Physical chemical characterization of drug substances

William H. Streng

During the development of new drug substances, researchers have typically focused on the biological properties, with less concern for the physical chemical properties. However, in order to understand the behavior of drugs in physiological systems, a broad range of physical chemical properties must be determined. Not all of these properties need to be determined before any clinical studies are initiated; some can be obtained throughout the development process. Others, however, are critical to the early evaluation of the compounds and should therefore be determined as early as possible. This review discusses the nature and timing of the various studies.

During the past 50 years, there has been an increased interest in obtaining information about the physical and chemical properties of new pharmaceuticals, by both industrial and academic researchers. The emphasis on understanding such properties is driven by both regulatory requirements and the recognition that understanding the properties can help in the design of 'better' compounds or aid the formulation development effort. There are many reasons for characterizing drug substances, but the four primary reasons are:

- to determine the structures of the compounds,
- to determine the properties of the pure substances,
- to determine the properties of the compounds in solution, and
- to explain and/or predict the behavior of the compounds under conditions not studied.

Most, though not all, drug substances are solids at room temperature, and therefore this review will be concerned primarily with the characterization of substances that are solids at room temperature. In most sections specific examples have not been included because over the past ten years comprehensive reviews have been written about most examples, and justice could not be done to them within the space of this review.

Characterization studies

The characterization studies naturally divide into three areas: spectroscopic properties, solid-state properties and solution properties.

Spectroscopic properties

Spectroscopic characterization studies are performed using NMR, MS, IR, FT-IR and UV-visible spectroscopy. The major objective of these studies is to elucidate the chemical structure of the compounds. Because spectroscopic tests are very specific and are related to specific instruments, such tests will not be described in detail. Briefly, NMR studies can be conducted with the solid substances or with solutions. Different probes can be used to explore interactions

William H. Streng, Hoechst Marion Roussel, Analytics, PO Box 9627, Kansas City, MO 64134-0627, USA. tel: +1 816 966 7106, fax: +1 816 966 5974, e-mail: williamstreng@hmri.com

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between different elements. The most widely used probes are hydrogen and ^{13}C. Several methods are used to generate the mass spectral data; they depend in part on the compound and under what conditions it will ionize. Infrared spectroscopic data can give an indication of specific functional groups on the molecule since different groups will have different IR spectra.

Depending on the stage of development of the compound, different studies will be conducted and different levels of accuracy and completeness will be necessary. The studies can be divided into those needed for compound screening, those needed for preclinical and clinical candidates, and those needed for late-stage candidates. In general, as the development progresses from screening to late stage, greater quantities of the compound will be synthesized at one time, and the synthesis procedure will become finalized. Because of the small quantities of the compounds available during screening and the fact that the compound might not be sufficiently pure for applications in later stages of development, characterization studies that can be performed using small quantities of compound and in a short period of time are conducted during this early stage. Studies that can be carried out rapidly are performed early because of the potentially large number of compounds encountered during screening.

Solid-state properties

Listed below are a number of studies that can be conducted to characterize solid-state properties:

- surface area
- particle size distribution
- hygroscopicity
- polymorphism
- solid-state stability
- intrinsic dissolution rate

Because such studies determine properties of the compounds as powders, they are useful in development of the solid dosage form.

Solution properties

The following studies are conducted to determine solution properties:

- association and dissociation constants
- complexation constants
- solubility as a function of pH
- solubility in selected solvents
- partition coefficients as a function of pH
- solution stability
- solution thermodynamics

These studies determine properties of the compounds that describe their behavior in solution and are, therefore, useful in the development of solution dosage forms and in understanding their pharmacological behavior.

Compound screening

During the early phases of development and before the selection of a specific compound for clinical studies, only a limited number of characterizations can be performed because limited quantities of the compounds are available and all tests have to be performed on a large number of compounds in a short period. The following studies can be performed during this stage of development:

- enantiomorphic composition
- partition coefficients at selected pH
- estimated dissociation constant
- solubility at selected pH
- preliminary stability

The information obtained from these studies establishes how a compound will behave under physiological conditions, which is a critical factor in the selection of a compound for further development. As is true in any research environment, if a compound has unexpected properties or behavior, and is showing pharmacological responses of interest, additional investigations would be conducted to further clarify and explain its behavior.

Enantiomorphic composition

Compounds with an asymmetric carbon center will exist as enantiomers[1–6]. It is well documented that the different enantiomers can have significantly different physiological activity; in fact, one enantiomer can be responsible for the desired activity while the other can be responsible for the unwanted side effects. If there is more than one asymmetric carbon, then the different isomers are referred to as diastereomers. Some cases in which isomers exhibit different pharmacological responses are listed in Table 1.

