development of rapid, sensitive, specific and convenient immunologic assay procedures for the measurement of serum and urine concentra-

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tions of various compounds of pharmacologic interest (Butler, Jr., 1973; Spector, 1973; Bidanset, 1974; Marks et al., 1974; Mulé et al., 1974b). It is the purpose of this review to describe the production of antibodies to drugs, the detection and immunologic characterization of such antibodies, their use in the development of specific immunoassays and, finally, the application of these antibodies in clinical and experimental studies.

2. Production of antibodies to drugs

2.1. General principles

Most drugs are relatively small compounds, with molecular weights less than 1000. Such molecules are not ordinarily immunogenic but, as delineated in the classic studies of Landsteiner (1945), these low molecular weight substances will, when conjugated as haptens to protein or polypeptide carriers, elicit the production of antibodies capable of reacting specifically with the free, unconjugated hapten.

2.2. Choice of a carrier

Almost any protein (including homologous plasma proteins) may be used as a carrier for an haptenically coupled drug. Serum albumins of various species have been most commonly used as such carriers for a number of reasons: their ready availability, low cost, high degree of immunogenicity, excellent solubility, and relative resistance to denaturation by the organic solvents and somewhat rigorous chemical conditions employed in some conjugation procedures. The functional groups in albumins or other protein carriers to which haptenic drugs or drug derivatives may be conjugated include: free amino groups (ϵ-amino of lysine and NH_2-terminal residues); free carboxyl groups (aspartic acid, glutamic acid and COOH-terminal residues); and phenolic (tyrosine), sulfhydryl (cysteine), imidazo (histidine), indolyl (tryptophan) and guanidino (arginine) functions. Drugs and drug derivatives have most frequently been coupled to the amino, carboxyl or phenolic groups of protein carriers (Beiser et al., 1968; Parker, 1972; Butler, Jr. and Beiser, 1973; Erlanger, 1973).

2.3. Choice of a conjugation method

The method selected for conjugation of the haptenic drug or drug derivative to a carrier must employ chemical conditions which do not cause significant structural alterations of the hapten and which do not produce sufficient denaturation of the carrier protein to render it insoluble. A number of such relatively gentle methods have been described for coupling small molecules to protein carriers via amino, carboxyl or hydroxyl groups in drugs or chemically related derivatives (Beiser et al.,

1968; Parker, 1972; Butler, Jr. and Beiser, 1973; Erlanger, 1973; Marks et al., 1974). For example, aliphatic amines can be coupled to carriers using water-soluble carbodiimides (Goodfriend et al., 1964), bifunctional diisocyanates (Haber et al., 1965a), or by conversion to *p*-nitrobenzoylamides followed by reduction to the *p*-aminobenzoyl derivative which, upon diazotization, can be coupled to the tyrosine residues of protein (Anderer, 1963). Aromatic amines can be diazotized and coupled directly to carriers (Hamburger, 1966). Drugs or derivatives with free carboxyl groups can be coupled to amino groups of proteins either by the mixed anhydride (Vaughan, Jr. and Osato, 1952; Erlanger et al., 1957) or carbodiimide (Goodfriend et al., 1964) method. Numerous methods have been developed for coupling compounds with free hydroxyl groups to carrier proteins. The hydroxyl groups of steroids react with succinic acid to form the hemisuccinate (Erlanger et al., 1957) which can then be coupled to a protein via its carboxyl group, using the mixed anhydride or carbodiimide methods, as described above. Phenols have been converted to active reagents by reaction with diazotized *p*-aminobenzoic acid (Weliky and Weetall, 1965); such *p*-aminobenzoate derivatives, like hemisuccinate derivatives, can then be coupled to the amino groups of protein via their functional carboxyl group. Compounds with vicinal hydroxyl groups can conveniently be coupled to amino groups of protein carriers by the periodate oxidation method (Khym, 1963; Erlanger and Beiser, 1964; Butler, Jr. and Chen 1967). These and other conjugation methods have been reviewed in detail recently (Beiser et al., 1968; Parker, 1972; Butler, Jr. and Beiser, 1973; Erlanger, 1973; Marks et al., 1974).

2.4. Characterization of drug–protein conjugates

Landsteiner (1945) and Erlanger (1973) have found that the incorporation of too much, as well as too little, hapten into a hapten–protein conjugate may lead to a poor antibody response; in their experience, 10 haptenic groups per molecule of carrier seemed optimal when serum albumin was used as the carrier. Hence, before one immunizes experimental animals with a newly synthesized drug–protein conjugate, it is usually desirable to determine the number of drug molecules one has conjugated to the protein (or polypeptide) carrier employed.

