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Allen C. Templeton
Stephen R. Byrn
Roy J. Haskell
Thomas E. Prisinzano
Editors

Discovering and Developing Molecules with Optimal Drug-Like Properties

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Editors

Allen C. Templeton
Analytical Sciences
Merck & Co.
Summit, New Jersey, USA

Stephen R. Byrn
Department of Industrial and
Physical Pharmacy
Purdue University
West Lafayette, Indiana, USA

Roy J. Haskell
Discovery Pharmaceuticals
Bristol-Myers Squibb
Wallingford, Connecticut, USA

Thomas E. Prisinzano
Department of Medicinal Chemistry
University of Kansas School of Pharmacy
Lawrence, Kansas, USA

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About the Editors

Allen C. Templeton is executive director of the Analytical Sciences organization within Merck Research Laboratories. He is responsible for the managing staff in the pursuit of scientific problem-solving for pharmaceutical product development. Before assuming his current position, Dr. Templeton held positions of increasing responsibility within Merck, including leadership roles in preformulation and formulation. Dr. Templeton earned his Ph.D. in analytical chemistry from the University of North Carolina at Chapel Hill. His research experience has been in the area of analytical and materials chemistry. He has published more than 50 articles, served as co-inventor on 11 patents, and authored more than 120 presentations in the area of pharmaceutical analysis. He has organized a number of symposia and training courses on diverse topics within the field of pharmaceutical characterization. Dr. Templeton is an active member in a number of professional organizations, including the American Association of Pharmaceutical Scientists (AAPS) and the American Chemical Society (ACS). He has served in a number of roles for AAPS and is most recently the secretary/treasurer of the Physical Pharmacy and Biopharmaceutics section. He was elected to the United States Pharmacopeia (USP) expert committee on physical analysis and has worked to revise a number of USP standard chapters. He is also currently serving on the Analytical Leadership Group for the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ). He is on the editorial advisory boards for the *Journal of Pharmaceutical Sciences*, *American Pharmaceutical Review*, and *Current Drug Delivery*.

Stephen R. Byrn is Charles B. Jordan Professor of Medicinal Chemistry in the Department of Industrial and Physical Pharmacy, Purdue University. Dr. Byrn set in motion the development of the field of solid state chemistry of drugs with his books and papers on the subject. He has also taught more than 100 short courses on solid state chemistry and pharmaceutical solids and has educated more than 50 Ph. D. students and postdoctoral fellows. Dr. Byrn has had numerous grants, including one of the first 13 from NIH Centers for AIDS Research. Dr. Byrn is cofounder of Purdue's graduate programs in regulatory and quality compliance. He is also

cofounder of the Purdue-Kilimanjaro School of Pharmacy graduate certificate program in industrial pharmacy and manufacturing in Moshi, Tanzania. Dr. Byrn has served as chair of the Pharmaceutical Sciences Advisory Committee to the FDA and chaired several USP committees. Dr. Byrn is also cofounder of SSCI, Inc. (Solid State Chemical Information), a cGMP research and information company now owned by Aptuit. Dr. Byrn is an elected Fellow of the American Association of Pharmaceutical Scientists (AAPS) and has received several awards for his research and entrepreneurial activities including the first AAPS David Grant Award for Research Achievement in Physical Pharmacy. The *Journal of Pharmaceutical Sciences* has a special issue dedicated to Dr. Byrn. His current research interests include strategies for accelerated drug development and the use of synchrotron X-rays for pharmaceutical research.

Roy J. Haskell is a Research Fellow in the Discovery Pharmaceuticals group of Bristol-Myers Squibb, where he is engaged in discovery support as well as the design and characterization of novel formulations. He received a Ph.D. in analytical chemistry from the University of Wisconsin–Madison. He joined The Upjohn Company and worked in the area of protein biophysics. As part of Pharmacia, his focus transitioned to formulating poorly soluble molecules and characterizing the formulations by which such compounds are delivered. Dr. Haskell's research interests include design and characterization of colloidal formulations, predictive modeling of physicochemical properties, sizing submicron particles, aggregation/precipitation mechanisms, the role of solubility and supersaturation in oral absorption, and the use nanotechnology in drug discovery and development. Dr. Haskell is the author of three patents and more than 30 publications and presentations.

Thomas E. Prisinzano received his Ph.D. in pharmaceutical sciences from the School of Pharmacy, Virginia Commonwealth University, in Richmond, Virginia. He was an Intramural Research Training Award Fellow in the National Institute of Diabetes and Digestive and Kidney Diseases in Bethesda, Maryland, and is currently Professor and Chair of the Department of Medicinal Chemistry at the University of Kansas. His research focuses on the development of novel agents to treat pain, substance abuse, and other CNS disorders through the identification, structure elucidation, and optimization of natural products. Dr. Prisinzano has received a number of awards including the D. John Faulkner Travel Award from the American Society of Pharmacognosy, the Jack L. Beal Award from the *Journal of Natural Products*, the Matt Suffness (Young Investigator) Award from the American Society of Pharmacognosy, the Joseph Cochin Young Investigator Award from the College on Problems of Drug Dependence, and the David W. Robertson Award for Excellence in Medicinal Chemistry from the American Chemical Society.

List of Contributors

John Baumann Bend Research, Bend, OR, USA

David M. Bender Small Molecule Design and Development, Eli Lilly and Company, Indianapolis, IN, USA

C. J. Benmore X-ray Science Division, Argonne National Laboratory, Lemont, IL, USA

Shobha N. Bhattachar Small Molecule Design and Development, Eli Lilly and Company, Indianapolis, IN, USA

Nathan Boersen Celgene Corporation, Summit, NJ, USA

Chad Brown Formulation Sciences, Merck & Co., West Point, PA, USA

Stephen R. Byrn Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN, USA

James DiNunzio Merck & Co., Summit, NJ, USA

Brett Duersch Analytical Development-Commercialization, Merck & Co., West Point, PA, USA

Kimberly A. Foster Discovery Pharmaceuticals, Bristol-Myers Squibb, Lawrenceville, NJ, USA

Sudhakar Garad Discovery Pharmaceuticals, Cubist Pharmaceuticals, Lexington, MA, USA

Roy J. Haskell Discovery Pharmaceuticals, Bristol-Myers Squibb, Wallingford, CT, USA

