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Modern drug analysis in biological fluids suitable for clinical pharmacokinetics*

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Current Scope of Clinical Pharmacokinetics

Therapeutic drug monitoring can provide valuable information to guide clinicians in achieving optimal treatment with selected therapeutic agents. Large intersubject variations in drug response are common. There are many factors that may cause variability in drug disposition and drug plasma levels using standard dosage regimens:

(1) Biometric factors: age, height, weight;
(2) Absorption and patient compliance;
(3) Distribution, cardiac failure;
(4) Elimination: hepatic and/or renal failure;
(5) Individual capacity in metabolizing drugs;
(6) Drug–drug interactions.

All these factors demand dosage adjustments for the individual patient and contribute to the observation that the correlation between drug plasma level and response is much better than that between the total dose and response among a given patient population. Pharmacokinetic–pharmacodynamic studies yield information on the therapeutic plasma concentration range of a drug that should be maintained during therapy to achieve maximum benefits. This leads to the concept of the 'target level' of a particular drug in plasma, which is important for the application of clinical pharmacokinetics in drug therapy. There are two ways by which 'target levels' can be utilized to optimize the individual drug dosage regimen: first, calculation of the dose based on predictive pharmacokinetic models for the individual patient; and second, inclusion of drug level monitoring to correct the predictive model for unaccountable intersubject variation.

Predictive pharmacokinetic models are particularly useful for drugs that are mainly eliminated by renal excretion, since good correlations between clinical kidney function tests and drug clearance have been established. Thus dosage adjustments, based on individual kidney functions, are an integral part of modern drug therapy with drugs like

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antibiotics, digoxin and lithium. Computer packages are now routinely available that simplify the application of clinical pharmacokinetics. However, a series of shortcomings is inherent in this process: our ability to predict individual pharmacokinetic parameters is still essentially nonexistent for most drugs that are eliminated by metabolism. The regulation of individual drug metabolizing capacity is too complex to allow the applications of predictive models.

The second approach, which includes actual drug level determinations, should ideally be based on the scheme shown in Fig. 1.

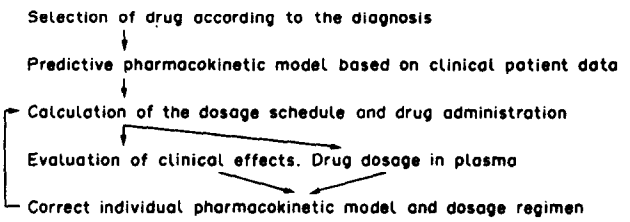


Figure 1

Determination of drug levels allows the rapid and safe attainment of target plasma levels, and in conjunction with observations of the clinical drug effects, should provide the safest approach to drug therapy.

Nevertheless, scepticism prevails among many clinicians, who believe that the cost of therapeutic drug level monitoring outweighs the potential benefits to the patient. Several factors, which may indeed seriously affect the interpretation of drug level data, are the basis for this scepticism:

(1) There is no clearly defined therapeutic concentration range or toxic concentration range. Optimum effects for an individual patient may at times be achieved outside the established therapeutic range. Moreover, drug level data are only aids, to be used with clinical observations, to optimize individual therapy. If, however, the clinical response cannot be readily monitored, use of drug 'target' levels may be the next best approximation to optimize therapy.

(2) The formation of active metabolites, frequently with unknown efficacy in human beings, complicates the interpretation of drug level data.

(3) Analytical assay error may be substantial. Moreover, different assay techniques are utilized in different hospitals, resulting in unpredictable differences in assay precision and specificity.

(4) Interpretation of drug level data depends on the following information: dosage regimen, time of last dose, and time of blood sampling. Information on these variables may be inaccurate or unavailable.

(5) The time of blood sampling of greatest value depends on the dose schedule and the half-life of the drug. Since the half-life may differ greatly between patients, blood samples may be obtained at points that do not allow kinetic interpretation of the data.

(6) Plasma level data may be improperly used to adjust dosage levels, or may be altogether ignored.

It seems, therefore, that therapeutic drug level monitoring can be successful only if the organization of sample and data collection, the interpretation of drug levels, and the implementation of appropriate therapeutic decisions are all supervised by suitably trained personnel.

Which drugs require individualized dosing? The first group consists of agents exhibiting an erratic or unpredictable dose–response relationship (e.g. theophylline).

Another group is represented by drugs which possess narrow therapeutic ranges in serum (aminoglycosides, cardiac glycosides, antiarrhythmic drugs).

Serum concentration data must be interpreted in the light of the clinical situation and with a working knowledge of the pharmacokinetic profile of the particular drug. A drug level determination is only one indication of the patient's therapeutic status and must be used in conjunction with the therapeutic range over which a drug has a high degree of efficacy and a low risk of dose-related toxicity. Thus, the therapeutic range is a statistical concept: it is the concentration range associated with therapeutic response in the *majority* of patients. The clinical application of therapeutic drug monitoring is, of course, limited to those agents demonstrating a correlation between a particular range of serum drug concentration and a beneficial therapeutic effect. Monitoring drug concentrations may also be useful in identifying drug interactions (e.g. enzyme induction of metabolism, modification of the distribution volume, competition for renal excretion).

It is also very important to complete a comprehensive requisition form for each request for therapeutic drug monitoring, including the time when the sample is drawn, the last time the drug was given, and the route of administration.

The expense involved is also to be considered when therapeutic drug monitoring is evaluated. Regardless of the method used in measuring serum drug concentrations the total cost may be US$20–25. Thus, while economic considerations are important, other factors determine the appropriateness of serum drug determinations. They include the analytical method (sensitivity, specificity, time taken, and the clinician's ability to utilize the serum level information to improve patient care.

Table 1
Summary of data points to be considered in developing requisition forms

1.	Patient identification
	1.1 Name
	1.2 Address
	1.3 Social Security, or other numbers
2.	Patient characteristics
	2.1 Age
	2.2 Sex
	2.3 Ethnic origin
	2.4 Size and habitus of patient
	2.5 Concurrent diseases afflicting patient and other pertinent clinical information
3.	Specimen information
	3.1 Time of specimen collection
	3.2 Number if part of a series of specimens
	3.3 Nature of specimen: specific body fluid submitted
	3.4 Preservatives or anticoagulants mixed with specimen
4.	Purpose of analysis
	4.1 TDM, overdose specimen, etc.
	4.2 Drug to be identified and/or quantitated
	4.3 Other drugs and/or interfering substances in specimens
5.	Dosage information
	5.1 Time of last dose, frequency of prescribed dose, quantity administered, and route of administration
6.	Clinical communications
	6.1 Source of specimen, referring physician
	6.2 Point to which information should be communicated
	6.3 Therapeutic and toxic concentrations of drug
7.	Processing data
	7.1 Time of receipt of specimen by laboratory
	7.2 Time of report by laboratory
	7.3 Identification of responsible scientist

Analytical Techniques

Introduction

In the last decade, the methodology for determining drug concentrations in body fluids has advanced dramatically. Relatively non-specific, time consuming and complex procedures requiring large sample volumes have been replaced by methods using microsamples and displaying improved sensitivity, specificity and simplicity.