The fact that lock and key conformations as exemplified in Table 1 can be found for almost every pharmacological

Table 1. Compounds with different enantiomeric or diastereomeric activity

Compound	Application	Stereoselective action
Etomidate	Intravenous anesthetic agent	All activity in *R*-isomer
Viloxazine	Antidepressant	ED_{50} *R*-isomer 50 × ED_{50} *S*-isomer
Isoetharine	Antiallergenic and β-adrenocepter-stimulating	(1*R*,2*S*) most active
Labetalol	Cardiotonic	*R*,*R*-isomer is a β_1-blocker, and *S*,*R*-isomer is an α-blocker; remaining two isomers are inactive
Methylphenidate	Analeptic	(2*R*,2′*R*) most active
Chlorpheniramine	Antihistamine	ED_{50} *R*-isomer 100 × ED_{50} *S*-isomer
Captopril	Antihypertensive	*S*,*S*-isomer active
Naproxen	Nonsteroidal anti-inflammatory	All activity in *S*-isomer
Warfarin	Anticoagulant	*S*-isomer more potent
Chloramphenicol	Antibiotic	D-isomer most active

activity has led to the general recognition that the most active compounds will exhibit stereospecificity when there are asymmetric carbon centers. Although it is not always possible to synthesize all of the possible enantiomers or diastereomers as pure compounds, this should be a goal of the synthetic chemist. Compounds that have hindered rotation about a bond can behave in the same way as enantiomers if there is a sufficiently high energy barrier to rotation. An example of this is iodixanol, an X-ray contrasting agent (Figure 1). It has been found that, because of hindered rotation, there are six enantiomeric pairs and four *meso* forms instead of the expected three racemates and three *meso* forms[4].

Partition coefficients

The ability of a compound to be absorbed or to be transferred through a membrane is related to its partition coefficient[7–9], which is defined as the equilibrium concentration of the compound in the organic solvent divided by the equilibrium concentration in the buffered aqueous solvent, when partitioned between two immiscible solvents.

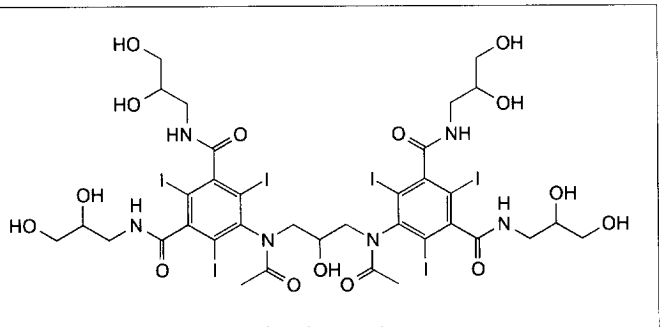

Figure 1. *Structure of iodixanol.*

The observed partition coefficient will be a function of pH for compounds that are weak acids or weak bases. This is because for these compounds the primary species in solution can be either charged or uncharged (neutral) depending on the pH of the aqueous phase. In general, when the compound is uncharged it will have a larger partition coefficient than when it is charged.

During compound screening, partition coefficients are often determined at a limited number of pH values. Because of this, partition coefficient values of different series of compounds are not always suitable for comparison, but values for compounds having similar structures can be compared. The organic solvent most often used is water-saturated n-octanol, and generally measurements are made with the aqueous phase buffered at pH 7. Other organic solvents that have been used include heptane, cyclohexane, carbon tetrachloride, toluene, benzene, natural product oils, ether, ethyl acetate, xylene, cyclohexanol, 1,2-dichloroethane, heptanol and nonanol. Besides equilibration of a sample between two immiscible solvents, other techniques have been used to arrive at parameters related to partition coefficients, including centrifugal partition chromatography[10,11], HPLC (Refs 12–14), microemulsion electrokinetic chromatography[15] and capillary electrophoresis[16]. In addition to these experimental methods, partition coefficients can also be calculated using QSAR (Refs 17–19). Whichever method is used, it is important to recognize the limitations of the technique and to know how to compare the calculated parameters to those of other compounds in a given series.

Dissociation constant

Because the ionic state of a compound is critical to both its *in vitro* and *in vivo* behavior, the dissociation constant

should be obtained very early during development. Often, approximate methods are used in compound screening to obtain values because of both the time required and the amount of drug substance needed. As a first approximation, values for compounds having a similar structure can be obtained from the literature. Techniques for determining dissociation constants utilizing capillary electrophoresis have been reported that require very little compound and can be conducted quickly[20–22]. Other methods include potentiometric titrations[23], HPLC (Ref. 24), UV-visible spectroscopy[25], NMR (Ref. 26), conductivity[27,28] and solution calorimetry[27,28]. Because of limited solubility or chromatography conditions, some of these methods require the use of co-solvent systems.