If the haptenic group has an absorption spectrum which can allow one to differentiate it from the protein carrier, the ratio between the molar extinction coefficient (Little and Donahue, 1968) of the conjugate and that of the hapten at an appropriate wavelength can be used to calculate the molar incorporation of hapten onto protein carrier. However, as Erlanger (1973) has pointed out, even if there is overlap in spectra between hapten and carrier, reasonably accurate estimates of molar incorporation of hapten can be made by determining differences in molar extinction coefficients between conjugate and carrier, and then comparing the difference with the molar extinction coefficient of the hapten (Erlanger et al., 1957; Butler, Jr. et al., 1962; Smith et al., 1970; Tigelaar et al., 1973). The extent of

haptenic incorporation into a hapten–protein conjugate can also be determined by various chemical analyses of conjugates (Goodfriend et al., 1964; Dumasia et al., 1973), extent of incorporation of radiolabelled drug into conjugates (Lewis et al., 1972; Cheng et al., 1973; Chung et al., 1973; Cook et al., 1973; Mahon et al., 1973), or by the decrease in free amino groups of carrier, following conjugation of a hapten to carrier by a method which utilizes the amino groups of the carrier (Erlanger et al., 1957; Erlanger, 1973).

2.5. Immunization

Ordinarily, drug–protein conjugates are suspended in complete Freund's adjuvant mixture (Freund et al., 1948) at a final concentration of 1 mg/ml or less; usually 1 mg or less is injected into experimental animals, most often rabbits or guinea pigs, at intervals of 1 week or more. Longer periods of time (8–16 months, in some instances) than are employed for protein antigens may be required to obtain anti-hapten antibody of optimal titer, specificity, and affinity (Smith et al., 1970; Jaffe et al., 1971). Vaitukaitis et al. (1971) have recently described a method which employs a small, divided primary immunizing dose together with *Bordetella pertussis* vaccine and which may be particularly useful when the quantity of hapten or conjugate is limited. These and other practical problems connected with raising antisera for use in immunoassays have been reviewed recently (Chase, 1967; Hurn and Landon, 1971; Parker, 1972; Hurn, 1974).

2.6. Detection of drug-specific antibodies

Since most animals immunized with drug–protein conjugates form antibodies with specificity for the carrier protein, the method chosen for detection of anti-drug antibodies must be one in which antibodies specific for the carrier will not also interact. The simplest and most direct methods for the detection of drug-specific antibodies without interference by carrier-specific antibodies involve the direct demonstration of binding of radioactively labeled drugs or drug derivatives by antibody. Such binding of radiolabeled drug by antibody can be demonstrated directly by equilibrium dialysis (Eisen, 1964) or indirectly by one of the many methods now available to separate antibody-bound drug from unbound drug; currently popular methods include the dextran-coated charcoal technique (Herbert et al., 1968) gel or membrane filtration (Haber et al., 1965b; Van Vunakis and Levine, 1974), electrophoresis (Yalow and Berson, 1964), and coprecipitation of drug with antibody by the so-called 'double antibody' method (Morgan and Lazarow, 1963; Van Vunakis and Levine, 1974). Since many drugs are bound to a significant degree by normal serum proteins (especially in undiluted serum), it is important to ascertain that binding of radiolabeled drug is not observed with appropriate dilutions of control sera from non-immunized animals and from animals immunized with unrelated antigens.

In the absence of radiolabeled drug, anti-drug antibodies can be demonstrated by other methods, including hemagglutination of drug–erythrocyte conjugates (Adler, 1974) and inactivation of drug–bacteriophage conjugates (Mäkelä, 1966; Dray et al., 1972). It is also possible to employ classic precipitin, complement fixation or passive hemagglutination methods to demonstrate the interaction of anti-drug antibody with conjugates in which the hapten is attached to a carrier antigenically unrelated to the carrier used for immunization; in this latter instance, it is particularly important to ascertain that the interaction with antiserum is specifically inhibited by free, unconjugated drug (Butler, Jr. and Beiser, 1973).

When antibodies to a carrier do interfere with the detection of anti-drug antibodies, such antibodies can usually be removed by prior absorption of antiserum with unconjugated carrier protein or with an appropriate insoluble immunoadsorbent containing the carrier protein (Butler, Jr. and Beiser, 1973). In the instance of one drug–albumin conjugate prepared by the carbodiimide method, native albumin did not completely remove anti-carrier antibody, but albumin polymerized with carbodiimide effectively removed the interfering antibodies (Adler and Liu, 1971). If one wishes to avoid the formation of anti-protein antibodies almost completely, one may take advantage of the fact that, while haptens coupled to homologous albumins are immunogenic, such conjugates usually do not elicit significant production of antibodies to the carrier (Butler, Jr. and Beiser, 1973).