Serum drug analysis as a clinical tool requires that the method selected be sufficiently sensitive and highly specific; and it must be sufficiently rapid to allow the result to be useful in the clinical management of the patient.

As important as selecting the most appropriate analytical method is deciding precisely *what* must be measured. Measuring only the drug administered may be of limited value and may even be seriously misleading. Often, concentrations of active metabolites must also be measured, depending upon the amount of active metabolite formed and/or its accumulation in the body (e.g. procainamide and NAPA).

Today there are many assay methods available for specific compounds. Many of these techniques are comparable, but each has its inherent advantages and limitations. An in-depth review of all the methods is beyond the scope of this paper, but many of the commonly used methods will be highlighted.

Current trends in analytical techniques for T.D.M.

Drug level monitoring is based on analytical techniques suitable for the quantitative detection of drugs and their metabolites in biological samples (qualitative methods, although often similar, will not be discussed here). The following methods are available:

high performance liquid chromatography (HPLC);
gas chromatography (GC);
mass spectrometry (MS);
thin-layer chromatography (TLC);
ultraviolet spectrophotometry (UV);
fluorescence (Fluor);
colorimetry (Col);
polarography (Pol);
radioimmunoassay (RIA);
enzyme immunoassay (EIA);
other immunoassays (IA);
protein binding assays (PBA) (excluding immunoassays);
radio receptors assays (RRA);
enzymatic assays (EA);
microbiological assays (Micro).

A review published in 1980 provided estimates of the applicability of various methods to the TDM of 75 important drugs: (1) GC (77%); (2) HPLC (70%); (3) MS (53%); (4) RIA (39%); (5,6) Fluor and Col (35% each); (7) TLC (32%); (8) UV (29%); (9) EIA (23%). The remaining methods were applicable to only 13% of the drugs at most. The prevalence of GC attests to its versatility. The even more widely applicable HPLC technique ranks only second because it was introduced to drug level monitoring several years later than GC: HPLC is expected to surpass GC as the most widely applicable method in the near future. Also, the ranking order obtained does not include the suitability of the various methods. For example, simple UV assays often lack the specificity required for clinical applications.

Another important development is the growth of immunological methods in T.D.M., particularly the non-radioactive methods (FIA, EIA, NIA, PACIA . . .). The major techniques used in clinical laboratories fall into three general classes:

(1) Chromatographic methods, combined with a variety of detection modes (HPLC, GC, TLC);
(2) Spectroscopic analyses (UV, Fluor, Col);
(3) Competitive protein binding assays (IA, PBA, RRA).

Mass spectrometry, in combination with GC or by direct analysis, accounts for a surprising 53% of the drugs. Many MS assays have been developed because of the potentially high sensitivity and specificity of this method, although the instrumentation is considerably more elaborate than that of other techniques. Since the development of highly sensitive and specific HPLC, GC and RIA, drug assays MS has become obsolescent, unless employed in special research or quality control situations. The various techniques will be discussed in more detail.

Collection of biological samples for analysis

The most readily accessible body fluids are blood, saliva and urine. While all these fluids are utilized for drug assays, plasma or serum measurements may yield a better correlation between drug concentration and effects. The analysis of drugs in whole blood should not be encouraged, since the erythrocyte/plasma concentration ratio is dependent on a number of variables that may limit the pharmacologic interpretation of the results. Blood samples should therefore be centrifuged to obtain either plasma or serum. There appears to be very little drug binding to fibrin, and assay results using plasma and serum are usually identical. Plasma specimens can be obtained by preventing coagulation with various agents, including heparin and the Ca^{2+} binding agents, EDTA, citrate and fluoride. Choice of the anticoagulant may affect assay results; similarly, blood collection tubes (e.g. plastic vacutainers) may release substances, such as plasticizers, that can also interfere with the assay. Most clinical laboratories now use serum for drug analysis. The composition of plasma and serum varies considerably with pathophysiological condition; therefore drug assays that are recommended for clinical use have to be validated in samples obtained from patients with a variety of disease states. Most notably, hyperlipidemia and hyperbilirubinemia are likely to interfere with many drug level assays.

All drug assays are designed to measure total concentration rather than the free fraction of a drug in serum, yet many drugs are highly bound to serum proteins. Albumin accounts for most of the drug binding, while globulins may significantly contribute in some instances. When defining the range of therapeutic serum concentrations of individual drugs, one considers only total drug concentrations; however, the inter-individual variability of drug protein binding may be large under normal conditions and even greater under pathophysiological ones (hyperbilirubinemia, hypoalbuminemia). The correlation between total drug level and response deteriorates under conditions of variable drug protein binding; phenytoin represents an example of clinically important binding variability, and drug level monitoring is best performed on the free drug fraction in the serum. Because of the additional analytical work-up required (e.g. ultracentrifugation or ultrafiltration) most clinical laboratories do not perform such assays.

Preparation and treatment of the sample before the analysis

Suitable preparation of biological specimens is decisive in the successful application of

an analytical technique. The preparation should be as simple as possible, yet allow the specific assay of a drug in the presence of numerous biological components. The extent of sample work-up is therefore largely determined by the selectivity of the analytical technique. Potentially interfering endogenous substrates need to be removed before analysis. A second objective of the preparation of biological specimens is to protect the analytical apparatus from contamination by lipids, proteins and undissolved particles. Biological sample preparation has to vary according to the technical demands of the various analytical instruments.