Aqueous solubility

The solubility of a compound must be determined at several pH values during compound screening to aid formulation activities and to ensure that the compound will be available under the conditions of the screening test. Usually, solubility is only tested in aqueous media; however, there may be a need to investigate co-solvent systems when the compound is insoluble over the pH range of the screening test. Serum pH is approximately 7, and therefore the solubility should be determined at this pH value. If the compound is to be tested via oral administration, then the solubility at a pH between 1 and 3 should also be obtained. In addition to these specific pH values, the solubility in water should be measured. The solubility of a compound will be dependent upon the concentration of the buffers if the solid material in equilibrium with the saturated solution is the salt form of the compound.

Stability

Preliminary information on the stability of a compound both as a solid and in solution should be obtained during compound screening. This information is needed in order to know how the compound must be handled and stored as well as how stable the compound will be in liquid dosage forms and under physiological conditions. It may be necessary to store the compound in a freezer, as for some proteins and peptides, or to keep the compound from low or high pH conditions if it is susceptible to hydrolysis. During the compound screening stage, these studies are not very complete and are focused only on gross instabilities. If a compound is found to be unstable, additional studies are needed to determine more precisely what the instability is dependent upon, such as light, pH, oxygen or temperature.

Preclinical/clinical candidate

During the preclinical and early clinical stage of development, information needed for filing with the regulatory agencies must be obtained in order to conduct the first clinical studies. Information on both the solid and solution properties of a compound should be obtained during this stage of development. The properties that should be determined during this stage are listed in Box 1. This information is needed for the development of the formulation, development of analytical methods, and to help in understanding the behavior of the compound under physiological conditions. Some of these studies are performed not just during this stage, but data are also obtained during the entire developmental process.

Hygroscopicity

The ability of a compound to pick up or lose water when exposed to a wide range of humidities is referred to as its hygroscopicity[29]. Because hygroscopicity is a measure of both thermodynamic and kinetic properties, there is no universally accepted definition. Generally, hygroscopicity experiments determine the amount of water sorption after equilibrium is attained, which is thermodynamic property. Occasionally, the rate of sorption is very slow, and equilibrium is difficult to reach. In such cases the kinetics of the process are used to describe the hygroscopicity.

Hygroscopicity is a function of both relative humidity and temperature, and therefore both variables should be investigated when characterizing a compound. Assuming all the sorbed water is on the surface of the particles, a calculation of the number of layers of water molecules required for the amount of water sorbed usually leads to an unrealistic number

Box 1. Properties to be determined during the preclinical and early clinical stage

Solids
Hygroscopicity
Preliminary polymorphism
Dissolution rate constants
Solid-state stability

Solutions
Partition coefficients as a function of pH
Solubility as a function of pH
Dissociation constant
Solubility in selected solvents
Modified solution stability

of layers of water on the particles. The excess water must be incorporated into the solid and not be just on the surface. A phenomenon called capillary condensation can occur in which water condenses within pores that are present on the particles. The condensation is a direct result of the energy related to curved surfaces and the effects of this surface energy on the water vapor pressure. The vapor pressure of water will be lowered when it is in a small curved pore resulting in the condensation of the water until the vapor pressure of the condensed water increases to that of the bulk water vapor.

Another phenomenon that can occur with highly soluble compounds is deliquescence. This is the adsorption of water on the surface of the particles to the extent that the compound is dissolved and a saturated solution is obtained. If the vapor pressure of the water in the saturated solution is less than the vapor pressure of the bulk water vapor, water will continue to condense out of the vapor phase. This can and does happen at relative humidities of less than 100%. As more water vapor is condensed, more compound will be dissolved until most or all is dissolved. A classification scheme for hygroscopicity has been proposed and is given in Box 2 (Ref. 30).

Polymorphism

The properties of a compound, both physical and chemical, can be affected by the form of the compound in its solid state[31–33]. Because the properties of a compound are affected by its form, regulatory agencies have become more demanding in the determination and characterization of polymorphs. There are three major solid-state forms: amorphous, polymorph and pseudo-polymorph.

An amorphous material has a non-ordered, random arrangement of the molecules in a solid phase. Polymorphic compounds exist in multiple crystalline arrangements with exactly the same molecular composition. The term pseudo-polymorph refers to different hydrates and solvates of the same compound. By this definition, polymorphs can be different crystalline arrangements with the same solvation; however, these compounds are usually referred to as pseudo-polymorphs, particularly when the compound can exist in the nonsolvated form.

In addition, there are two types of solid-state phase transitions; enantiotropic and monotropic. An enantiotropic phase transition is one that is reversible, whereas a monotropic phase transition is irreversible. Different enantiotropic forms are stable under different conditions, whereas forms that undergo a monotropic phase transition have only one thermodynamically stable form under all attainable conditions. This does not mean that the unstable form cannot be a useful one because the activation energy for the conversion to the stable form can be high. Thermodynamic rules for enantiotropic and monotropic phase transitions have been formalized by Burger[34] and are presented in Table 2. It must be kept in mind that a change in the morphology or crystal habit of a form as seen under a microscope does not necessarily mean that there is a change in the crystal packing or arrangement of the molecules in the unit cell, which would imply a polymorph. Crystallization requires growth in three directions and a change in the solvent system can result in changes in the growth rates for the three directions; therefore, the shape of the crystal can change while the crystal structure remains the same.