2.7. Specificity of antibodies to drugs

The specificity of antibodies for a drug is usually determined by comparing the capacity of the non-radiolabeled drug and various structurally related compounds to interfere with the interaction between drug and antibody as measured by one of the methods described above. It is particularly important to ascertain that related compounds in serum, urine or tissue do not interact before the antibody is used in the development of an immunoassay (Parker, 1972; Butler, Jr. and Beiser, 1973). For example, antibodies to digitalis glycosides react with steroid hormones (Butler, Jr. and Chen, 1967); therefore, it was necessary to demonstrate that concentrations of steroid hormones encountered in human sera did not inhibit the binding of [^{3}H] digoxin by anti-digoxin antibodies before the radioimmunoassay method could be used clinically to measure serum digoxin concentrations (Smith et al., 1969, 1970).

It is important to recognize that antibodies to a given drug will usually react with metabolites of that drug. It may occasionally be possible to remove some of the antibodies which cross-react in this manner. It should be remembered, however, that absolute specificity of antibodies for a given molecule will rarely, if ever, be observed (Beiser et al., 1968). Cross-reactivity with drug metabolites may not represent a major disadvantage if, in practice, serum concentrations of 'immunoreactive' drug correlate well with values obtained by other methods and with the clinical state of the patients studied. For example, such a correlation does exist in the case

of digoxin; this correlation, however does not necessarily apply to other drugs because digoxin is somewhat unusual in that it is not extensively degraded in man, and in that several of its major metabolites are both pharmacologically and immunologically active (Butler, Jr., 1972).

Antibodies to a given drug will often react with related drugs of the same class, but this cross-reactivity should not constitute a major problem clinically if it can be established with certainty that the patient is receiving a given drug and has not recently received a chemically related agent (Butler, Jr., 1972).

If problems are encountered in obtaining antibodies of satisfactory specificity for use in an immunoassay procedure, it is important to remember that different individual animals may produce antibodies which vary greatly in specificity; one should, therefore, examine several antisera before selecting the one with optimal specificity. It also may be useful to recall that the specificity of anti-hapten antibodies appears to be directed primarily against that portion of the hapten molecule furthest from the site of conjugation to the carrier; antibodies of different specificity can usually be obtained if the hapten is coupled to the carrier via a different functional group (Parker, 1972; Butler, Jr. and Beiser, 1973; Erlanger, 1973).

2.8. Affinity of drug-specific antibodies

Antiserum to a given drug usually contains a heterogeneous population of anti-drug antibodies with different avidities or association constants. In general, antisera with high average intrinsic association constants are most useful in the development of immunoassay procedures of optimal sensitivity, rapidity and reproducibility (Parker, 1972). The determination of association constants is therefore useful in the selection of antisera for use in immunoassay work; it is, however, not essential since the avidity of antibodies may be inferred from a variety of measurements of hapten–antibody interactions (Hunter, 1973).

Recent studies have also called attention to the fact that dissociation constants of drug-antibody complexes may be important when the adsorbent used to separate free from antibody-bound drug in an immunoassay procedure competes with antibody for drug molecules which dissociate from antibody during the separation step of the immunoassay (Meade and Kleist, 1972; Smith and Haber, 1973).

2.9. Titer of drug specific-antibodies

Titer is generally defined as the greatest dilution of antibody which will produce a given degree of binding of a stated amount of a drug (Hunter, 1973). The higher the titer, the more determinations one can perform with a given volume of antiserum; for example, anti-digoxin sera frequently can be used at dilutions theoretically great enough to allow for the performance of 200,000 digoxin determinations with 1 ml of antiserum. Titer is, however, far less important than specificity and affinity in the choice of an antiserum for use in an immunoassay procedure; a

high-titered antiserum will be much less useful than a lower-titered serum with greater specificity or affinity.