John Higgins Discovery Pharmaceutical Sciences, Merck & Co., West Point, PA, USA

Grace Ilevbare Discovery Pharmaceutical Sciences, Merck & Co., Kenilworth, NJ, USA

- Akash Jain** Discovery Pharmaceuticals, Cubist Pharmaceuticals, Lexington, MA, USA
- Susan M. Jenkins** Metabolism and Pharmacokinetics, Bristol-Myers Squibb, Wallingford, CT, USA
- David Johnson** Pharmaceutical Commercialization, Merck & Co., West Point, PA, USA
- Filippos Kesisoglou** Biopharmaceutics, Merck & Co., West Point, PA, USA
- Michael H. Kress** Process Chemistry, Merck & Co., Rahway, NJ, USA
- Michael Lowinger** Discovery Pharmaceutical Sciences, Merck & Co., Rahway, NJ, USA
- William Marinaro** Formulation Sciences, Merck & Co., Summit, NJ, USA
- Patrick Marsac** Formulation Sciences, Merck & Co., West Point, PA, USA
- Craig McKelvey** Merck & Co., Inc., Formulation Sciences, West Point, PA, USA
- Michael McNevin** Formulation Sciences, Merck & Co., Summit, NJ, USA
- Elaine Merisko-Liversidge** Pharmaceutical Research & Development, Alkermes, Waltham, MA, USA
- Robert Meyer** Pharmaceutical Commercialization, Merck & Co., West Point, PA, USA
- Amitava Mitra** Biopharmaceutics, Merck & Co., West Point, PA, USA
- Michael Morgen** Bend Research, Bend, OR, USA
- Justin Moser** Formulation Sciences, Merck & Co., West Point, PA, USA
- Dawn D. Parker** Discovery Pharmaceuticals, Bristol-Myers Squibb, Wallingford, CT, USA
- Thomas E. Prisinzano** Department of Medicinal Chemistry, University of Kansas, Lawrence, KS, USA
- Alfred C. F. Rumondor** Formulation Sciences, Merck & Co., Summit, NJ, USA
- Joyce Stellabott** Formulation Sciences, Merck & Co., West Point, PA, USA
- Stephanie A. Sweetana** Small Molecule Design and Development, Eli Lilly and Company, Indianapolis, IN, USA
- Allen C. Templeton** Analytical Sciences, Merck & Co., Summit, NJ, USA
- Karen Thompson** Formulation Sciences, Merck & Co., West Point, PA, USA
- Ching Kim Tye** Discovery Pharmaceuticals, Bristol-Myers Squibb, Lawrenceville, NJ, USA

David T. Vodak Bend Research, Bend, OR, USA

James A. Wesley Small Molecule Design and Development, Eli Lilly and Company, Indianapolis, IN, USA

Dan Zhang Formulation Sciences, Merck & Co., Summit, NJ, USA

Part I
Strategy and Tactics that Enable Discovery

Chapter 1

Developability Assessment and Risk Management During Drug Discovery

Sudhakar Garad and Akash Jain

1.1 Introduction

The pharmaceutical industry is seeing insufficient revenue and profit growth due to high development costs, long development and approval times, fewer new product launches, lack of rich pipelines, and loss of revenues to generics due to patent expiration (Cockburn 2004; Frank and Seiguer 2003). The lack of rich pipeline in the industry is caused by the high attrition rates due to inadequate physicochemical and biopharmaceutical attributes, acceptable safety, and sub-marginal efficacy in preclinical and clinical studies, all of which also contribute to increasing drug development costs (Subramaniam 2003; Rosiello et al. 2013). Because of rapidly rising drug development costs, there is an enormous pressure to cut cost and streamline development (Watkins 2002; Paul et al. 2010). While different companies are dealing with these challenges differently, the industry can benefit substantially by enhancing the discovery–development interface. The knowledge gained during the candidate selection phase will be useful in fast development of optimal formulation and clinical approaches for clinical studies, and consequently will help shorten the development timelines for new chemical entities (NCEs). This will directly benefit not only the industry, but also the society, as new and better lifesaving drugs can reach patients sooner (Venkatesh and Lipper 2000).

In such a scenario, the reduction of attrition rate and thus reduction in the development cost and timeline for a product pipeline can be achieved by strengthening the efficacy and toxicity screens, and establishing a developability screen to enable selection of developable compounds to the clinic.

Efficacy and toxicity screens involve dosing the drug in a variety of animal models. A general schematic of the most common in vivo studies performed during

S. Garad (✉) • A. Jain

Discovery Pharmaceuticals, Cubist Pharmaceuticals, 65 Hayden Avenue, Lexington, MA 02421, USA

e-mail: sudhakar.garad@cubist.com

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different discovery stages is shown in Fig. 1.1. Selection of the right formulation principles is critical for the proper functioning of efficacy and toxicity screens, especially for NCEs with poor solubility and/or with potential for variable systemic exposure. For such NCEs, an inadequate formulation principle may limit their systemic exposure through limited solubilization or precipitation in the gastrointestinal tract or in the blood stream (from a solubilized formulation), thus leading to low and variable exposure and false safety interpretation. Additionally, sub-marginal exposure may also lead to poor efficacy, and thus could lead to rejection of a potential blockbuster. For example, blockbuster drug products such as Cyclosporine (Neoral[®]), Telaprevir (Incivek[®]) and Lopinavir/Ritonavir (Kaletra[®]) are excellent case studies where the poor solubility of respective APIs (active pharmaceutical ingredients) was successfully overcome by selecting the right enabling formulation technology. Therefore, the success of such efficacy and toxicity screens for NCEs depends a lot on proper selection of suitable formulations, which eventually will lead to objective go/no-go decisions before the NCEs enter into full development.

Developability screens are equally important as the efficacy and toxicity screens. Developability assessment typically involves physicochemical characterization of NCEs, characterization and selection of the most suitable/stable solid form of API, development of formulations for robust PK, efficacy and safety assessments in preclinical species, biopharmaceutics (e.g., factors limiting absorption and bio-availability, food effects, etc.) and drug delivery options for further development of NCEs. The data package based on *in silico*, *in vitro* and *in vivo* evaluation of multiple formulations/delivery technologies allows the selection of a biopharmaceutically optimized formulation/delivery strategy, and consequently, yields more reliable information on developability of NCEs moving into clinic and commercialization.

