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Analytical techniques in dissolution testing and bioavailability studies*

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Abstract: The rate and extent of absorption of a drug into the bloodstream is an important quality characteristic of a dosage form. *In vivo* bioavailability and *in vitro* dissolution studies are important in the development and ultimately in the quality control of a dosage form. The integrity of these tests is dependent on the analytical methods used. The advent of very potent drugs used in low dosage and the development of novel drug delivery systems require that the most sophisticated methods are used.

This paper surveys recent trends in analytical advances that are useful in dissolution and bioavailability testing and suggests some future directions.

Keywords: *Dissolution testing; bioavailability; stable isotopes; immunochemical techniques.*

Introduction

The rate and extent of absorption of a drug into the systemic circulation (bioavailability) is an important determinant of the clinical efficacy of a drug. Absorption that is too rapid may lead to blood levels that cause side-effects whereas absorption that is too slow may lead to sub-therapeutic blood levels. Consequently considerable effort is expended to fully characterize the *in vivo* bioavailability of a drug in a given dosage form. These studies require sensitive and selective assays in order to measure the very low levels found in blood, often in the $\mu g\ ml^{-1}$ or $ng\ ml^{-1}$ range. Furthermore, the nature of bioavailability studies requires the analysis of large numbers of samples; this places ease and speed of analysis at a premium.

In vivo bioavailability studies are useful in the characterization of dosage forms in research and development work. They are not, however, appropriate for evaluating the performance of dosage forms for quality control purposes. For this and other reasons, *in vitro* dissolution tests have been developed to monitor the performance of the drug release characteristics of production lots. Attempts have been made, sometimes successfully, to correlate *in vitro* test results with *in vivo* bioavailability. This area of research, while interesting, is well beyond the scope of the present paper.

In the study of both *in vivo* bioavailability and *in vitro* dissolution modern analytical techniques play important roles. The present paper discusses a few techniques and

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concerns that are of current interest. Emphasis is placed on the latest findings and techniques that hold unique promise. A complete coverage of the analytical techniques useful in dissolution and bioavailability testing would constitute a book and is far beyond the scope of this review.

Dissolution Testing

The dissolution experiment can be conducted in a variety of ways. Usually the volume of the dissolution medium is fixed; agitation is provided by one of two devices, a rotating mesh basket (USP apparatus 1) or a rotating paddle (USP apparatus 2). In flow-through methods agitation is produced by the flowing solvent; in such methods the volume of dissolution fluid is not fixed and depends upon the flow rate and the length of the experiment.

In the analysis of the dissolution fluid for the dissolved drug several considerations are important. First, the method must possess sufficient sensitivity; this becomes a major concern with very potent drugs where the total amount dissolved may be less than 1 mg or where the release is slow, e.g. with a sustained-release dosage form. In the latter case, the amount dissolved early in the test may be very small. In addition to possessing the requisite sensitivity, the methods must be free from interference which may arise from undissolved particles or soluble materials in the excipients.

The two most widely used methods used in dissolution testing are ultra-violet spectrophotometry and high-performance liquid chromatography. The principal problems associated with UV measurements are caused by interference from undissolved particulates (light scattering) or from UV absorbing materials in the excipients. The former problem can often be eliminated by the use of appropriate filters. Filters also remove undissolved drug so that dissolution does not continue in the sample. Filters of relatively inert materials of small pore size are commercially available from a variety of sources. When using a filter, two factors must be considered: extractables from the filter bed and adsorption of the analyte by the filter. These factors can be evaluated by a simple experiment in which a standard solution of the analyte is analysed before and after filtration. Some results of this type of experiment are shown in Table 1. Note that for two of the filters, extensive losses of the analyte occur for the lower strength tablet with smaller losses for the higher strength tablets. This is consistent with adsorption of the drug on the filter with the adsorptive sites becoming saturated as more drug is passed through the filter. This problem can sometimes be avoided by flushing sample through the filter before taking the analytical sample.