During this stage of development it is important to conduct a microscopic investigation of the material available. Whenever possible, an attempt should be made to obtain several batches of the material under investigation, which were synthesized using different, as well as the same, processes. Hot-stage microscopy will tend to overestimate the number of polymorphs. However, as a first indication, this is the preferred technique because it is fast and requires only small quantities of material. To confirm the results, other tests that require small amounts of material, such as IR spectroscopy, differential scanning calorimetry (DSC),

Box 2. Hygroscopicity scale[a]

Class 1
Non-hygroscopic: no water sorption at relative humidities less than 90%. The sorption of water after one week above 90% relative humidity is less than 20%.

Class 2
Slightly hygroscopic: no water sorption at relative humidities less than 80%. The sorption of water after one week above 80% relative humidity is less than 40%.

Class 3
Moderately hygroscopic: water sorption does not increase more than 5% at relative humidities less than 60%. The sorption of water after one week above 80% relative humidity is less than 50%.

Class 4
Very hygroscopic: water sorption occurs at relative humidities as low as 40%. The sorption of water after one week above 80% relative humidity is greater than 50%.

[a]After Ref. 30.

Table 2. Thermodynamic rules for polymorphic transitions (I is the higher melting form)[a]

Enantiotropic	Monotropic
Transition temperature < melting temperature of I	Transition temperature > melting temperature of I
I is stable above transition temperature; II is stable below transition temperature	I Always stable
Transition reversible	Transition irreversible
Solubility of I higher than II below transition temperature; solubility of II higher above transition temperature	Solubility of I always lower than II
Transition II→I endothermic	Transition II→I exothermic
$\Delta H_f^I < \Delta H_f^{II}$	$\Delta H_f^I > \Delta H_f^{II}$
IR peak I before II	IR peak I after II
Density I < density II	Density I > density II

[a]After Ref. 34.

differential thermal analysis (DTA) or thermal gravimetric analysis (TGA), can be conducted. Another test that does not require much material is X-ray powder diffraction, which is a very good method for looking at polymorphs because the diffraction patterns obtained are directly related to the spacing of the molecules in the unit cell.

Dissolution rate constant

In a particular solvent medium, the dissolution rate of a compound is dependent upon its form and particle size distribution. Methodology has been developed to measure the dissolution rates of compounds as powders or compressed into disks. In Figures 2 and 3, the cells used to determine these rates are shown. A flow system is used when determining the rates of powders, and the system has a fixed solution volume when determining the rates of disks. As shown, the dissolution solutions are passed through a spectrophotometer to quantitate the amount of compound dissolved. Any analytical procedure can be used to quantitate the amount of compound dissolved; however, the use of a spectrophotometer has proven to be very convenient.

Whenever there are different forms of a compound, the dissolution rates should be measured in different media to ascertain whether there are any significant rate differences that could affect bioavailability. Dissolution studies typically use water, pH 7 buffer and pH 1 buffer as a medium. If powders are studied, it is necessary to check the particle size distribution. The particle size distribution should be the same when comparing different compounds or different forms of one compound, because differences in surface area may result in differences in the dissolution rates. In order to eliminate particle size and shape effects, compressed disks can be prepared from the powders. It is not always possible to prepare such disks, but when it is, care must be taken to compress the disks using the same compression force for all of the different forms.

Solid-state stability

During preclinical/clinical development more information must be obtained on the solid-state stability of the compounds. Not only is it necessary to have actual stability data on the pure compound under the actual conditions intended for storage, packaging and handling, but stability information on mixtures of the compounds and excipients that may be used in the preparation of formulations is also needed.