3. Immunoassay procedures

3.1. Principles

Immunoassay methods for the measurement of drugs are based upon the ability of drugs to inhibit the reaction between drug-specific antibodies and the corresponding drug–carrier conjugate or the corresponding labeled hapten. Using principles delineated by Berson and Yalow, increasing concentrations of a known standard solution of the drug are incubated with constant predetermined amounts of drug-specific antibody and of drug–carrier conjugate, or of labeled drug, under conditions of antigen excess. A standard curve is then constructed, upon which decreasing amounts of drug–antibody interaction can be shown to correspond with increasing concentrations of drug. If the biological fluid to be assayed (a) does not interfere with the drug–antibody reaction, (b) does not degrade the drug, and (c) does not contain substances which crossreact significantly with the drug antibody, the concentration of the drug in that biological fluid can be determined from the degree to which it inhibits the reaction between antibody and drug–carrier conjugate or labeled drug, when compared with a simultaneously performed standard curve (Yalow and Berson, 1964; Parker, 1972; Hunter, 1973; Ekins, 1974).

3.2. Methods

Inhibition of complement fixation (Hamburger, 1966) or of passive hemagglutination (Adler, 1974) have been used to measure drugs. However, because of their greater sensitivity, precision, rapidity and convenience, greater experience has been obtained with immunoassay methods which employ radioactively or physicochemically labeled drugs or drug derivatives.

Until recently, most of the studies with labeled haptens employed radioactively-labeled drugs or drug derivatives. Since a high specific activity is generally required for optimal sensitivity in radioimmunoassay procedures, ^{3}H-labeled drugs have generally been more useful than ^{14}C-labeled compounds. Since ^{3}H and ^{14}C are both beta-emitting isotopes, their use necessitates the employment of liquid scintillation counting techniques which are expensive and cumbersome, and which may be associated with problems of chemilluminescence and variable counting efficiency when biological fluids are studied (Butler, Jr., 1972; Smith and Haber, 1973). The development of methods which can be used for the radioiodination of drugs and drug derivatives (Oliver et al., 1968; Parker, 1972; Hunter, 1974; Van Vunakis and Levine, 1974) has permitted the use of simpler gamma-counting procedures and should permit the more rapid introduction of drug radioimmunoassays into general and clinical investigative use.

Many methods are available for the separation of free, unbound radiolabeled drug from antibody-bound radioactivity in the development of radioimmunoassay procedures (Hunter and Ganguli, 1971; Parker, 1972; Hunter, 1973; Ratcliffe, 1974). These include electrophoresis, gel filtration, adsorption systems (e.g., dextran-coated charcoal, membrane filtration), solvent and salt precipitation systems, solid-phase antibody procedures and the double antibody method. Because of its convenience and rapidity, the dextran-coated charcoal method of Herbert et al. (1968) has been widely used in drug radioimmunoassay procedures. It involves the almost instantaneous adsorption of non-antibody-bound drug onto charcoal particles and its rapid centrifugal separation from antibody-bound radioactivity; thus, it is not useful in instances wherein radiolabeled drugs or drug derivatives are not effectively adsorbed to the charcoal nor in instances in which the dissociation constant of a drug–antibody complex is great enough to cause a temporally related variability in results as dissociated drug is progressively adsorbed to charcoal as a function of the duration of the charcoal incubation step in the immunoassay procedure (Smith and Haber, 1973). Because of such problems with adsorption methods, many newly developed radioimmunoassay procedures have employed either the double antibody method or a solid phase antibody procedure. Theoretically, 'immunoradiometric' assay procedures employing radioiodinated antibody (Woodhead et al., 1974) may also be used, but extensive experience has not yet been obtained with such procedures in the assay of drugs.

To eliminate some of the technical problems associated with the use of radiolabeled drugs in clinical laboratories or in automated equipment, physicochemically-labeled drugs have recently been employed in the development of non-isotopic immunoassay procedures. For example, spin-labeled drugs have been used in an immunoassay procedure which takes advantage of the fact that the mobility of free radicals of spin-labeled drugs in the free, unbound state differs from their mobility when bound to specific antibody, as measured in an electron spin resonance spectrometer. This technique has proved useful in screening biological fluids in the detection of opiates and other drugs of abuse (Leute et al., 1972a, b; Schneider et al., 1974). Another, perhaps more useful method, is based on the fact that anti-drug antibodies will inactivate drug–enzyme conjugates in a reproducible manner which is readily quantifiable by simple spectrophotometric assays of enzymatic activity; increasing quantities of drug will reduce the degree of enzyme inactivation in a reproducible and predictable manner which can be used as the basis for a simple immunoassay procedure (Engvall and Perlmann, 1971; Rubenstein et al., 1972; Schneider et al., 1973, 1974).