1.2 Developability Assessment Group (DAG)

1.2.1 Background

The organizational structure of the function performing developability assessment varies significantly from one pharmaceutical company to another (Kerns and Di 2002; Fiese 2003; Balbach and Korn 2004; Sun et al. 2004; Balani et al. 2005; Singh 2006; Maas et al. 2007). Developability Assessment Groups (DAGs) could reside in either discovery or development parts of an organization. In either case, the key enabler for their success is strong cross-functional collaborations, especially with medicinal chemists, biologists, pharmacologists and ADME/PK/Tox groups on the discovery side and technical development teams (API and Drug Product) and clinicians on the development side. Other key enablers for DAGs include *in silico* modeling tools, state-of-the-art analytical capabilities, solid-state characterization

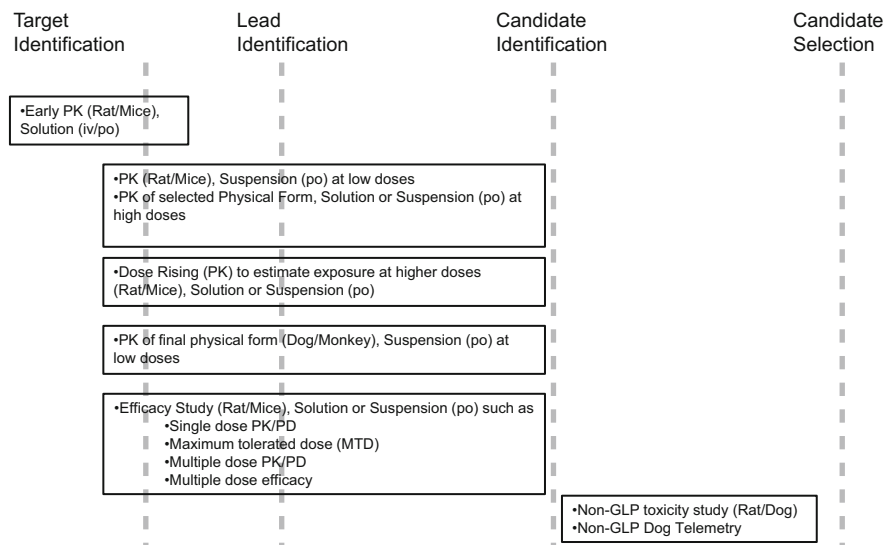


Fig. 1.1 General and most common sequence of in vivo studies during candidate selection. Reprinted from Saxena et al. (2009) with permission from John Wiley & Sons

technologies, automation and high-throughput platforms and strong understanding of biopharmaceutics and drug delivery.

1.2.2 Roles and Responsibilities

In principle, the DAGs can take a two-pronged approach on NCEs depending on complexity of biological target and chemical modification space available to medicinal chemists:

Pharmaceutics—Build right developable properties in molecule (e.g., pK_a , solubility, stability, permeability, etc.), identify developable solid forms (e.g., salts, stable polymorphs, co-crystals) and selection of optimal delivery strategy (e.g., route of administration, formulation principle).

Enabling Technologies—Solubility and bioavailability enhancement (multiple technologies for parenteral and oral delivery), modified release (improve therapeutic index), and targeted drug delivery (local, site of inflammation/infection, etc.).

In scenarios where both of the above-mentioned approaches are unsuccessful in identifying NCEs with desired profile, a timely feedback can be provided to discovery teams to consider terminating further efforts on such targets. This is a great mechanism to derisk NCEs as soon as possible based on their biopharmaceutical, technical, and preclinical safety evaluation. Another key responsibility of

DAGs is to act as a liaison between discovery and technical development functions and ensure seamless transfer of physicochemical properties, solid form, biopharmaceutics and technical information on NCEs in a timely manner. This is an extremely value-adding step during transition of NCEs from discovery to development as it enables rapid decision-making on deliverables (API and Drug Product for GLP tox and first-in-human studies), avoids loss of critical information and helps minimize redundancies between different technical functions.

1.2.3 Deliverables

Based on authors' experience, the key deliverables of DAGs can be summarized as follows:

- (a) Intellectual input to discovery project teams during hit-to-lead and lead optimization stages to build the right physicochemical and biopharmaceutical properties into the lead candidates. Such properties include but are not limited to pK_a , $\log P/D$ and addition of functional groups to improve solubility and/or to reduce very high lattice energies due to strong intermolecular interactions.
- (b) Physicochemical and solid-state characterization of NCEs (melting point, solubility, pK_a , $\log P/D$, permeability, etc.).
- (c) Formulation development for pharmacokinetic, pharmacological and toxicological studies using conventional approaches such as solutions, suspensions, etc., or using non-conventional delivery approaches such as spray-dried dispersions, nanosuspensions, etc.
- (d) Generate *in silico* predictions of biopharmaceutical performance using tools such as GastroPlus™, maximum absorbable dose, etc.
- (e) Recommendation on stable physical form and clinical formulation.
- (f) Recommendation to terminate non-developable candidates as soon as possible thereby enabling research colleagues to move on to new scaffolds/targets.
- (g) Risk assessment with mitigation plan for the selected development candidates.
- (h) Enabling technologies for NCEs (e.g., targeted delivery, modified release, etc.).

The creation and implementation of DAGs in multiple organizations, over the last decade or so, has yielded a new paradigm for transition of NCEs from discovery into development. The above listed deliverables traditionally, once a candidate was handed over from discovery to development, would take approximately 6–9 months for completion and utilize ~30–100 g of API. In addition, if any developability issues were identified, it was almost too late to provide the feedback to discovery teams. With formation of DAGs, similar deliverables can now be achieved in approximately 2–4 months with ~2–10 g of API (Table 1.1). These significant savings in time and material have been made possible by development of automated and high-throughput platforms. The early intervention of DAGs in discovery programs helps provide timely feedback on developability issues and suggests appropriate measures well before a candidate is nominated for full development.