The high inherent specificity and sensitivity of immunoassays usually allows the direct detection of drugs in very small samples (<100 µl serum). High performance liquid chromatographic methods require at least protein precipitation in order to protect the column against the deposit of proteins on the stationary phase. Alternatively, a small precolumn, into which plasma samples can be injected without any preparation, can be used. Such precolumns filter out the protein fraction and have to be changed regularly. Protein precipitation methods are rapid and include mixing the sample (serum, urine) with water-miscible organic solvents or acids ($HClO_4$, trichloroacetic acid). Of the organic solvents, acetonitrile yields a protein precipitate that can be readily centrifuged into a small pellet. Choice of the protein precipitating reagent may affect assay results by potential coprecipitation of varying amounts of the drug, a possibility that has to be tested in each case. Use of protein precipitation alone without further work-up is rapidly rising because of the increased application of HPLC. However, sensitivity is usually limited to the micrograms per milliliter range when using UV detection, since only small aliquots of the biological sample can be taken for analysis without overloading the column or causing assay interferences.

Organic solvent extractions are still by far the most useful methods for the preparation of biological samples for subsequent analysis. Many drugs are lipophilic, while a majority of the small molecular weight constituents of serum and urine are polar. Thus a single solvent partitioning step removes many impurities, allowing the analysis of larger aliquots of the biological sample and, thereby, greater sensitivity. Removal of polar, nonvolatile substances is also needed to protect GC columns from unwanted contaminations. Three major variables should be considered in the design of suitable solvent extraction procedures: the organic solvent, the pH of the aqueous phase, and the volumes of the organic and aqueous phases. A higher pH is often desirable, since many endogenous substances are acidic and are not extractable at alkaline pH.

Special types of solvent extraction include ion-pair extractions (methotrexate) and extractive alkylations (oxazepam, 6-mercaptopurine). The second approach involves the simultaneous extraction and chemical derivatization of the drug. This technique is important for a drug that might otherwise be difficult to extract because of its amphoteric polar nature. Sample preparation by chromatographic techniques using small columns is becoming more frequent (Extralut®, Minicon®, . . .). These techniques lead to substantial economy in solvents and time; the sensitivity is also improved because the purification and the concentration of the original sample is better than with repeated extractions.

Chromatographic techniques used in T.D.M.

GC and HPLC are the two most frequently used techniques for therapeutic drug monitoring.

Gas chromatography (GC). There are two basic modes of gas chromatography, gas–liquid partitioning (GLC) and gas–solid adsorption (GSC). Adsorption gas chromatography (GSC) employs an adsorptive solid column packing material and is mainly applied to gases and highly volatile compounds, e.g. volatile anaesthetics and ethanol.

Partitioning GLC is performed either on columns packed with an inert, microparticulate support material that is coated with a liquid phase, or with long capillary tubes coated on the inside wall with the liquid phase. Capillary GC columns are highly efficient, since turbulence of the carrier gas is minimized. With a column length of more than 100 m, capillary GC offers the greatest separation potential at present. Packed columns, on the other hand, are quite versatile and are sufficient for most applications.

The major limitations of GC are the requirements for a sufficiently high vapour pressure and for thermostability at GC temperatures (100–350°C). Polar functions (OH, NH_2, COOH, etc.) reduce the vapour pressure and may prevent effective GC analysis. Therefore many drugs have to be derivatized prior to GC in order to lower their vapour pressure. Thermolytic reactions during GC separation are quite common (e.g. carbamazepine, sulfonamides, phenobarbital, chlordiazepoxide, . . .) and should be carefully checked. Biological samples contain large amounts of polar nonvolatile materials such as peptides, sugars and amino acids, which have to be removed prior to GC in order to prevent column degradation. The detectors most frequently used in drug level monitoring are the flame ionization (FID), nitrogen–phosphorus (NPD) and electron capture (EC) detectors. While the sensitivity of the FID is high, assay sensitivity is usually limited to injected drug amounts in the nanogram range, since the FID is nonselective and measures any organic compound capable of flame ionization. Thus FID sensitivity is background noise limited, normally providing a usable assay range above 100 ng drug/ml serum. The NPD yields greater assay sensitivity for nitrogenous drugs in biological samples because of greater selectivity. Electron capture detection is not only more sensitive than the FID but also more selective than the NPD, in this case for compounds with high electron affinity. Only a few drugs possess sufficient electron affinity to permit sensitive EC detection. However, polyfluorinated derivatizing reagents convey excellent sensitivity toward EC detection as well as suitable GC characteristics for the derivative; these reagents are frequently employed for drugs with low therapeutic or active plasma concentrations ($\leq$10 ng/ml) (clonidine, digoxin). The lower limit for detection of a drug in serum may be as little as 100–500 pg/ml (clonazepam, clonidine, morphine).

Because of the many steps involved in GC sample preparation, an internal standard is needed to correct for extraction losses and injection inaccuracies. An internal standard should be a close chemical congener subject to the same derivatization reaction. It also should be added at a concentration similar to that of the analyte and well separated not only from the drug but also from other interfering peaks.

In conclusion, although GC analysis has been somewhat over-shadowed by recent HPLC developments, there now seems to be a renewed interest in GC. During 1978–1982 several drug assays were published that are at least comparable to those obtained with HPLC in speed, sensitivity, precision, and specificity for therapeutic drug level monitoring. These excellent GC assays are often based on rapid microextraction techniques, capillary columns and the NPD.

High performance liquid chromatography (HPLC). High performance liquid chroma-

tography (HPLC) is increasingly becoming attractive as an analytical tool in T.D.M.

Chromatography with a liquid mobile phase (LC) is more appropriate for most compounds of biological origin than gas chromatography (GC) because these compounds are not usually volatile, but open column methods are too slow for analytical work, and quantitative interpretation of thin layer chromatograms is not easy. HPLC methods are faster, need less sample preparation or derivative formation and are more specific than previous ones. This aspect is often emphasized when it is important to detect and determine drugs together with their metabolites in biological fluids. In earlier work metabolites, generally more polar and more difficult to extract than the original drug, were often not identified. Reversed phase chromatography exploiting ion pair equilibria is the most widely used technique in this context.

The advances made in the speed, flexibility and sensitivity of these methods have been brought about by the combination of the study of fundamental principles and design of equipment. The sophistication of the theory has led to a simplification of procedures, and methods which do not require elaborate sample preparation or derivative formation have in many cases replaced gas chromatographic procedures. In other cases, procedures including trace enrichment through gradient elution and pre- and post-column derivatization are being developed to meet the ever increasing demand for sensitivity and specificity.

In routine work, where standard substances are available, retention data are adequate for identification so that analyses can be carried out with simple equipment to give easily evaluated results.

Identification can be made more reliable by exploiting the specific properties of detectors, and ultimately by combination with mass spectrometry. Reversed phase HPLC (RP) is the most widely used mode of chromatography today in drug level monitoring. There are four reasons for this:

(1) Separation based on lipophilicity is advantageous, since many drugs are more lipophilic than potentially interfering endogenous substrates.