In some cases the drug is in sufficient quantity or has a very high absorptivity which allows for direct UV measurement. In these cases interference caused by light scattering is low. Several approaches have been made to automate the collection of absorbance data to provide real-time dissolution profiles. The most common approach is to pump the dissolution fluid through the spectrophotometer cell and then back into the dissolution flask. By the use of a sample cell changer and computer acquisition of the data, six dissolution flasks can be monitored simultaneously. A concern in this method is that the pumping of the dissolution fluid does not create excessive agitation thus causing higher dissolution rates.

Another approach is to place a fibre optics probe in the dissolution flask and to measure the absorbance directly. Figure 1 shows the results of a study comparing the dissolution profile of a prednisone tablet by the continuous flow method of monitoring

Table 1
Effect of filtration on recovery of drug standard solutions

	Relative bias* 1.0 mg tablet	5.0 mg tablet	20.0 mg tablet
Filter type Cellulose acetate–cellulose nitrate ester, 0.45 μm	−25%	−6.1%	−1.6%
Polysulfone, 0.45 μm	−0.7%	−0.4%	−0.3%
Acrylic copolymer cast on nylon substrate, 1.2 μm	−13%	−2.3%	−0.5%
Polytetrafluoroethylene, 1.0 μm	+3.9%	−0.3%	−0.3%
Nylon 66	−1.9%	−1.4%	−0.9%

*Filter bias in phosphate buffer for 15-ml samples using a 10-ml preflush.

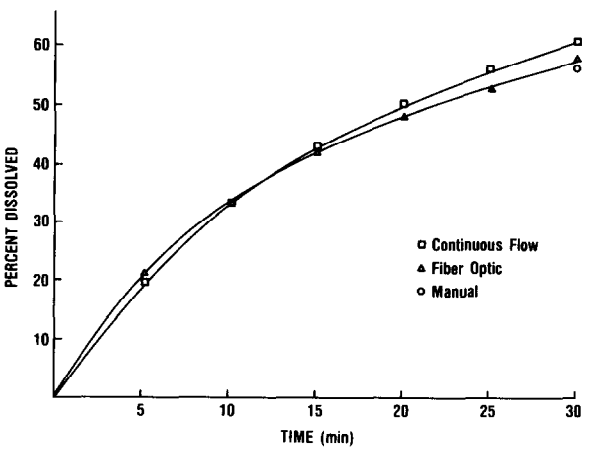

Figure 1
Comparison of prednisone tablet dissolution monitored by flow-through cells (UV spectrophotometer) and by a fibre optic probe.

the absorbance and by a fibre optics probe. A single time point (30 min) was measured manually for reference. The profiles obtained are not significantly different suggesting that fibre optics probes constitute a viable method. The fibre optics probe offers unique possibilities when coupled with robotics. A single probe coupled to a programmable robotic arm could be used to monitor absorbance in a large number of flasks without the use of elaborate fluid pumping systems. Furthermore, the residence time of the probe in the flask would be short and hence would minimize flow disturbances. This is an area of active research in the automation of dissolution testing. A recent study using robotics [1] demonstrated that this technique is feasible; the results were comparable with those by manual methods of analysis.

Little need be said about the use of HPLC for the analysis of the dissolution fluid. As a general technique it has the desired sensitivity and selectivity and can be easily automated. The Disoette sampling system (Hansen Research) uses an autosampler tray to collect samples of dissolution fluid. These trays can be placed directly into an autosampler for analysis. Much of the separative power of HPLC is not needed for the analysis of dissolution fluids. Because the drug need only be resolved from the excipients which are frequently eluted at the solvent front, the elution time may be shortened considerably by the use of stronger solvents in the mobile phase. This reduces the total time needed to analyse a set of dissolution samples. This aspect is very important in a high volume laboratory operation.

Bioavailability Testing

A complete treatment of the analytical techniques used in bioavailability testing is beyond the scope of this review. Consequently only a few topics have been selected. These include: stable isotopes in bioavailability testing; immunoassays without radio-isotopes; direct injection (HPLC) of serum or plasma samples; immunosorbent techniques for sample preparation and novel derivatization strategies. Emphasis is placed on recent work to illustrate evolving areas of research.