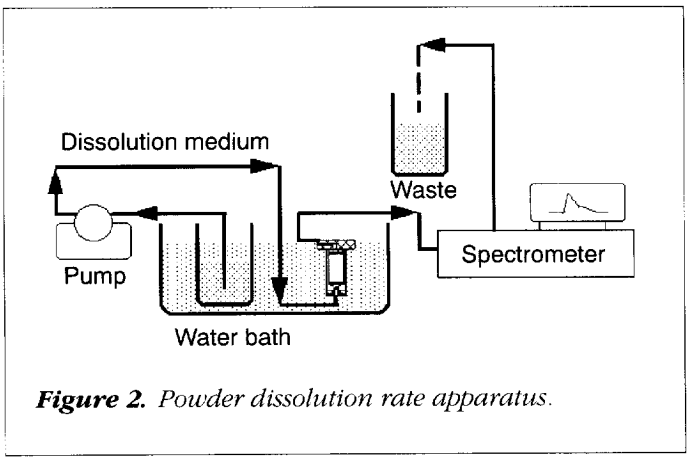

Figure 2. *Powder dissolution rate apparatus.*

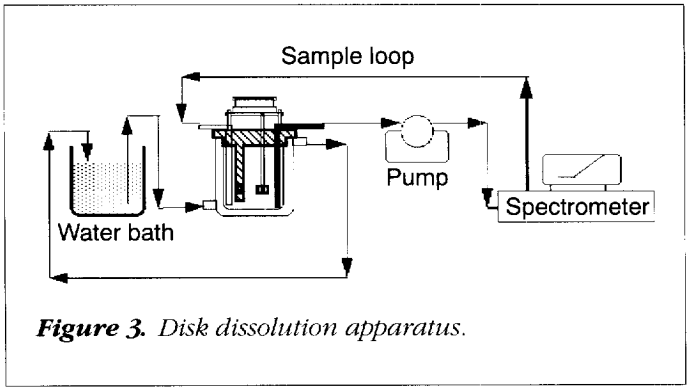

Figure 3. *Disk dissolution apparatus.*

Investigations into the compound stability are not restricted to the compounds alone or in simple mixtures with one or two excipients. They should include studies of the actual formulations and the effects of processing encountered during the manufacture of formulations. These latter studies include such factors as granulation fluid, lyophilization, and freeze–thaw cycling. Much of what is required in terms of stability data for the pure compound and the formulation is dictated by the regulatory agencies. Stability information is required for the following conditions:

- at the temperatures the product is to be stored
- in the containers the product is to be packaged
- at elevated temperature and humidity
- under temperature-cycling conditions
- of the polymorph or pseudo-polymorph
- of the superstructure (secondary, tertiary, quaternary) of proteins

The regulatory agencies demand assurances that all compounds will be sufficiently stable during the course of any clinical study; that is, the chemical assay should not drop below certain pre-established levels, usually 90% of theory. Typically, when stability is discussed it is usually the chemical stability that is considered, but an area that has recently gained more prominence is the physical stability of the compounds. Solid-state physical stability is concerned with, in addition to polymorphic or pseudo-polymorphic composition, the secondary, tertiary and quaternary structure of proteins and peptides. Changes in the superstructure of proteins can occur during the lyophilization process or during other freeze–thaw processes[35,36].

The solid-state chemical stability can be studied using any of the conventional procedures. Some of these, because of their nature, must be long term – as long as five years – whereas it is possible to conduct other studies in much shorter time by using elevated temperatures. Usually, chromatographic methods that can separate the original compound from the degradation products are used for such studies. If the degradation products are known, or if it is only necessary to determine if there is an interaction, for example in excipient compatibility studies, then DSC or micro-Watt calorimetric methods[37] can be used.

More sophisticated methods to investigate the solid-state physical stability of the compounds include spectroscopic procedures (such as X-ray, NMR and IR), calorimetric procedures (such as DSC and micro-Watt solution calorimetry) and optical microscopy.

Partition coefficients as a function of pH

More details concerning the solution behavior of the compounds should be obtained during the preclinical/clinical phase. A complete partition coefficient profile over a wide pH range and at different concentrations of the compound should be determined. Even though the pH of serum is approximately 7.4, the pH that a drug will encounter when ingested orally can be as low as 1.0 (Refs 38,39) and as high as 8.0 (Ref. 40). Over this wide pH range, the partitioning behavior of the compound can change significantly because the predominant species in solution can change. Such a change will affect its transport through a physiological membrane.

Another important reason for determining the partition coefficient over a large pH range is to aid the formulation of liquid dosage forms, particularly ointments, emulsions or other multiple liquid-phase systems. The distribution of the compound in the multi-phase system will be directly related to its partitioning behavior. It is also known that compounds can aggregate in either phase[41–43], and the partitioning data can be used to determine the degree of aggregation.

As mentioned previously, there are many different methods to obtain partition coefficient values. Although some methods are good for generating values for a series of compounds with similar structures, they may not yield values that can be compared with values for compounds with different structures. The safest approach is to use a conventional equilibrium method in which the compound is placed in a container containing two phases, usually n-octanol and an aqueous buffer. The system is agitated for a minimum of 30 min. to facilitate equilibrium. After reaching equilibrium, the two phases are analyzed and the partition coefficient calculated for the specific buffer pH. A phosphate buffer system can be used over the pH range of interest, although the buffering capacity is not very great at some pH values.

It is common practice to place a relatively high concentration of sodium chloride (0.05–0.10 M) in the container. The sodium chloride provides a common ion that will preferentially associate with the compound as an ion pair when the compound is charged. Knowing the partition coefficients as a function of pH, the intrinsic partition coefficients for the compound can be calculated. If the partition coefficients are determined using several different total concentrations of compound, the data can be used to determine whether the compound is aggregating in either solvent.