(2) Since polar compounds are less well retained on the column, the polar endogenous substrates elute prior to the lipophilic drugs. Usually, no further UV absorbing material elutes after the drugs, thus allowing rapid analysis of multiple samples. Furthermore, organic solvent extraction may not be necessary, as is the case with normal phase HPLC.

(3) Several chemically different reversed phase column packings are applicable to a surprisingly large number of drugs.

(4) In conjunction with ion-pair chromatography, a variety of polar ionic and nonionic drugs can be analysed by reversed phase HPLC (e.g. gentamicin).

Reversed phase HPLC is capable of directly measuring therapeutic serum concentrations of the following drugs without organic solvent extraction: anticonvulsivants, chloramphenicol, indomethacin, nitrofurantoin, penicillins, quinidine, sulphonamide, theophylline and warfarin.

Ion-exchange chromatography depends upon the exchange of ions between the mobile phase and the ionic sites of the packing, for example, sulphonic acids and quaternary ammonium groups. Both cationic and anionic drugs can be analysed (e.g. acetaminophen, methotrexate, procainamide). The actual mode of separation may not depend exclusively on the ionic strength of the drug, with lipophilicity also contributing to chromatographic behaviour.

Exclusion chromatography is based on the molecular size of the solute. It is potentially useful for the separation not only of macromolecules but also of small (<1000) molecular

weight substances. Gel permeation and gel filtration are synonyms for exclusion chromatography. Steroids are commonly separated on Sephadex LH-20 (Pharmacia, Uppsala) at ambient pressure, using eluents. The HPLC assay of acetaminophen can be performed on a polyamide column as an example of exclusion chromatography.

Of the many existing and potentially useful HPLC detection systems, only four have actually been employed for the analysis of drugs in biological fluids. These are the flow cell detectors based on UV absorbance, fluorescence and electrochemical reactions, and MS.

The sensitivity of electrochemical flow cell detectors can match that of a fluorescence detector (<1 pmol injected), yet few examples are available of the assay of drugs in biological samples (e.g. methyldopa, tetracyclines). The future use of this detector system will certainly include the analysis of several drugs with suitable redox potentials.

In summary, HPLC is one of the most useful techniques in drug level monitoring. Technical advances in the near future are likely to include the development of columns with a substantially larger number of theoretical plates for the analysis of complex mixtures, and the enhancement of existing detection systems by electronic and statistical means and computerization. The use of the mass spectrometer as an on line detector (LC/MS) is also in progress.

Immunoassays for drugs

The first immunoassay for a drug — digoxin — was described in 1967 and in the next 3 years only four more assays were added to the repertoire. Then the flood gates opened and new assays began to appear in the literature at the rate of 15–20 per year. Very few of these assays have, however, progressed beyond the initial description or research stage and only a miniscule proportion of all the drug assays described have found a regular place in the clinical laboratory repertoire.

There are several reasons for this:

(1) Technical difficulties concerned with regular production of reagents (especially antisera) suitable for incorporation into reliable immunoasssay kits. Because of the low immunogenicity of most drugs, even when coupled to large proteins under ideal conditions using suitable spacer groups and optimum substitutions antibody titres are seldom very high.

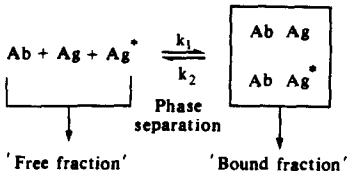
(2) Few clinicians recognize the potential advantages of monitoring blood drug levels as a guide to treatment. Consequently demand is likely to be much smaller than the true need.

(3) The number of drugs shown conclusively to be worth measuring clinically as a guide to therapy is still pitifully small.

(4) Few of the immunoassays currently available are well suited to clinical — as opposed to research — needs. Drug assays, to be of real value, must be available within minutes, or at the most hours, of collection of the blood sample. In contrast, in diagnostic procedures such as those involving the measurement of hormones timing is generally much less critical.

General principle. Since radioimmunoassays were the prototypes of all subsequent types of immunoassay, they can be used to illustrate the general principles.

Equation (1)



(1)

(where Ag represents the analyte, Ag* its radiolabeled form, Ab the specific antibody, and k_1 and k_2 are the association and dissociation constants respectively) describes the fundamental reaction upon which all competitive techniques are based. The antigen (Ag) component is either a pure standard preparation of the analyte or the analyte contained in the biological sample undergoing analysis. The labelled antigen (Ag*) is clearly not identical to Ag but must display closely similar immunoreactive characteristics. In practice the total antigen (Ag + Ag*) competes for a predetermined, limited number of binding sites on the specific antibody, partitioning themselves according to their relative abundance in the mixture. Because most antibodies used in RIAs display high affinity towards their respective antigens attainment of equilibrium is generally quite rapid. In many cases where the drug concentration is relatively high (nmol/l–μmol/l) an immunoassay can thus be completed in only a few minutes.

There are two species of labelled material in the incubate when equilibrium has been reached. One is bound to the antibody (Ag* Ab) and one is not — the 'free' fraction. The two species must be separated from each other without disturbing the equilibrium, and the radioactivity in one or both of them determined. The amount of label bound to the antibody varies according to the ratio of the concentrations of the labelled and unlabelled antigens, an increase in the latter producing a decrease in the amount of antibody-bound label. By adding increasing amounts of pure antigen to a series of incubates containing fixed amounts of label and antibody, a dose–response curve can be constructed.

Antiserum preparation. The antiserum is undoubtedly the main determinant of the capabilities of an immunoassay. The most important properties are its specificity and affinity for the antigen.

Antibodies are produced in animals in response to challenge by an immunogen, i.e. a substance capable of provoking an immune response. Immunogens are normally substances of high molecular weight and in order to get a small molecular weight compound, such as a drug, to act as an immunogen it must first be covalently linked to a carrier protein, in which case the drug behaves as a 'hapten'. Several factors are involved in the production of drug–protein conjugates suitable for the production of antibodies for use in a radioimmunoassay. These include (i) the point of attachment of the hapten to the carrier, (ii) the number of haptens per carrier molecule, and (iii) the type of link between hapten and carrier.

The carrier is invariably a protein, so conjugation is generally effected by attaching the hapten, by any one of an increasing number of conjugation reactions, to either the free -COOH or $-NH_2$ groups in the protein.