3.3. Problems in application of drug immunoassay methods to the study of biological fluids

It is most convenient when an immunoassay procedure can be carried out with untreated serum, plasma or other biological fluid. In some instances, however, prior

treatment or separatory procedures may be necessary to inactivate substances which degrade the drug being studied. In other cases, prior extractions or separatory procedures may be necessary to concentrate a drug, to remove it from a normal binding site on a plasma protein in the test serum, or to separate the drug from certain of its metabolites or from other structurally related compounds also capable of inhibiting the drug–antibody interaction (Parker, 1972; Butler, Jr. and Beiser, 1973). In radioimmunoassay procedures, the presence of a radioisotope (administered for diagnostic or therapeutic purposes) in a patient's serum constitutes a potential source of error, if the presence of the isotope is not known to the laboratory; in radioimmunoassay procedures employing liquid scintillation counting methods, chemilluminescence produced by urine and certain sera (especially from azotemic patients) may also interfere with assay results. Neither of these latter sources of error in radioimmunoassay procedures, however, should cause a problem if proper control procedures are carried out (Butler, Jr., 1971; Smith and Haber, 1973).

3.4. Stability of reagents

Although antibodies are stable for many years if properly stored in concentrated form, deterioration of antisera may occur if appropriate precautions are not taken. Deterioration of radiolabeled drugs or drug derivatives also may occur with time; this is particularly true in the case of radioiodinated derivatives of drugs (Kirkham and Hunter, 1971). Similarly, great care must be exercised in the preparation and storage of the drug standards used in the construction of standard immunoassay curves because results in unknown specimens will be calculated on the basis of values obtained with these standards of predetermined, known concentration. (Bangham and Cotes, 1974).

4. General applications of drug immunoassays

The development of radioimmunoassay methods as well as of other new assay methods of great sensitivity and specificity has added greatly to our knowledge of the pharmacokinetics (absorption, compartmental distribution, degradation and excretion) of many important drugs and their metabolites (Levy and Gibaldi, 1972). With the increasing availability of information in this area, much has been learned about previously obscure processes which contribute greatly to the long-recognized but hitherto poorly understood individual variability in drug dosage requirements and in susceptibility to the toxic effects of drugs (Azarnoff, 1973; Butler, Jr. and Lindenbaum, 1975). In this regard, drug assay methods of many types have made it possible to study drug–drug interactions (Solomon et al., 1971) and to identify clinically important differences in the biologic availability of different preparations of the same drug (Lindenbaum et al., 1971; Brodie and Heller,

1972; American Pharmaceutical Association, 1973), in the genetic capacity to metabolize certain drugs (La Du, 1972; Vesell, 1972) and in the serum protein binding (Davison, 1971) and renal excretion (Bennett et al., 1973) of other important drugs.

Since, in many instances, immunoassay procedures can be carried out on microliter volumes of unextracted serum, such methods often offer significant advantages over some of the other new techniques for the measurement of drugs at the submicrogram level. In addition, their convenience and rapidity are major advantages in the clinical laboratory where the rapid analysis of large numbers of specimens is highly desirable in the evaluation of drug therapy and in the prompt determination of appropriate dosage schedules for individual patients being treated with a given drug. However, as with all drug assay procedures, drug immunoassay methods will yield useful information only if it can be established, experimentally or clinically, that the drug concentrations in the specimens being assayed bear some relationship to a pharmacological effect of, or the clinical response to, that drug (Brodie and Reid, 1971; Vesell and Passananti, 1971; Koch-Weser, 1972). In this latter connection, the time at which the specimen is best obtained with respect to the last dose of the drug is quite important and must be established before the immunoassay procedure can be used properly (Butler, Jr., 1972; Koch-Weser, 1972).

5. Specific drug immunoassays

Table 1 lists drugs to which antibodies have been elicited, but for which immunoassay procedures have not been described. Immunoassays have been described for the drugs listed in table 2. These immunoassays will be described individually in this section.

5.1. Analgesic drugs

An analogue of fentanyl, carboxyfentanyl, has been synthesized and conjugated to bovine γ-globulin by the carbodiimide method. Rabbits immunized with carboxyfentanyl–protein conjugates formed antibodies capable of binding [^{3}H] fentanyl, as assessed by the ammonium sulfate precipitation method. Unlabeled fentanyl and its metabolites are capable of inhibiting the binding of [^{3}H] fentanyl by antibody, suggesting that this antibody will be useful in the development of a radioimmunoassay method for the measurement of fentanyl in biological fluids and tissues (Henderson et al., 1974).

5.2. Antibiotics

The nitro group of chloramphenicol has been reduced to form the amine, which could then be diazotized and coupled to protein carriers. Rabbits immunized with