Stable isotopes in bioavailability testing

The use of labelled compounds as internal standards in GC/MS assays is well known. In practice, the analyte is labelled with deuterium, ^{13}C and/or ^{15}N. The labelled compound becomes the internal standard and has essentially the same extraction and chromatographic properties as the analyte. In this section the focus will be placed on another aspect of labelled drugs, i.e. use as a biological internal standard. In these experiments the labelled drug (either as an oral solution or as an intravenous injection) is given to the subject at the same time as the unlabelled drug being studied. Serum samples are collected as a function of time and both forms of the drug are analysed by GC/MS. Ratios of the blood levels of each form are used to calculate the appropriate pharmacokinetic parameters. By the use of ratios much of the inherent biological variability is removed since both forms of the drug experience the same absorption, distribution, metabolism and excretion (ADME) processes. Consequently fewer subjects are needed in order to obtain the desired statistical significance.

A standard premise in the work is that the labelled portion of the drug is not involved in metabolic transformations. To test the pharmacokinetic equivalence of both forms they are administered simultaneously as oral solutions. If both forms experience the same ADME processes, the serum level profiles should be superimposable. This is illustrated in Fig. 2 for a study of ibuprofen (d_0 and d_4) in beagle dogs [2] where the profiles are virtually indistinguishable. In such studies another labelled form of the drug is often used as an internal standard. In the above example, ibuprofen-d_7 was used as the internal standard. Whenever labelled drugs are being analysed or used as an internal standard it may be necessary to make isotopic corrections depending on the spacing of the mass of each moeity, natural abundance of the isotopes causing some overlap of the masses. In a recent study employing metoprolol labelled both with deuterium and ^{13}C, it was found that correction factors of up to 1% were required in some cases [3].

The improved statistics obtained by this method are illustrated in a study of methyltestosterone administered as a tablet and the trideutero-analogue administered as

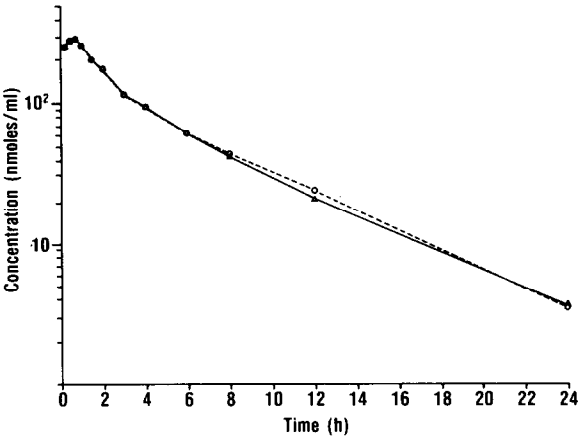

Figure 2
Demonstration of equivalences of ibuprofen (△) and ibuprofen-d_4 (O) in a beagle dog.

an oral solution [4]. In a study of eight subjects the relative standard deviation for the area under the blood-level curve (AUC) calculated conventionally was 38% while the same parameter obtained using ratios was only 19%.

Labelled drugs can also be used to determine the absorption kinetics for dosage forms that have prolonged absorption profiles such as those obtained with sustained-release tablets. Results from the administration of an oral solution of a labelled drug and a sustained-release tablet are shown in Fig. 3. The elimination kinetics can be determined directly from the oral solution curve and then used to determine the absorption kinetics from the sustained release curve. This has the advantage of not requiring a crossover study and hence requires fewer subjects.