Table 1.1 Key activities and deliverables of Developability Assessment Groups (DAGs)

Hit to lead (5–20 mg)	Lead optimization (50–500 mg)	Candidate selection phase (2–10 g)
pKa	Weight-based purity	Batch characterization
Solubility (pH) and simulated fluids	Solid-state characterization (DSC, TGA, XRPD, DVS)	Solid form selection salt/co-crystal, polymorph selection
Stability (pH)	Preliminary salt/crystallization profiling	Formulation and condition-of-use stability for DC-enabling studies
PAMPA (GI, BBB)	In-process analytical method development	Analytical method development and validation
Formulation for PK/acute tolerability	Formulation for efficacy/toxicity studies	Impurity characterization
HPLC purity	Dosing/delivery strategies	Establish standards and specification
	Biopharmaceutics	Initiate GMP and GLP validation
		Clinical formulation recommendation

For a DAG to be successful and effective, it is very important to have the right balance of expertise in the group. Scientists in such groups generally have background in areas such as pharmaceuticals and biopharmaceutics, solid state and material science, analytical science and drug delivery. In addition to the technical expertise, competencies in cross-functional communication, working in team environment, and strong dedication and passion to bring new development candidates into clinic for unmet medical needs are critical in the role of a developability assessment scientist.

While the authors believe that the most optimal organizational structure is to have a DAG integrated within the discovery organization, several other organizational structures can function equally well, as long as there is close cooperation between a DAG and discovery teams. The DAG is not only responsible for building and selecting developable molecules but it is also responsible for identifying enabling drug delivery technologies for clinical and commercial products. This broad spectrum role of DAGs adds tremendous value to an organization in increasing or maximizing the potential of safe and efficacious candidates, especially, when it is very difficult to find NCEs for novel mechanisms. The emphasis in this chapter is not so much on the organizational structure, but on the valuable information that is generated by DAGs at various stages of drug discovery and development process with automated tools, minimum amount of material, resources and utilization of cutting edge technologies.

1.3 Developability Assessment of New Chemical Entities (NCEs): Methods and Best Practices

1.3.1 Biopharmaceutics-Based Molecular Design

Biopharmaceutical properties are usually secondary for any given molecule during early discovery stages as most time and effort is spent in identifying molecules with the right balance of potency and safety. However, based on lessons learned from failures and delays in advancing candidates with poor biopharmaceutical profile through clinical development, major pharmaceutical organizations are looking to build biopharmaceutical properties into molecules during early discovery stages (Saxena et al. 2009, Zheng et al. 2012). A few important properties are discussed in this section.

1.3.1.1 Ionization

Ionization constant, pK_a , is a useful thermodynamic parameter to monitor the charge state of NCEs. Based on their pK_a , all NCEs can be classified into four major classes—acid, base, neutral, and Zwitterion. Each of these classes of molecules represents different challenges and opportunities in their biopharmaceutical performance. Molecules with acidic pK_a (pK_a 3.5–6.0) predominantly exist in their ionized and solubilized form at physiological intestinal pH (5.5–7.4). However, it has been reported and well documented that ionized form of a molecule tends to have poor passive permeability (Thomayant et al. 1998). As both the ionized and unionized form of a molecule exist in a dynamic/equilibrium process in vivo, the authors believe that a sufficient fraction absorbed (F_a) is achieved in vivo from acidic molecules. Basic molecules demonstrate a much larger range of pK_a (2.0–9.0) depending on the type of functional groups. Strongly basic molecules (pK_a above 6.0) are considered ideal for improving solubility and dissolution rates and to minimize in vivo precipitation (and related complications) in the physiological pH range for both i.v. and oral delivery. Such molecules readily form salts with acidic counter ions and remain solubilized at the site of absorption. On the other hand, weakly basic molecules (pK_a below 6.0) require stronger acidic counter ions for salt formation and demonstrate improved solubility/dissolution rates only at pH 4.0 and below. At physiological pH for absorption (pH 5–7), the weakly basic molecules rapidly dissociate from their salt forms and convert to poorly soluble neutral forms resulting in poor and/or variable absorption and bioavailability. Weakly basic molecules also pose a significant challenge in developing stable dosing solutions for parenteral delivery due to risk of precipitation upon injection (Jain et al. 2010). Zwitterionic molecules are the most complex in their physicochemical and biopharmaceutical behavior. Such molecules typically demonstrate high variability in oral absorption and bioavailability (Thomas et al. 2006). In addition, their pK_a values and solubility profile are highly sensitive to presence of additional functional

groups in vicinity of the ionizable groups. For instance, the presence of a strongly electron-withdrawing group next to the ionizable group can lead to a considerable shift in its pK_a and results in poor solubility/dissolution. Given a preference, it is better to avoid Zwitterions especially for development, unless they demonstrate high intrinsic solubility across the entire pH range or high potency such that absolutely minimal doses are required in preclinical as well as clinical studies.

1.3.1.2 Intermolecular Interactions

Intermolecular interactions of NCEs (e.g., charge–charge, charge–dipole, dipole–dipole, van der Waal’s dispersion, π – π stacking, hydrogen bonding, etc.) can significantly impact their crystallinity and solid-state properties which in turn can have a profound impact on their solubility, dissolution, precipitation, recrystallization behavior and ultimately their biopharmaceutical performance (Thomoyant et al. 1998). It is therefore advisable to minimize exploration of chemical scaffolds with very high lattice energies (e.g., π – π stacking of planar aromatic rings). Solid-state properties such as melting point and/or heat of fusion can serve as useful surrogates to estimate the lattice energies.

To summarize, it is always worthwhile to spend the extra time and effort during lead optimization stages in building some of the key biopharmaceutical properties into a molecule. This investment during discovery stages eventually pays off in development as it lays the foundation for design of a robust drug product strategy that can help clinical teams accelerate and shorten development timelines.

1.3.2 Physicochemical Characterization

A thorough understanding of physicochemical properties of a NCE is a prerequisite to prediction of biopharmaceutical behavior as well as screening and development of solid form and formulations for preclinical and clinical studies. Developability assessment teams are able to generate a robust package of physicochemical characterization data using minimal amounts of time and material, largely due to availability and constant evolution of *in silico* prediction tools and high-throughput screening technologies. This section will describe the methods and best practices in characterization of physicochemical properties such as pK_a , $\log P/D$, solubility/dissolution rate, permeability, and *in vitro* metabolism.