Solubility as a function of pH

There are several reasons for determining solubility profiles of compounds as a function of pH (Refs 44–48). First, the dissolution rate of a compound is dependent, in part, on its solubility. Second, it is necessary to know the solubility profile as a function of pH when developing a liquid dosage form. Finally, if the experimental procedure is carefully designed, the data can be used to determine the pK_a, uncharged (neutral) species solubility and pK_{sp} (the solubility product of the charged species with the appropriate counter ion) of the compound.

Box 3. Solvents and solvent systems for solubility studies

Ethanol
Hexane
Isopropyl alcohol
Methanol
Corn oil
Propylene glycol
75/25 Propylene glycol/water
50/50 Propylene glycol/water
25/75 Propylene glycol/water
Polyethylene glycol 300 (PEG 300)
75/25 PEG 300/water
50/50 PEG 300/water
25/75 PEG 300/water
Water

If the compound is a weak acid or weak base the solubility will be dependent upon the pH of the solution. For such compounds, the theoretical relationships between the solubility and pK_a, uncharged solubility and pK_{sp} have been reported[47,48]. Also, when the compound is a weak acid or base, a salt can be formed over a select range of pH values, and, therefore, the solubility will be dependent upon the counter ions that are present. As discussed above, whenever solubilities are determined over pH regions in which the solid material in equilibrium with the solution is a salt form of the compound, both the counter ion and the concentration of the counter ion should be mentioned along with the solubility of the compound.

It is tempting to initiate solubility studies using buffers at selected pH values because this can provide useful information relating to a specific formulation or physiological condition. However, the problem with using buffers is that the solubility will depend on the buffer species concentrations, as mentioned, when the salt form is the solid material in equilibrium with the solution. The solubility information from this type of study will therefore be specific for the buffers used and little, if any, understanding will be obtained that can be used to predict the behavior of the compounds under conditions not studied.

Several methods can be used to obtain solubility profiles. The more traditional approach is to place a specific amount of compound into several ampoules and to add different amounts of a strong acid or base. The ampoules are then sealed and equilibrated for five to seven days (shorter equilibration times have been used, but fully saturated solutions are not always obtained), and the solutions are then assayed using a stability indicating method. Knowing how much compound was placed in each ampoule and how much strong acid or base added, the data can be used to determine the pK_a, uncharged (neutral) species solubility and pK_{sp} of the compound. The parameter values determined in this way can be very accurate and precise. In addition, the equilibrium constants can be determined using activity coefficients and are therefore thermodynamic constants.

There are two very real problems with this approach. First, if the compound exists as pseudo-polymorphs, the most stable form might not be the form used in the experiment and inconsistent results can be obtained when the conversion to the more stable form is not rapid. Second, if the compound is very unstable the values obtained for the 'saturated solubilities' can be low and therefore incorrect solubilities would be reported.

Recently, a pH-stat method has been shown to give good reproducible results for solubility measurements[49]. The procedure involves placing a quantity of compound that will exceed the expected solubility over the entire pH range of the experiment into a reaction beaker and adding the desired amount of solvent. A pH-stat titration is then conducted using either a strong acid or a strong base as titrant. Titrant is added to obtain either a fixed pH change or a known volume addition. After establishing equilibrium, a sample of the solution is assayed and another increment of titrant is added and the procedure repeated. The data obtained can then be used to calculate the pK_a, uncharged solubility and the pK_{sp} of the compound. The major advantage of this procedure is that it can be completed in much less time than the traditional ampoule method and therefore the stability of the compound will not be as much an issue. Potential disadvantages to this approach are that the solubility obtained can be that of a pseudo-polymorph that is not the stable form under the experimental conditions and it is more difficult to make activity coefficient corrections in the calculations.

Dissociation constants

The fact that the ionization of a compound plays a critical role in the behavior of the compound both physiologically and in relationship to the formulation makes the dissociation

constant a critical parameter to obtain. During preclinical/clinical development, the pK_a of the compound should be re-determined or the value that was obtained during compound screening should be evaluated for accuracy and precision. At this stage it may be important to determine the value at several temperatures, depending on the formulation and the temperature conditions to which it may be exposed. Also, determining the temperature dependence of the dissociation constant will permit the calculation of the enthalpy and entropy for the dissociation and thereby provide information on the solute and solvent interactions.

Common techniques that have been accepted as providing accurate and precise values for equilibrium constants include potentiometric titrations and UV-visible spectrophotometric methods[50]. Calorimetry[51], conductivity and solubility can also be used. Activity coefficients can be included in each of these techniques, resulting in thermodynamic values. Often the method of choice will depend on the availability of material and the solubility and stability characteristics of the compound. It is not uncommon for the uncharged (neutral) species of the compound to have extremely low solubilities and therefore techniques that require moderate or high concentrations might not be applicable. On the other hand, the use of solubility methods to determine the pK_a of compounds that are very soluble is generally not recommended. Finally, if a compound is very unstable over a certain pH range or under specific conditions, it might be necessary to use techniques that can be performed quickly or do not require exposure of the compound to the unstable environment.