So far much of the discussion has centred on the use of GC/MS as the analytical method for these studies. In fact, the inability to analyse some drugs by this method has

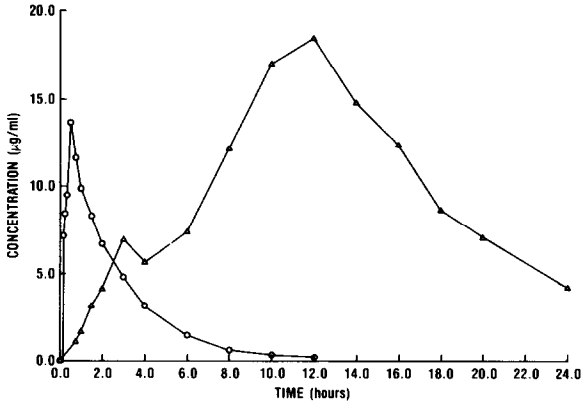

Figure 3
Comparison of blood level profiles for deuterated drug given as an oral solution (O) and as a sustained release dosage form (△).

restricted the use of stable isotope studies. Recent advances in LC/MS, in particular, thermospray ionization [5], show promise for the extension of stable isotopes studies. Earlier methods of interfacing the LC to the mass spectrometer were plagued with problems of handling the solvent flows typically used in HPLC.

Thermospray LC/MS has several unique advantages. These include the ability to introduce up to 2 ml min^{-1} of aqueous HPLC eluent into the MS and to use HPLC eluents containing high percentages of water. This coupled with the use of volatile ionic modifiers opens the possibility of analysing most drugs since reversed-phase HPLC is widely used in pharmaceutical analysis.

Immunoassays without radioisotopes

Radioimmunoassays (RIA) have grown to occupy a very important place in the tools of pharmaceutical analysis. The specificity achieved through the antibody–antigen reaction and the sensitivity achieved through radioactivity measurements have made RIA techniques very popular for the analysis of drugs in biological fluids, particularly for drugs at very low levels. The desire to reduce the level of radioactivity in the analytical laboratory has spurred research into immunochemical methods that employ other analytical techniques. In a method for the determination of carbamazepine (CBZ) in serum [6] a phosphorescent label, erythrosine, is covalently bound to CBZ. The long-lived phosphorescence is detected after pulse excitation in a time-resolved luminescence spectrometer. An *in situ* chemical deoxygenator, sodium sulphite is used to reduce oxygen quenching. Sheep antibodies to CBZ are used to provide the antigen–antibody complex; reduction of the phosphorescence due to antibody binding of the antigen is the basis for the analysis.

In another recent method, a digoxin–alkaline phosphatase conjugate was employed as the labelled hapten [7]. A digoxin antibody was prepared by standard methods and immobilized in a polystyrene cuvette. A sample containing digoxin and a fixed amount of the labelled hapten was incubated in the cuvettes at room temperature. The contents of the cuvette were removed by aspiration and the cuvettes were washed to remove unbound hapten. An enzyme substrate solution containing phenyl phosphate was added to the tube. After a fixed incubation period the amount of phenol released was analysed either by flow injection analysis or HPLC, both with electrochemical detection. This method correlated well with a RIA method for digoxin and had sufficient sensitivity for the expected range of concentrations.

Chemiluminescence has also been used to determine progesterone where the labelled hapten was an acridinium-9-carboxylate conjugate of progesterone [8]. After the antibody–antigen reaction and subsequent isolation of the complex, dilute sodium hydroxide/hydrogen peroxide was added to initiate the chemiluminescence which was integrated over a period of 10 s.

The development of this type of assay will be spurred on by the development of highly specific monoclonal antibodies; this is an area of intense research activity. With such selective complexing agents, the limiting factor may well be the ingenuity of the analytical chemist.

Direct injection (HPLC) of serum and plasma samples

A typical crossover bioavailability study will generate hundreds of samples. In virtually all cases, extensive sample pretreatment is undertaken to concentrate the analyte or to remove endogenous interference. In many instances, however, the drugs are present in

sufficient concentration to allow direct analysis if the serum or plasma components can be removed.

One approach uses direct injection with micellar liquid chromatography. In this technique, the powerful solubilizing properties of non-ionic and ionic surfactants are used to completely solubilize serum proteins [9]. Since these surfactants are present in the mobile phase they can also significantly affect retention of the analyte. This approach has been successfully used for the determination of acetaminophen (paracetamol), carbamazepine, phenobarbitone, phenytoin, procainamide and quinidine [10] and quinine, morphine, codeine and propranolol [11]. Both ultraviolet and fluorescent detection were employed in these assays. Although this approach will be useful for drugs at relatively high concentrations, it will be less useful for those drugs with very low serum levels.