1.3.2.1 Ionization Constant (pK_a)

As noted above, the ionization constant, pK_a , is a commonly used thermodynamic parameter in drug discovery to understand binding mechanisms and to predict ADME properties of NCEs due to the pH gradient of 1.0–7.0 in the human GI

tract (Avdeef 2003). A number of important properties such as lipophilicity, solubility pH profile, permeability, and human Ether-à-go-go-Related Gene (hERG) binding affinity are modulated greatly by pK_a . While pK_a can be successfully predicted by *in silico* tools owing to the high dependence on the molecular structure of NCEs, this prediction may not be accurate unless the corresponding model has been parameterized to account for the novel chemical space spanned by many discovery programs, thereby justifying the need for *in vitro* determination. A range of experimental approaches with varying throughput, cycle time, sample requirement, and costs are available for pK_a determination (Wan and Ulander 2006; Wang & Faller 2007; Wang et al. 2007). Some of the commonly used methods are potentiometric titration (Avdeef 2003), capillary electrophoresis (CE) (Cleveland et al. 1993; Ishihama et al. 2002), Spectral Gradient Analyzer (Box et al. 2003), and Sirius T3 (Sirius Analytical).

Potentiometric Titration

In the potentiometric titration method, a potentiometer records the pH changes with a glass electrode, caused by introducing a known volume of titrant to the well-mixed solution of a NCE. This methodology is tedious as it requires a lengthy process due to long and repetitive equilibrium steps after titrant additions. However, the pK_a values obtained are reliable and therefore, this methodology is considered to be the gold standard despite a number of limitations. This methodology is only suitable for compounds with good solubility as potentiometric titration requires concentrations in the range of 0.1–1 mM. Although cosolvents have been used to circumvent poor solubility, it still requires several titrations to extrapolate the data from different water and cosolvent mixtures to aqueous solution (zero cosolvent concentration). An additional problem with potentiometric titration is the increasing numbers of NCEs in early discovery are delivered as salt forms with protogenic counter ions, like acetate, fumarate, titrate, etc. Finally, this methodology requires materials in milligram scale, and therefore, it is more useful in late discovery.

Spectral Gradient Analyzer

This assay works effectively with poorly soluble NCEs by using cosolvent in the media. The pK_a data measured using this “rapid-mixing” approach correlate well with those from the potentiometric titration method. In addition, this method is useful for measuring pK_a for early discovery compounds as it requires a small amount of material, and allows for high throughput/automation. The limitation is that it is suitable for ionizable compounds, which induce a change in the UV spectra scan. In other words, not only is a UV chromophore required, but also the chromophore may have to be located close enough to the ionization center within an NCE. This technique is based on a continuously flowing pH gradient and a UV-DAD

(diode array detector). It was developed recently and allows for a much higher throughput (Box et al. 2003). This method establishes a stable time-dependent pH gradient by rapidly mixing acidic and basic buffers, during which drug candidates pre-dissolved in organic solvent are introduced at different pH conditions. This fast method allows full characterization of ionizable groups for 70 % of the discovery output and usually gives high quality data that are consistent with those obtained by potentiometric titration (Garad et al. 2009). Neither this “rapid mixing” nor the potentiometric titration approach is compound-specific, so compounds with purity or stability issues or counter ions containing similar UV chromophore may be problematic for this approach.

1.3.2.2 Lipophilicity ($\log P/D$)

Lipophilicity, as expressed by the logarithm of partition coefficient or distribution coefficient ($\log P$ or $\log D$) of NCEs between a lipophilic phase (e.g., octanol) and aqueous phase, is a valuable physicochemical property. Compounds with very high ($\log P/D > 6$) or very low lipophilicity ($\log P/D < 1$) can present significant developability challenges such as poor formulability, poor permeability and absorption, accumulation in tissues/organs, etc. Therefore, a right balance of lipophilicity in any compound is essential prior to its selection as a candidate for development. Multiple techniques can be used to determine lipophilicity of the compound. While shake-flask is the conventional method for $\log P$ (or $\log D$) determination, the dual-phase potentiometric titration approach is also widely accepted, particularly during late drug discovery phase (Avdeef, 2003). For NCEs lacking an ionizable group, HPLC $\log P$ technique, also known as eLog P can be applied (Lombardo et al. 2000). A variety of techniques that are suitable for early discovery include liposome chromatography, immobilized artificial membrane (IAM) chromatography, capillary electrophoresis (CE) (Avdeef, 2003), and artificial membrane preparations (Wohnsland & Faller 2001).

1.3.2.3 Solubility/Dissolution

Solubility of NCEs is one of the most important physicochemical properties that govern its ADME, pharmacological and biopharmaceutical profile. Poor solubility and dissolution rates are the most common rate-limiting factors for oral drug absorption, especially for NCEs with high permeability (Ku 2008). A variety of solubility and dissolution measurements are performed throughout the discovery and development stages of a NCE (Hariharan et al. 2003; Vertzoni et al. 2005; Kibbey et al. 2001; Glomme et al. 2005; Avdeef 2007; Galia et al. 1998; Ingels et al. 2002; Lind et al. 2007). The objectives and methods for such measurements differ widely depending on the stage of the project and intended use of the data. A few examples of typical solubility experiments include high-throughput measurement in two or three buffer system during early discovery stages to rank order

Table 1.2 Solubility and dissolution experiments in discovery and early development of NCEs

Experiment	Application (s)
HT-solubility	Guide SAR during early discovery stages
Shake-flask solubility	pH solubility profile
Solubility in biorelevant media	Prediction of maximum absorbable dose, GastroPlus™ simulations, build IVIVC, food effect prediction
Organic solvents	Salt and polymorph screening, crystallization process development
Solubility in cosolvent, surfactant, complexing agents, lipids	Develop solubilized formulations for preclinical and clinical studies; develop dissolution media for release testing
Intrinsic dissolution rate	Rank-order solid forms (e.g., salts, co-crystals, polymorphs)
Non-sink dissolution	Rank-order of prototype solid forms and formulations (e.g., solid dispersions, salts, etc.)
Dissolution (type II apparatus)	Release testing for solid dosage forms; biorelevant testing and food effect prediction for solid dosage forms

compounds and develop SAR, solubility measurement of an API in organic solvents to develop a crystallization process or measuring kinetic solubility of API in biorelevant media to predict maximum absorbable dose. Solubility experiments can be performed under a variety of conditions such as equilibration at room temperature (e.g., 24–48 h), kinetic solubility at 37 °C (e.g., 1–4 h), temperature cycling in organic solvents or two-step solubility screen in biorelevant media to identify supersaturation-enhancing excipients. A detailed list of solubility and dissolution experiments typically performed during discovery and early development stages is shown in Table 1.2.