Before injecting the immunogenic conjugate into animals it may first be mixed with an oil-containing adjuvant, forming stable emulsion and increasing the probability of a good immune response. Whilst most investigators have used Freund's complete adjuvant, equally good results can be obtained using a Marcol 52 based material which is non-ulcerogenic.

Phase separation techniques. A method of separating the free from the antibody-bound antigen without disturbing the antigen–antibody equilibrium is essential for all radioimmunoassays and some other I.A.

There is no single method of phase separation which is equally suitable for all assays and several different types of separation procedure have been developed. The list of separation methods is sufficiently large to supply at least one that is suitable for any particular radioimmunoassay. Within each group modified procedures have often been developed to suit specific peculiarities.

Chromatographic techniques are generally too cumbersome for routine analysis of many samples, but may be extremely useful in the initial stages of assay development. Adsorption methods are widely favoured since they are usually simple, rapid and cheap. Usually the 'free' antigen fraction is adsorbed onto the material selected, leaving the antibody-bound fraction in solution. Particulate charcoal is the most widely used adsorbent, especially for the assay of small molecules, but is rarely used in untreated form, since it is also capable of binding antibody-bound antigen. In order to try and alleviate this difficulty charcoal is generally coated with dextran before use in the radioimmunoassay, the molecular weight of the dextran being determined by the molecular size of the antigen involved.

The main alternative to adsorption of 'free' antigen is the precipitation of the antibody-bound fraction. This can be achieved by physico-chemical (salt precipitation) or immunological (double antibody) means. The latter, whilst time-consuming and expensive, is generally advantageous when only low avidity antisera are available. The precipitating, so-called 'second' antibodies are usually raised in large animals (monkeys, goats or sheep) by immunizing them with purified immunoglobulin fractions from animals of the species in which the 'first', i.e. drug-specific, antiserum was produced. The 'second' antiserum must be titrated against the amount of immunoglobulin in the first specific antiserum to establish the correct volume to be added to produce an insoluble matrix.

Increasingly popular are types of phase separation involving the use of insoluble supports to which either 'first' or 'second' antibodies are covalently attached. The solid phase used in such systems can be almost any insoluble material capable of making suitable links and include cellulose, high molecular weight dextrans and even the plastic tubes in which the assays are carried out. The use of such solid phase antibodies can greatly simplify assay procedures providing they retain their avidity and do not leach off the matrix.

Immunoassay techniques. At present time, the growing interest in T.D.M. has permitted the development of many immunoassay techniques using sophisticated apparatus for label detection. The main immunoassay techniques are:

R.I.A.: Radioimmunoassay
E.I.A.: Enzyme immunoassay
- E.M.I.T. (Syva)
- E.L.I.S.A. (Boehringer)
- E.F.I.A. (Dade)

F.I.A.: Fluoroimmunoassay
- S.L.F.I.A. (Ames)
- F.P.I.A. (Abbott)
- F.I.I.A. (Syva)

N.I.A.: Nephelometric immunoassay
- R.N.I.A.
- N.I.I.A.

P.A.C.I.A.: Particle counting immunoassay

L.I.A.: Luminescence immunoassay
- Chemoluminescence
- Bioluminescence.

We shall now review the main characteristics of each method.

Radioimmunoassay. The labelled competitor is radioactive. A high-specific activity label is a *sine qua non* for the development of a sensitive radioimmunoassay. In practice this means either a multiple tritium substituted molecular species of the analyte or an analogue of the substance to which a ^{125}I or ^{131}I tag has been attached. Whilst the latter type of label seldom presents difficulties in the radioimmunoassay of protein and large peptide molecules, problems do arise with low molecular weight compounds due to differences between the immunoreactivity of the labelled product and the original hapten. Both ^{3}H and ^{14}C-labelled materials which have half-lives of several years, have similar, though not always identical, immunoreactivities to the native hapten, and do not suffer radiation damage unless they are of extremely high specific activity. They are, therefore, ideal for development work leading to the establishment of a functional assay. Their main disadvantage — especially those based on ^{14}C — is that they have low specific activities, thereby reducing the sensitivity of the assay and the need to employ scintillation counting. In order to overcome these difficulties, labels incorporating shorter half-life γ-emitting radionuclides such as ^{125}I have received increasing attention, and are the basis of most of the established drug radioimmunoassays. In some cases the drug may possess a structure, e.g. a phenolic ring or a histidine residue allowing direct iodination. This may, with some of the larger drugs, produce an immunoreactive label. However, in most cases direct iodination of low molecular weight compounds results in such radical alterations in molecular size and shape as to render the compound non-immunoreactive with the antibody. To overcome this difficulty the practice has grown up of attaching drugs to other molecules which are capable of being iodinated directly.

Radioimmunoassay permits quantitative estimation of a great variety of biologically active compounds, but it does have certain drawbacks: beta-emitting isotopes, for example, necessitate the use of expensive scintillants and liquid scintillation counters, and often need long counting times. The radioactive material must be used and disposed of safely and is costly. Compounds labelled with gamma-emitting isotopes have a relatively short half-life.

Non-radioactive immunoassays. For all these reasons, alternative types of label have been investigated in an attempt to retain the advantages of specificity and sensitivity of immunoassay without using radiolabels. Labels that have been investigated include enzymes, fluorescent groups, stable free radicals, metals, erythrocytes and bacteriophages, all of which can be attached either to the hapten or to the antibody. A potential advantage of many (though not all) the alternative labels is their amenability to homogeneous assays, in which it is not necessary to separate antibody-bound from free hapten. This opportunity does not exist for radioimmunoassay since binding of antibody to an isotopically-labelled antigen does not affect the radioactivity of the isotope.

(a) Enzyme immunoassays (E.I.A.)

Although several different types of E.I.A. have been used to measure drugs they can, generally speaking, be divided into those that have substituted an enzyme for a radiolabel in an already well-established radioimmunoassay procedure, and those which are novel and utilize the fact that the enzyme label is a protein with special physical and chemical characteristics.

Competitive enzyme immunoassays are analogous to classical radioimmunoassays in which an enzyme-labelled antigen competes with unlabelled antigen for binding to a limited quantity of specific antibody sites. Free antigen is separated from antibody-bound antigen and the enzyme activity in either fraction measured and related to the concentration of unlabelled antigen.

(i) Heterogenous enzyme immunoassay. In the immunoenzymometric assay the antigen to be measured is permitted to react with an excess of labelled antibody. After incubation excess solid-phase antigen is added to the mixture and binds any antigen-free labelled antibody present. Following separation of the solid phase, enzyme activity is measured in either the supernatant or the solid phase. The amount of enzyme activity present in the supernatant is directly related to the amount of antigen originally present in the sample (E.L.I.S.A.).