A different approach is to use two columns for the analysis. The first column contains an aqueous gel (e.g. TSK G2000 SW) which separates entities on the basis of differences in molecular weight. The smaller drug molecules are retained and pass on to an analytical column for final separation. The serum proteins are not retained on the first column and are removed by rinsing. This method has been used for the analysis of cefmetazole, warfarin, carbaquone and ketamine in plasma [12].

Immunosorbent techniques in sample preparation

Immunosorbents have been classically used in RIA in the form of antibodies adsorbed or bound to a solid surface. The same technique has been successfully used for purification of analytes before chromatographic analysis. Solid-phase antisera have been used to rapidly and selectively remove 17-beta-oestradiol [13] and progesterone [14] from plasma. Once the analyte is bound to the antibody, other components can be washed off. Finally, the analyte is eluted from the antibody and analysed. Although this technology is not widely used, the availability of specific monoclonal antibodies should spur rapid development in this area.

A variant of this approach is the double antibody precipitation technique. In a study of plasma levels of arbaprostil [(15R)-15-methylprostaglandin E_2], rabbit *anti*-arbaprostil serum was added to plasma samples containing arbaprostil [15]. Goat *anti*-rabbit serum was subsequently added and the mixture was allowed to incubate overnight. The immunoprecipitate was isolated by filtration and the arbaprostil was extracted with ethyl acetate and reacted with panacyl bromide prior to analysis by HPLC. Once the necessary antisera had been prepared this approach could be used to analyse a large number of samples in much less time than that required with conventional purification by extraction.

Novel derivatization strategies

Derivatization of drugs to produce enhanced response or separations is widely used in the analysis of drugs in bioavailability studies. Some new approaches deserve comment since they point to ways of increasing the power of HPLC and GC assays. In a recent study on cannabinoids [16], a resin was used to extract the drug directly from plasma. After removal of the plasma and washing of the resin, a solution of the derivatizing reagent, pentafluorobenzyl bromide, was added to the resin. After a period of incubation, the resin was washed and the derivatized cannabinoid was eluted by washing with methylene chloride and ether which were then evaporated to dryness; the derivatized drug was then determined by GC with electron capture detection. This resin-

mediated reaction offers an opportunity to remove plasma components and to remove excess derivatizing reagent while the drug is still bound to the resin. This approach, in effect, increases the sensitivity by removing sources of interference that limit detection.

Post-column reactions to form derivatives are widely used in HPLC. Post-column derivatization has the advantage of not altering the chromatographic behaviour of the drug but it frequently has the disadvantage of causing band broadening due to reagent mixing effects. Recently, online electrochemical reagent production was used for the analysis of some phenothiazines [17]; potassium bromide in the mobile phase was converted to bromine in a post-column electrochemical cell. The active bromine then oxidized the phenothiazines (thioridazine, mesoridazine and sulforidazine) to produce highly fluorescent products. Simple deproteination of the plasma samples with acidified methanol was all that was necessary in the preparation of the sample.

Drugs are frequently eliminated from the body as glucuronide conjugates. If both the free and conjugated drug levels are desired, a portion of the sample is treated with a glucuronidase enzyme prior to further work-up. In a novel approach to avoid this problem, workers [18] immobilized beta-glucuronidase on controlled-pore glass beads and placed these in a post-column reactor for the analysis of the glucuronides of fenoldapam. Although the glucuronides are electrochemically inactive, the hydrolyzed parent drug is readily detectable at +0.6 V. Surprisingly, the reactor was fully active in concentrations of methanol up to 25% in the mobile phase. Above that concentration the activity decreased and the activity was totally lost when the concentration of methanol was 50%.

These few examples illustrate the potential for further developments in the area of derivatization. Perhaps in the future isolation of the analyte by immunochemical techniques will be coupled with post-column reactions to produce species that can be detected selectively at low concentrations. This combination could easily bring the pg ml^{-1} range into reach of virtually any investigator.

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