An important consideration for developability of a NCE is its tendency to precipitate under physiological conditions. Depending on its solid form and pH-dependent solubility, a compound may precipitate out in stomach fluids (pH ~2) or in small intestinal fluids (pH ~5–7) during its transit in the gastrointestinal tract. The precipitated compound may dissolve differently in the gastrointestinal fluids than the original form. Occasionally, for compounds with pH-dependent solubility, certain forms/salts of a compound may dissolve in stomach fluids, and upon gastric emptying in the intestine may remain dissolved, thus yielding supersaturated solutions. In such cases, the compound may show much higher bioavailability. Thus, it is critical to evaluate dissolution characteristics of various solid forms before selecting a final solid form for further development. Dissolution studies should be conducted either with suspension formulations or with compounds filled in capsule with or without formulation excipients. The physiological conditions can be simulated by using buffers (pH 1, 2, 4.5, and 6.8) and/or simulated fed/fasted gastric and intestinal media (Jantratid et al. 2008) at 37 °C either as single step or pH-shift method (Mathias et al. 2013). The presence of formulation excipients could help simulate a solid dosage form and can reflect the effects of formulation excipients on dissolution rate. For compounds with poor

solubility, dissolution studies should be conducted in simulated gastric and intestinal media to determine the potential for food effect.

Solubility and dissolution data are often used to select and/or rank-order NCEs during lead optimization stages. However, due to unknown efficacious clinical doses at such early stages of discovery, it is difficult to drop or terminate NCEs based on poor solubility values. In addition, for poorly soluble NCEs (typically with aqueous solubility <100 $\mu\text{g/ml}$), there are a number of formulation technologies (see Sect. 1.3.4.4) that could be explored to improve the solubility/dissolution rates and eventually their in vivo absorption and bioavailability. Early screening of such formulation technologies in combination with human PK/PD and dose predictions is essential for a successful developability assessment of poorly soluble NCEs.

1.3.2.4 Permeability

Permeability plays a vital role in the absorption of orally delivered NCEs. Permeability is a complex phenomenon and it involves multiple mechanisms across the GI mucosa (Artursson & Tavelin 2003). Hence, it is important that a developability assessment scientist identify permeability behavior of NCEs early during lead optimization stages and provide timely feedback to project teams, especially when the teams are considering an oral route of administration. Unlike poor solubility, there are only few formulation or delivery technologies that can overcome poor permeability of NCEs and provide significant improvement in their oral absorption or bioavailability (Maag 2012; Maher and Brayden 2012).

One of the simple parameters that correlate well with permeability is the molecular weight of any given NCE. As per Lipinski's rule of 5, any NCE with molecular weight <500 typically demonstrates medium to high passive permeability and NCE with molecular weight >500 usually tend to exhibit poor to medium or almost no passive permeability. Another in silico model is the one developed by Egan and coworkers (2000). Their absorption model is based on polar surface area (PSA) and calculated $\log P$ ($C \log P$). Based on their analysis, it was shown that majority of highly permeable marketed drugs were populated in an egg-shaped zone of the absorption model and therefore, specified as "good" molecules. On the other hand, drugs with poor permeability or those considered as substrates for efflux transporters were scattered outside the egg-shaped zone and denoted as "poor" in the model. Interestingly, an exception to this egg-shaped model is the blockbuster drug—Lipitor.

In addition to the in silico methods, a number of in vitro models were developed to predict permeability as well as to assess the contributions of active transporters in the permeation process (Hämäläinen & Frostell-Karlsson 2004; Balimane et al. 2006). A few important assays, commonly being used in the last few decades, for measuring permeability of NCEs with high speed and reliability/reproducibility are described as follows.

PAMPA

Parallel artificial membrane permeability assay (PAMPA), pioneered by Kansy et al. (1998), utilizes a chemical membrane immobilized on a 96-well filter plate and samples are analyzed by a UV plate reader. Distinct membrane models were established by Kansy et al. (1998), Avdeef et al. (2001), Faller (2001) and Sugano and coworkers (2001) to mimic passive diffusion of NCEs across the GI tract (Garad et al. 2009). Avdeef (2003) introduced the “double-sink” model that simulates the concentration and pH gradient across the GI membrane. Cosolvents (Ruell et al. 2004) or excipients (Kansy et al. 1998; Sugano et al. 2001; Liu et al. 2003; Bendels et al. 2006) were employed in PAMPA to overcome the low solubility issues frequently encountered in early discovery. Quantification using LCMS (Balimane et al. 2005; Wang & Faller 2007; Mensch et al. 2007; Cai et al. 2012) or HPLC (Liu et al. 2003) drastically improved the sensitivity and robustness of PAMPA by extending the limit of detection for NCEs with low solubility. It also prevents interference originating from impurities with high solubility and/or strong UV chromophore. PAMPA ultimately offers a fast and relatively cost-effective method to estimate permeability for NCEs absorbed by passive diffusion mechanisms.

Caco-2

The human colon adenocarcinoma (Caco-2) cell permeability model exhibits morphological (e.g., tight junction and brush-border) and functional similarities (e.g., multiple transport mechanisms) to human intestinal enterocytes (Ungell & Karlsson 2003; Englund et al. 2006), thereby serving as the gold standard for *in vitro* permeability assessment in industry (Artursson & Tavelin 2003; Lennernas and Lundgren 2004). Caco-2 cells, which extensively express a variety of transport systems beyond P-glycoprotein (Pgp), are amenable to investigate the interplay among different transport systems and differentiate the relative contributions from passive and active transport mechanisms to the overall permeability across the human GI tract (Ungell 2004; Steffansen et al. 2004). While the conventional protocol (e.g., 21-day cell culturing) is essential to assure the full expression of transporters, the Caco-2 models using accelerated cell culturing procedures (e.g., 3–7 days) are not suitable for studying active transport of NCEs due to inadequate expression of transporters (Liang et al. 2000; Alsenz & Haenel 2003; Lakeram et al. 2007). Scientists (Yamashita et al. 2002; Miret et al. 2004) are still debating whether the short-term Caco-2 culturing system is appropriate to rank permeability, with concern about its poor correlation with human absorption data and inability to differentiate medium permeability compounds (Miret et al. 2004; Sugano et al 2010).