Solubility in selected solvents

Unlike the obvious need to determine the ionization constants and the aqueous solubility, the need to determine the solubility of a compound in solvents other than water might not be as apparent[52–55]. It becomes clear when it is understood that not all liquid formulations are in pure water but often will be mixtures of several solvents. The solvents used can be either miscible or immiscible and, if they are immiscible, the distribution of the compound between the phases will be dependent upon the solubility of the compound in each phase. There are several other reasons for determining the solubility in selected solvents: during the synthesis of the compound non-aqueous solvents are usually used; co-solvents are often used in the analytical methods used to assay the compound; and, finally, by knowing the solubility in several non-aqueous solvents, it is possible to predict the solubility in solvent systems not studied.

Some solvents and solvent systems that are commonly used to study the effects of solvent upon the solubility of a compound are given in Box 3. In this table both water-immiscible solvents and co-solvent mixtures are listed. Many compounds will exhibit a maximum solubility at a specific dielectric constant for similar solvent systems[56]. It is, therefore, possible to determine the solubility of a compound experimentally in several mixtures of a co-solvent system and obtain a good estimate of the composition of a second co-solvent system that will provide the maximum solubility.

It is of interest to be able to predict the solubility of a compound in solvent systems not studied. One approach has been to determine the Hildebrand solubility parameter for the compound, and use it with the Hildebrand parameters for different solvents to calculate the solubility in solvents not studied. As originally derived by Hildebrand[57], the theory did not apply to semipolar crystalline compounds, but now has been extended to include these types of compounds[58,59]. The change in solubility with dielectric constant was shown to be similar to the change in solubility with solvent solubility parameter, and an equation relating the solvent solubility parameter to the dielectric constant has been proposed[60]. This explains why there is a specific dielectric constant resulting in a maximum solubility when studying co-solvent systems.

Modified solution stability

During preclinical development, more information concerning the solution stability of the compounds is obtained[61]. Solution degradation products are determined as part of the development of the analytical methods. Studies are conducted to stress the compound under different solution conditions such as temperature, pH, peroxide, light and solution formulating excipients. Because the analytical methods need to separate the degradation products from the compound of interest, the conditions under which degradation can occur must be determined. This information is also critical in the solution dosage form development in order to formulate at the optimum pH and to determine if stabilizing excipients such as chelating agents and antioxidants must be incorporated into the formulation.

Late-stage candidate

In general, it is not possible to generate all of the characterization data required or needed for drug substances when they are initially filed with regulatory agencies for the first clinical studies. While clinical trials are being conducted, the studies listed in Box 4 should be completed. The solid

phase studies, particularly surface area and particle size distribution, require having studied different sizes and distributions of particles in order to establish specifications based on clinical tests, or it must be shown that the same particle size or distribution can be manufactured consistently. Because of the need to have multiple batches of the compound synthesized, this information cannot be obtained until the final clinical studies are being conducted. The solution stability studies cannot usually be completed until this late stage because of the time required to complete the studies. It is not uncommon to need up to two years to evaluate the rate constants and determine the degradation products and reaction mechanisms.

Box 4. Physical chemical properties determined during clinical testing

Solids
Surface area
Particle size distribution
Polymorphism

Solutions
Complexation constants
Aggregation constants
Detailed solution stability

Surface area

The surface area of a compound[62,63] can be used as a control check to determine if the same particle size distribution is being produced from batch to batch. This is important because the formulation as well as dissolution and bioavailability can be affected by the size of the particles.

The specific surface area can be obtained directly from the measurement of a volume of gas (usually N_2, He or Ar) that can be adsorbed onto the surface of 1 g of material using the BET (Brunauer, Emmett and Teller) equation. Experimentally, a monolayer of gas is adsorbed onto the surface of the particles. Given that the volume of gas adsorbed and the volume of one molecule of gas are known, the total surface area can be calculated. Studies that measure the desorption, as well as the adsorption, of the gas can be used to determine whether the particles have pores or cracks that contribute to the surface area because there will be a hysteresis loop present in the isotherm. Another technique that can be used to determine the surface area of a compound is air permeability. This method measures the resistance to flow of air through a plug of compacted powder. The results from either of these methods can be interpreted in terms of a mean particle size. Usually the particles are assumed to be spherical when performing this latter calculation.

Particle size distribution

There are many methods available for determining the particle size of a compound[64,65]. In addition to the methods used to determine surface area, the following methods can be used to determine either a mean particle size or a particle size distribution: Coulter counter, laser light scattering, optical microscopy, sedimentation and sieving. Drug substances usually have particle size distributions greater than 0.1 μm. However, sometimes it is necessary to measure particles with size distributions of <0.1 μm. In this case, dynamic laser light scattering can be used when the particles are as small as 1–2 nm.