(ii) Homogeneous enzyme immunoassay (E.M.I.T.). In contrast to the foregoing 'substitutional' enzyme immunoassays, a homogeneous enzyme immunoassay has been described. The omission of a phase separation step is an enormous advantage in clinical use where speed is often essential. The principles of the E.M.I.T.® technique (developed by the Syva Corporation) depend upon the fact that binding of antibody to an enzyme-labelled drug inhibits enzyme activity either by sterically preventing access of the substrate to the catalytic site or by inducing conformational changes in the enzyme which prevents catalysis. Free drug competes with enzyme-labelled drug for antibody with the result that enzyme activity is uninhibited and proportional to the amount of free drug originally present. Because each enzyme molecule is capable of catalysing the conversion of an extremely large number of substrate molecules from an optically inert to optically absorbant substance the E.M.I.T.® system is (at least theoretically) extremely sensitive. In reality, the sensitivity of the method is limited by other factors, notably the antibody–antigen binding characteristics and the presence of background signals. It is, however, an extremely effective method of measuring the concentration of drugs in human serum with remarkable precision and accuracy.

(iii) Characteristics of enzyme immunoassay. The performance of an enzyme immunoassay can be evaluated in terms of sensitivity, precision, accuracy, cross-reactivity and practicability like that of a radioimmunoassay. The use of an enzyme label may introduce further complications, however; serum and plasma are more likely to affect the enzyme reaction but this can often be minimized by diluting the sample whenever possible, by measuring enzyme activity on the washed insoluble (bound) phase and by careful choice of enzyme. The measurement of enzyme activity requires the addition of substrate and usually a reagent to terminate the assay which may decrease the precision of the assay compared with radioimmunoassay. The choice of separation method is more limited than with radioimmunoassay due to the large molecular size of the enzyme, but methods that are suitable include the double antibody technique and the use of solid phase antigens or antibodies. Critical comparisons have been made of the relative sensitivity, precision, accuracy and practicability of the different types of enzyme immunoassay, and they have been compared with other types of immunoassay. Claims

have been made for greater sensitivity of enzyme immunoassay over radioimmunoassay but have not been substantiated. Nevertheless, some heterogeneous enzyme immunoassays are of comparable sensitivity to the corresponding radioimmunoassay and are certainly sufficiently sensitive to permit measurement of many clinically important drugs in biological fluids. Results obtained using enzyme immunoassay usually correlate well with those obtained using other immunoassay or reference techniques and both intra- and inter-assay coefficients of variation are generally within acceptable limits (i.e. ±7%). Homogeneous assays are generally much less sensitive than heterogeneous assays but, as already observed, have sufficient sensitivity to enable them to be used clinically with the added advantages of simplicity and rapidity.

(b) Fluoroimmunoassay

Various types of fluoroimmunoassay have been developed, some of which require no separation step. The merits, in addition to its amenability to homogeneous assay, of fluorometric measurement are the stability and freedom from hazard of the fluorescent labelled materials, the moderate cost and wide availability of the equipment required and the high potential sensitivity.

The fluorescent label may function in a manner analogous to a radiolabel, i.e. the activity of the fluorophor is unaffected by its attachment to the antibody so a separation step is needed. Of more interest are assays in which the properties of the label are altered by binding to antibody: these require no separation step and may be both rapid and easily automated.

(i) Polarization fluoroimmunoassay. Polarization fluoroimmunoassay is based on the fact that when plane polarized light falls on an antigen labeled with a fluorophor, molecules with their long axes parallel to the plane of light are preferentially excited. If they also rotate between excitation and light emission the resultant fluorescence is non-polarized. The degree of depolarization depends on the rate of rotation and this, in turn, is inversely proportional to molecular size. The binding of a low molecular weight labelled antigen to antibody produces a large complex which, unlike the native antigen, is only capable of rotating slowly so that much of the emitted fluorescence is still polarized. In an immunoassay unlabelled antigen present in the reaction mixture competes with the fluorescent-labelled antigen for antibody binding sites so that the extent of the depolarization of the emitted fluorescence is proportional to the concentration of antigen originally present in the solution.

Fluorescence polarization immunoassays are usually far less sensitive than radioimmunoassays and limited to the measurement of haptens and low molecular weight antigens, although recently introduced purpose-built instruments have to some extent improved the sensitivity. This is currently sufficient to permit measurement of many therapeutic agents in plasma without prior extraction. The gentamicin assay is fast, precise, independent of serum effects and yields results that are in agreement with previously established gentamicin assay methods. In the fluorescence polarization assay for phenytoin, non-specific binding of the fluorescein-labelled ligand to serum proteins occurs causing an increase in the polarization signal. This interference can be overcome by proteolytic degradation of the proteins present in the serum samples and by the use of purified immunoglobulins isolated from the phenytoin antiserum. Assay reagents and the equipment necessary to use them are available commercially (TDX, Abbott).

(ii) S.L.F.I.A., Ames TDA®. A second type of homogeneous fluoroenzyme immunoassay utilizing a totally different principle was described (1977) and became

commercially available as the Ames TDA® system a few years later. In this type of assay, drug is covalently bound to the substrate of an enzyme, so as to render it unavailable for hydrolysis when the drug–substrate complex is bound to antibody but freely available when it is not. If, in addition, only free substrate — and not its conjugate — is fluorogenic, then the amount of fluorescence developed during the reaction will depend upon the quantity of substrate–drug conjugate displaced by unlabelled drug from the antibody binding sites. In this type of assay the number of fluorescent molecules available for measurement is equal to the number of molecules of drug to be measured: this imposes limitations upon even the theoretical sensitivity of the assay. This is, in practice, almost invariably more than adequate for measuring most of the drugs for which a clinical need has been established. Like EMIT® the Ames TDA® system is accurate, precise and easy to use providing appropriate instrumentation is available (Fluorostat and Optimate).

(iii) Fluorescence enhancement and quenching assay (SYVA). Fluorescence enhancement and quenching immunoassays are based on the fact that when a fluorescent labelled antigen binds to antibody it may undergo an alteration in its fluorescent properties. This can take the form of either a quenching or enhancement of its fluorescence. In 1977, serum gentamicin was determined by a quenching fluoroimmunoassay technique based on the observation that the intensity of fluorescence produced by fluorescein-labelled gentamicin is reduced by binding to gentamicin antibodies. The effect is diminished by the presence of unlabelled gentamicin which competes with the label for antibody binding sites. Differences in fluorescence are fairly small and, as serum samples have an intrinsic fluorescence, this 'blank' value must be measured separately and subtracted from the total signal in order to obtain results that correlate well with other assay techniques. No separation phase is required.