Today, the cumbersome long culturing procedure can be readily handled by automation (Saunders 2004; Wang & Faller 2007) and therefore Caco-2 has been successfully validated and widely implemented in 24- or 96-well plate formats to assess the permeability and drug interaction with GI-related transporters (Ungell &

Karlsson 2003; Kerns et al. 2004; Marino et al. 2005; Balimane et al. 2004). Similarly to PAMPA, precaution should be taken when dealing with low solubility compounds in discovery to select agents inert to the permeability and transport process of the Caco-2 model (Yamashita et al. 2000). Introduction of bovine serum albumin (BSA) to the basolateral compartment is useful to mimic the *in vivo* sink condition and to help minimize the non-specific binding of NCEs to the cells and labware but the effect appeared to vary greatly upon the mechanism by which NCEs are transported across the monolayer (Saha & Kou 2002; Neuhoff et al. 2006).

The “bi-directional” approach is typically utilized to assess the transport mechanism in the Caco-2 model where permeability is measured from “apical” to “basolateral” compartments [absorptive permeability, $P_{app}(A-B)$] and in the reverse direction [secretory permeability, $P_{app}(B-A)$] (Artursson & Tavelin 2003; Lennernas & Lundgren 2004; Ungell 2004; Steffansen et al. 2004; Hochman et al. 2002; Varma et al. 2006). NCEs that function as substrates for efflux transporters are one of the major concerns in discovery as they may significantly limit molecules from absorption into the enterocytes and GI membrane and eventually retard the exposure (Varma et al. 2006). Historically, efflux ratio (ER) has been utilized to identify NCEs with potential efflux issues

$$ER = \frac{P_{app}(B - A)}{P_{app}(A - B)} \quad (1.1)$$

While the classification boundary may vary from lab to lab, NCEs with $ER \gg 1$ are characteristic of potential efflux substrates and those with $ER \approx 1$ are dominated by passive mechanism(s). Once oral absorption of a drug candidate or scaffold is limited by efflux-dependent GI permeability, Caco-2 mechanistic studies may help establish SAR, allowing for dialing out the efflux issue by optimization (Hochman et al. 2002). Experimentally, one can identify the transporters (e.g., Pgp) that NCEs may serve as substrates for and also the potential enhancement in oral absorption when primary transporters are inhibited. In addition, ER and $P_{app}(A-B)$ can be assessed at elevated NCE concentrations to investigate the potential of saturated transporters at high dose and eventually build IVIVC for highly soluble or formulated NCEs (Hochman et al. 2002).

As Pgp is, by far, the most prevailing one among transporters in human intestinal enterocytes, one frequently initiates the first transporter mechanistic study by applying potent Pgp inhibitors such as Cyclosporin (first generation), Verapamil and Valspodar (secondary generation), Zosuquidar/LY335979, and Elacridar/GF120918 (third generation) (Kuppens et al. 2005; Nobili et al. 2006). Troutman and Thakker found that ER is unable to properly characterize the Pgp-inhibition-mediated enhancement of absorptive permeability due to the asymmetric behavior of Pgp substrates in absorptive and secretory transport (Troutman and Thakker 2003a). Instead, absorption quotient (AQ) is recommended to better predict how Pgp-facilitated efflux activity attenuates intestinal permeability *in vivo*.

$$AQ = \frac{P_{Pgp}(A - B)}{P_{PD}(A - B)} = \frac{P_{PD}(A - B) - P_{app}(A - B)}{P_{PD}(A - B)} \quad (1.2)$$

where $P_{Pgp}(A-B)$ is absorptive permeability (or apparent permeability from apical to basolateral direction) attributed to Pgp activity and $P_{PD}(A-B)$ is absorptive permeability measured in the presence of Pgp inhibitor(s).

AQ differentiates the absorptive permeability from secretory transport and offers a more relevant approach to quantify the functional activity of transporters such as Pgp observed during absorptive permeability. For a potent transporter (e.g., Pgp) substrate, inhibition of transporter (or Pgp) activity usually leads to drastically enhanced absorptive permeability [$P_{PD}(A-B) \gg P_{app}(A-B)$] and thereby $AQ \approx 1$. On the other hand, comparable absorptive permeability values are anticipated (in the presence or absence of transporter inhibitor) for a weak transport substrate [$P_{PD}(A-B) \approx P_{app}(A-B)$] or $AQ \approx 0$.

MDRI-MDCK

The Madin-Darby canine kidney (MDCK) model originating from dog kidney with different expression of transporters than human intestine (Balimane et al. 2000; Irvine et al. 1999) has commonly been utilized for permeability evaluation of NCEs using the passive diffusion mechanisms (Ungell & Karlsson 2003). Recent developments using Pgp-transfected multidrug resistance-1 (MDRI)-MDCK model (Bohets et al. 2001) allows for estimating the contributions of efflux transporters with reduced cell culturing cycle time (3–5 days). By regulating the level of Pgp expression, the sensitivity of NCEs to Pgp in the MDCK assay may be amplified, although its relevance to GI physiology has yet to be established.

Given the advantages and limitations of each approach, the latest consensus appears to favor a strategy that combines all three approaches with *in silico* models to ensure high quality assessment of permeability in early discovery (Kerns et al. 2004; Balimane et al. 2006; Faller et al. 2007). PAMPA should serve as a fast and high-throughput permeability ranking tool in particular for scaffolds using passive diffusion mechanisms. The Caco-2 model should be applied to challenging scaffolds involving active transport mechanisms or with higher molecular weight (e.g., >600). The former (potential substrates/inhibitors for efflux transporters), most likely, will exhibit “medium” to “high” permeability in PAMPA but poor *in vitro*–*in vivo* correlation. Caco-2 mechanistic studies are valuable to identify the major transporters such as Pgp, MRP2 and BCRP and to appraise the impact of shutting-down active transporters via either inhibitory (Varma et al. 2003) or saturation mechanisms (Bourdet & Thakker 2006). MDCK, expressed with a specific transporter, may be ideal to tackle the impact of an individual transporter subsequent to Caco-2 transporter assays (Varma et al. 2005).