Polymorphism

During the later stages of development of a compound, a concerted effort should be made to determine whether polymorphs or pseudo-polymorphs can be formed. It is generally recognized[66] that almost all organic compounds exist in several solid-state forms, and it is the task of the investigator to discover under what conditions these other forms can be prepared. Attempts should be made to make different polymorphs and pseudo-polymorphs by crystallizing at different temperatures and using different solvent systems, exposing the solid material to different temperatures and humidities, and stressing solid samples by grinding or compressing. As already discussed, any number of techniques can be used to determine if different forms of the compound have been prepared.

Complexation constants

Most pharmaceutically active compounds are designed to interact with other compounds in order to be efficacious. Because of this, there is a very real probability that the active compounds can interact with metals and compounds that are not factors in their activity. They can interact with other components in the formulations, administration vehicles, administration sets, and/or in the physiological environment. The strength of the interactions and the sensitivities to low concentrations of the metals or compounds can be obtained from measurement of their complexation constants[67]. Many methods have been used to determine complexation constants, including spectroscopic, potentiometric, chromatographic, solubility, conductivity and dialysis techniques. If it is suspected that the active compound might interact with other compounds, studies should be conducted to determine if the interactions occurring are sufficiently

strong to affect the activity. Also, should other interactions be found, it may be necessary to conduct additional *in vitro*, *in vivo* or *in situ* studies to try and determine their pharmacological effects.

Aggregation constants

The aggregation of compounds not only includes the formation of dimers and trimers but also the larger associated complexes found in micelles[68–73]. Associations of this type probably occur more often than is realized, particularly when the aggregation numbers are small. This is because the deviation from expected behavior is not necessarily great or the parameters calculated, such as pK_a or partition coefficient, that can be used as an indication of association, are in the expected range for the compound structure. Usually it is necessary to obtain profiles such as solubility as a function of pH, solubility as a function of temperature, conductivity as a function of concentration, heat of dilution as a function of concentration, or light scattering as a function of concentration in order to determine if self-aggregation is occurring.

It is important to know when a compound is aggregating in solution not only because this provides information regarding its intrinsic properties but also because properties such as stability, partitioning, equilibrium constants and solubility can be affected. Changes in these properties can have a direct effect upon the analytical methods, formulation and pharmacological behavior of the compound. Because aggregation is dependent upon the solvent system, when formulations require co-solvents this phenomenon should be studied in the multi-component systems.

Solution stability

It is apparent from any chemical kinetics textbook addressing the solution stability of compounds that almost all biologically active compounds are unstable in solution[74–76]. It is therefore critical that detailed stability studies be performed and, at the very least, the effects of pH, oxygen and temperature on the stability investigated. In addition to these studies, the effects of other components in the formulation, such as sugars, buffers or preservatives, should be studied. Although instability is often presumed to be due to major components, it is not uncommon for stability to be affected by the presence of trace metal impurities in an excipient, which can catalyze a reaction. Also, there can be a low level of a component in equilibrium with a major component; for example, the aldehyde form (0.024%), which is in equilibrium with the hemiacetal form of dextrose, was found to interact with an amine functional group of an antibiotic[77]. As indicated, degradation mechanisms can be very complex and are usually species-dependent[78,79]. Therefore, in aqueous solution, the reaction rates must be determined as a function of pH.

If the instability of the compound is so great that it precludes the development of a dosage form, it is sometimes possible to add excipients that can stabilize the formulation. Several approaches have been used to stabilize liquid formulations, including the formation of hydroxypropyl-β-cyclodextrin complexes[80], the introduction of antioxidants[81] and placing surfactants in the formulation[82]. When stabilizers are added to formulations, the reformulated dosage forms need to be evaluated for their stability.

Conclusion

A tremendous amount of work must be completed before an approval can be obtained to market a new pharmaceutical product. Although not all of the information has to be obtained during the early phases of development, the sooner the data are generated and the behavior of the compound under different conditions is understood, the easier it is to make decisions concerning the development of the compound. As mentioned, it is not only the purpose of these investigations to determine the behavior of the compound under specific conditions but to be able to use this information to predict how the compound will behave under conditions not studied. The intent of this review, therefore, has been to summarize the major types of investigation that must be conducted during the course of development in order to make known the time, talent and financial resources that must be invested to develop a compound.

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In short...

Following meetings with the Center for Biologics Evaluation and Research and the FDA, **Chiron Corporation** and **Biomira Inc.** have confirmed plans to progress to a large, international multi-centre Phase III clinical trial in North America and Europe to evaluate efficacy of the therapeutic vaccine THERATOPE® [STn-KLH; see Koganty, R.R. *et al. Drug Discovery Today* (1996) 1, 190–198] in women with metastatic breast cancer who have responded to first-line chemotherapy. Phase I/II study results have suggested that the vaccine has minimal side effects and produces an immune response to both the active ingredient in the vaccine and the relevant cancer-associated antigen. These studies have also demonstrated an apparent increase in survival for women with metastatic breast cancer who were treated with vaccine.