Fluorescence energy transfer immunoassays are based on the principle that when suitable light absorbing molecules are brought into close proximity to appropriate fluorescent molecules showing extensive spectral overlap, there is a transfer of energy between them. Energy transfer occurs within the antigen–antibody complex when, for example, fluorescein-labelled antigen is used as donor molecule and rhodamine-labelled antibody as the acceptor. The quenching of fluorescence by the donor molecule consequent upon its binding to antibody can be inhibited by competition from unlabelled antigen, i.e. the substance to be measured, and can be used to measure the concentration of drugs. Energy transfer fluorescence immunoassays are, therefore, potentially very broadly applicable since they are suitable for the measurement of both low and high molecular weight compounds. A new instrument has been developed for this application: the Syva "Advance".

(c) Nephelometric immunoassay

N.I.I.A. (Beckman). Principle: Drug in the patient's sample inhibits the rate nephelometric reaction between the specific antibody and a drug–protein conjugate. As the concentration of drug in the sample increases, the amount of drug conjugate bound to the antibody decreases. Therefore, the resulting rate of formation of light-scattering complexes decreases with increasing drug concentration (Fig. 2).

This method of 'rate nephelometric inhibition' is fast: it provides quantitation of drug in less than 60 sec. It is easy: a standard dilution sequence applies to all samples. A special instrument has been developed for this purpose: the ICS analysis II (Beckman) which can be modified to provide complete automation up to 6 drug assays.

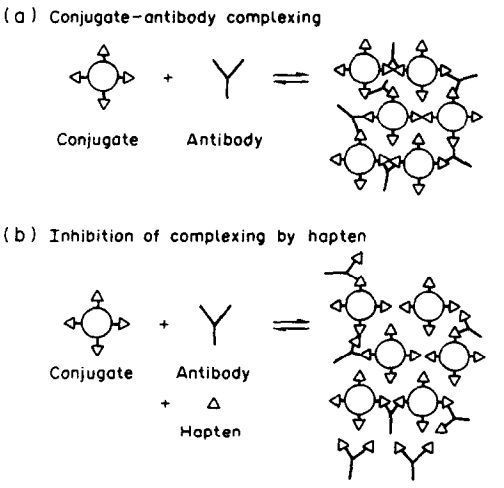


Figure 2

(d) Particle counting immunoassay (P.A.C.I.A.)

In 1956 Singer and Plotz proposed the titration of rheumatoid factor (RF) by agglutination of polystyrene beads coated with human γ-globulin. The use of these particles, usually of 0.8 μm diameter, called 'latex', has since become increasingly popular as a serological method to determine antibody (Ab), antigen (Ag), and immune complexes (IC).

The advantages of the latex fixation test are the facility with which proteins can be adsorbed to the particles and the relative stability of the suspension compared with biological materials such as erythrocytes. The limitations of the method are the difficulty of determining the end point precisely by eye and the risk of nonspecific agglutination or inhibition by factors unrelated to the Ag-Ab system of interest. The particle counting immunoassay (P.A.C.I.A.) technique, directly derived from the latex fixation test, avoids these two problems and possesses two additional advantages, a marked increase in sensitivity and full automation ensuring precision, speed and ease of use. These qualities make P.A.C.I.A. a serious competitor for radio- or enzyme immunoassays in both their experimental and routine clinical uses.

The P.A.C.I.A. is based on the principle that the number of free particles decreases during the agglutination reaction. By counting the unagglutinated particles with a blood cell counter, it is possible to evaluate the extent of the reaction in a much more sensitive and precise way than by the naked eye. To avoid nonspecific reactions, various procedures adapted to each substance to be determined have been introduced. P.A.C.I.A. was easily automated because it is an apparently homogeneous system; separation is done electronically rather than by such techniques as filtration or centrifugation. A new instrument for P.A.C.I.A. ('Impact') is to be marketed by Acade (Brussels). The determination of digoxin by P.A.C.I.A. has been published, and other drug determinations are under development.

(e) Luminescent immunoassays

Luminescent (chemiluminescence and bioluminescence) immunoassays have attracted attention due to their potentially high sensitivity. In one variant a covalent complex of a chemiluminescent compound (a luminol derivative), a protein and a steroid hapten, was

used as a labelled antigen analogous to the radio-label in competitive radioimmunoassay. The chemiluminescent label was bound by hapten-specific antibody and displaced by unlabelled steroid. Antibody-bound steroid was separated from the free steroid using a second antibody. Chemiluminescence was generated by oxidizing the label by hydrogen peroxide and copper acetate at high pH and the emitted light measured in a liquid scintillation counter. The assay was sensitive to 0.5 ng testosterone but probably could have been improved upon.

Other authors reported a homogenous luminescence assay based on the luciferin–luciferase system which utilized ATP according to the scheme shown below.

$$\text{ATP} + \text{luciferin} \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{adenyl luciferin} + \text{pyrophosphate} \qquad (2)$$

$$\text{Adenyl luciferin} \xrightarrow{O_2} \text{adenyl oxyluciferin} + \text{light}. \qquad (3)$$

ATP conjugated to the ligand, 2,4-dinitrobenzene, remained active as a substrate for luciferase but when the conjugate was bound to the ligand-specific antibody the intensity of peak light emission was markedly reduced. The addition of *N*-(2,4-dinitrophenyl)-β-alanine could reduce this inhibitory effect and provide an assay which, though not as sensitive as some radioimmunoassays, would nevertheless be sufficiently sensitive to measure many drugs at therapeutic concentrations in blood serum.

Advantages of the immunoassays. The advantages of immunoassay over other techniques for measuring drug concentrations in biological fluids are that (i) they are usually exquisitely sensitive permitting measurements to be made on very small volumes of sample; (ii) they can generally be carried out on urine, plasma and saliva without prior extraction and purification; (iii) they are chemically gentle and can, therefore, be used to measure unstable or labile substances; (iv) they are potentially universally applicable to all classes of compound; (v) they are often quick and technically easy to perform, once the reagents have been prepared, and lend themselves readily to automation; (vi) they can be developed to produce assays that are remarkably specific or non-specific according to the preference of the investigator and the purpose for which the assay is designed. Virtually absolute specificity can be achieved, if desired, by combining solvent extraction or chromatographic separation with an immunoassay.