1.3.2.5 Oxidative Metabolism

Metabolism is an important intrinsic property that drives the clearance or elimination of NCEs. Two of the major sites of metabolism for orally administered compounds are in the gastrointestinal tract (sometimes referred to as pre-systemic metabolism) and the liver (also known as first-pass metabolism). Finding the right balance of metabolic stability is critical during developability assessment of NCEs as high metabolism (or rapid clearance) could result in poor bioavailability and poor efficacy whereas very low metabolism, along with entero-hepatic circulation, could lead to prolonged half-life and accumulation of NCEs in the body resulting in undesirable side-effects. Metabolism is a highly species-dependent phenomenon. Metabolic rates can vary significantly among different species, due to the presence of unique metabolizing enzymes in each species, strain and gender (Martignoni et al. 2006). For example, CYP3A4 is the most important metabolizing enzyme in humans and current literature suggests that more than 50 % of the marketed drugs are metabolized by CYP3A4 enzyme. However, CYP3A4 is not found in any animal species other than humans and monkeys as described in Table 1.3 (Martignoni et al. 2006; Emoto et al 2013). Typically, rodents have a higher metabolic rate than dogs, monkeys, and humans. However, the rate and extent of metabolism and ranking of species can vary significantly from one chemical structure series to another. In particular, for compounds that exhibit significant species differences in their metabolism, it is very difficult to extrapolate the PK parameters in human and obtain any reasonable estimate of human dose. In such instances, it is advisable to conduct micro dosing or exploratory study in human as soon as possible. Screening of metabolic stability in multiple animal species early in drug discovery is very useful to guide structural modification and selection of compounds for in vivo studies. Metabolite identification is also very helpful for bioanalytical and pharmaceutical scientists to understand metabolically labile as well as chemically labile sites in a NCE. Metabolism is a predominant factor that differentiates oral absorption from oral bioavailability. For instance, a compound could have complete absorption (~100 % Fa or fraction absorbed) through gastrointestinal tract upon oral dosing based on its high solubility and permeability. However, the oral bioavailability for same compound could be only ~20–30 % due to its high first-pass metabolism prior to reaching systemic circulation. This is an extremely important consideration while developing formulations for poorly soluble NCEs. Majority of the solubility enhancing formulation technologies can potentially maximize the absorption (or fraction absorbed Fa%) of a given NCE, but not necessarily their oral bioavailability. The collection and use of data relevant to metabolism is more thoroughly covered in Chap. 4.

Table 1.3 Species-dependent CYP3A enzymes (*m* male specific, *f* female specific)

Human	Mouse	Rat	Dog	Monkey
3A4	3A11	3A1/3A23	3A12	3A4
3A5	3A13	3A2m	3A26	
3A7	3A16	3A9f		
3A43	3A25	3A18m		
	3A41	3A62		
	3A44			

1.3.3 Solid-State Characterization

Solid-state characterization assays and technologies (Table 1.4) are well known to most scientists responsible for solid form selection in support of CMC development activities. Thermogravimetric analysis (TGA), Differential Scanning Calorimetry (DSC), Powder and single-crystal X-ray diffraction are commonly used tools to characterize the solid forms of any given NCE using small amounts of material. Advanced calorimetric methods such as modulated DSC (mDSC) provide higher sensitivity and can measure the heat of fusion of crystalline solids as well as glass transition events in amorphous solids. Hot stage and infrared microscopy are useful tools to study solid form conversions (e.g., polymorphs). Dynamic vapor sorption (DVS) is a routinely used instrument to measure hygroscopicity behavior of solid forms and provides useful information for solid form selection activities.

1.3.3.1 Polymorphism

Polymorphism studies require special attention during developability assessment of NCEs because changes in physical form during development could result in significant delays and often, it is very difficult to reproduce the original form with optimal physicochemical and biopharmaceutical performance. The authors' experience suggests that the larger the scale of a crystallization step and longer the processing time, greater is the likelihood that such process will generate the thermodynamically preferred physical form. The thermodynamically preferred polymorphic form can be ascertained from a simple bridging experiment where two polymorphic forms are placed on a microscope slide in contact with a common solvent. In this case, the low-energy form grows at the expense of the high-energy form. Hot-stage microscopy and IR microscopy are useful instruments in monitoring these conversions. In some cases, the low-energy form may be obtained only through the use of hazardous solvents and/or procedures and hence may not be practical for large-scale production. Therefore, a thorough evaluation of polymorphic space and its impact on physicochemical, biopharmaceutical and processability of a given NCE is an important component of developability assessment.

Table 1.4 Solid-state characterization assays and technologies

1	Crystallinity: XRPD, DSC, Tg, TGA, IR, and microscopy
2	Single-crystal X-ray: structure/unit cell
3	Topography: scanning electron microscopy
4	Particle size analysis: by laser light scattering
5	Polymorphism and salt/co-crystal screening: manual, HTS
6	Bulk density, elasticity
7	Rationale for selecting the preferred crystalline form
8	Particle size recommendation for biopharmaceutics performance or manufacturability of a dosage form

1.3.3.2 Amorphous Solids

Amorphous materials have become more prevalent in the development pipeline as a result of the “hydrophobic” nature of NCEs originating from discovery. Such compounds are neither hydrophilic nor lipophilic and present unique challenges in their formulation development, especially, overcoming their poor solubility. Generation and stabilization of amorphous solid forms, using polymeric matrices in solid dispersion for example, provides an attractive formulation approach to improve the dissolution, solubility and oral bioavailability of these hydrophobic NCEs. APIs can also be developed as amorphous forms if supported by detailed understanding of the amorphous system and robust scalable process for manufacturing these forms. This approach becomes inevitable especially when NCEs are difficult to crystallize due to molecular complexity or presence of trace impurities that act as crystallization inhibitors. Amorphous materials, with increased dissolution rate and aqueous solubility are chemically reactive and more hygroscopic than crystalline material (Byrn et al. 1999; Hancock and Parks 2000; Hancock and Zografi 1997). Amorphous materials exist in either the glassy state below their glass-transition temperature (T_g) or as a super-cooled liquid above their T_g . Although the physical properties differ between each amorphous state, Arrhenius relationships are applicable below the T_g and allow for extrapolation to ambient storage/handling conditions. The ratio of T_g , in Kelvin, to the melting point of a crystalline material is a constant of 0.72 (range 0.59–0.84). This is a useful rule of thumb to estimate feasibility and likelihood of developing an amorphous API or formulation for a given NCE. Analysis of amorphous forms of drug candidates should include, in particular, the measurement of T_g (most commonly using mDSC) and any changes in water solubility, hygroscopicity, and solid-state stability relative to the crystalline form. Water solubility may be the most difficult parameter to measure for an amorphous material because of rapid crystallization (Hancock and Parks 2000). In summary, the know-how and experience in development and characterization of amorphous APIs as well as solid dispersion formulations has progressed significantly in last decade and more than a dozen products have been launched in recent past using these technologies. These are discussed in Chap. 3.