It is often not appreciated, during the development of heterogeneous immunoassays, how important the type of phase separation used is in determining the specificity, as well as the sensitivity, of the final assay. Separation techniques based on the use of dextran-coated charcoal are very popular but can only be employed with high avidity antisera. Other agents are often used for phase separation, however, especially when non-isotopic labels are employed or when antibody avidity is relatively low.

In most drug immunoassays the hapten–antibody complex reaches equilibrium rapidly so that incubation times as short as 30 min are common. Occasionally, however, longer incubation is necessary: this depends to some extent upon the affinity of the antiserum but mainly upon the concentration (in the final incubation mixture) of the drug. The higher the concentration of drug the shorter the incubation time necessary.

Isotopically labelled haptens of all kinds deteriorate with time. This is especially true of γ-labelled haptens, but it also applies to tritiated compounds and manifests itself as

loss of immunogenicity — checks on the purity of the label should, therefore be made from time to time by subjecting it to TLC or HPLC, and purification carried out if necessary to restore immunoreactivity. Enzyme labels are stable for 1–2 years at least and some fluorescent labels are stable indefinitely. This is undoubtedly a great advantage when immunoassays are prepared as commercial kits.

Specificity for a single compound may be difficult or impossible to achieve by immunoasssay, though no more than with any other analytical technique. Group specificity, on the other hand, is generally relatively easily achieved and may be all that is required; it may actually be more useful than absolute specificity. Specificity depends mainly on the binding characteristics of the antibody but may also be influenced by non-specific factors, some of which may be unpredictable. Others may reasonably be forecast, such as those that may arise when structural analogues or metabolites of the compound to be measured are also present in the analyte. Non-specific impairment of hapten–antibody complex formation is especially common when the affinity of the antiserum for the hapten is low or the concentration of hapten is small. Although there is considerable disagreement about the best procedure to use for measuring cross-reactivity, the concept of cross-reactivity is a useful one for comparing different antisera and assay performances. Antibody specificity can, to some extent, be tailored to meet the use to which it will eventually be put as part of an immunoassay.

Standardization. Numerical data from immunoassays are obtained in terms of the standards used. If, however, two or more drugs (or a drug and its metabolites) have identical or similar cross-reactivity characteristics, the result can only be recorded in terms of drug 'equivalents'. When the cross-reactivities of two similar drugs are different, but measurable, it may be preferable to have an antiserum that detects both providing there are no occasions when the two drugs would be given concurrently.

Quality Control in Drug Measurement

The importance of inter-laboratory quality control has already been established for a number of drugs which are widely measured in clinical laboratories. For example, the average coefficient of variation for plasma digoxin measurements in the optimum therapeutic range of 0.9–2.6 nmol/l was between 16 and 25% over a period of 12 months in about 40 different laboratories using various radioimmunoassays. Some laboratories used kits and others self-produced or bought-in-bulk reagents. None of the assay procedures was consistently more or less precise than any of the others and whilst some kits showed consistent bias from the mean, most showed only random errors.

In the international phenytoin quality control scheme (1975–80) those laboratories that used EMIT® kits returned the smallest proportion of 'unacceptable' results indicating the advantages of well standardized reagents and simple technology in obtaining reliable analytical data.

The results of an external quality control scheme have shown that UV spectrophotometry is also unsatisfactory for theophylline determination. It is interesting to note, however, that all the other methods (high performance liquid chromatography, gas chromatography, thin-layer chromatography and EMIT®) were judged to be satisfactory. It was concluded that the 'operator' was the key factor in determining the reliability of drug assay results, i.e. most assay techniques would be satisfactory in a laboratory employing conscientious staff and good internal quality control procedures.

Table 2
Phenytoin estimations according to method during the first 4 years of a quality control scheme

Method	No. of laboratories	No. of results	Outside 95% confidence limits (%)
GLC			
No derivative	34	691	9.3
Derivative	51	904	5.2
Spectrophotometry	10	138	30
TLC	3	83	16
EMIT	8	68	2.9

From the results of four national surveys of antibiotic assays made by *microbiological* methods, it was shown that doubling dilution broth techniques were not sufficiently accurate to give clinically useful serum gentamicin results. These surveys also emphasized the necessity for intra-laboratory quality control, as it was shown that reproducibility (for individual laboratories) was generally too poor to permit the identification of any specific technical factors which could be contributing to inter-laboratory variation. The immunological methods have now largely replaced the older microbiological techniques.

It appears that good internal quality control is necessary to obtain good results in an interlaboratory comparison. The internal quality control is obtained by:

(1) Repeated analysis of patient samples: duplicate or triplicate;
(2) Use of pooled serum for quality control;
(3) Statistical analysis of the results obtained each week in routine work;
(4) Statistical analysis of within-batch and between-batch standard deviation for each method in use in the laboratory.

The impact of analytical error will depend also on the drug concerned and is related to the patient's actual serum concentration and to the upper and lower limits of the 'therapeutic' range. It is clear that it is necessary to distinguish between sub-therapeutic, therapeutic and toxic concentrations but in addition it should be possible to discriminate between concentrations within the therapeutic range where increasing the dose is likely or unlikely to induce toxicity. Good reproducibility is also necessary at sub-therapeutic concentrations for monitoring therapy in patients on low doses, or who are possibly non-compliant or have an unusually rapid rate of drug metabolism.

Conclusions

The measurement of drugs in blood has already made a significant contribution to the rationalization of drug therapy and the continuing extension of immunoassays into this field will undoubtedly enable many more laboratories to offer a diagnostic and therapeutic drug measurement service. To ensure the proper interpretation of the data furnished by therapeutic drug monitoring, close collaboration between the physician and laboratory is essential. The challenge is to coordinate the laboratory data with the clinical situation to aid proper interpretation of the results. The clinical laboratory involved with therapeutic drug monitoring must be willing to participate actively in the care of the patient. This may entail providing drug assays outside routine laboratory services. In

addition, the laboratory must have personnel capable of evaluating the patient's entire therapeutic status; thus, some background in pharmacology and pharmacokinetics is needed. A general appreciation of the pharmacokinetics of a given agent and an understanding of the general principles of therapeutic drug monitoring permits the physician to individualize each patient's therapy. Used properly, serum level concentrations can enhance the clinical benefit of certain widely used agents, while reducing the incidence of drug-related toxicity.

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