Research Article

Incorporation of Physiologically Based Pharmacokinetic Modeling in the Evaluation of Solubility Requirements for the Salt Selection Process: A Case Study Using Phenytoin

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Abstract. In the pharmaceutical industry, salt is commonly used to improve the oral bioavailability of poorly soluble compounds. Currently, there is a limited understanding on the solubility requirement for salts that will translate to improvement in oral exposure. Despite the obvious need, there is very little research reported in this area mainly due to the complexity of such a system. To our knowledge, no report has been published to guide this important process and salt solubility requirement still remains unanswered. Physiologically based pharmacokinetic (PBPK) modeling offers a means to dynamically integrate the complex interplay of the processes determining oral absorption. A sensitivity analysis was performed using a PBPK model describing phenytoin to determine a solubility requirement for phenytoin salts needed to achieve optimal oral bioavailability for a given dose. Based on the analysis, it is predicted that phenytoin salts with solubility greater than 0.3 mg/mL would show no further increases in oral bioavailability. A salt screen was performed using a variety of phenytoin salts. The piperazine and sodium salts showed the lowest and highest aqueous solubility and were tested *in vivo*. Consistent with our analysis, we observed no significant differences in oral bioavailability for these two salts despite an approximate 60 fold difference in solubility. Our study illustrates that higher solubility salts sometimes provide no additional improvements in oral bioavailability and PBPK modeling can be utilized as an important tool to provide guidance to the salt selection and define a salt solubility requirement.

KEY WORDS: bioavailability; oral absorption; pharmacokinetic; physiological model; solubility.

INTRODUCTION

Oral bioavailability is often influenced by factors such as the physicochemical properties, intestinal permeability and metabolism of drugs. Among the physicochemical properties of poorly water-soluble drugs, solubility is considered the most critical factor affecting oral bioavailability. In past decade, poor solubility has been a growing cause of low oral bioavailability in the early development stage (1–10). Therefore, there have been continuous efforts in industry to improve the solubility of drug candidates. Despite these efforts, it is often difficult to incorporate solubility into a drug candidate while retaining adequate potency (1,2,5). Formulation-based approaches that improve both solubility and dissolution rate have been widely used in both preclinical and clinical studies to improve oral exposure. Approaches

such as the use of pro-drugs, inclusion complexation, nanoparticles, co-solvents, micelles/emulsions, salts, co-crystals, and amorphous solids to deliver poorly soluble compounds orally have been widely reported (1–15).

Of the formulation approaches, the use of pharmaceutical salts is the most common method to improve both solubility and dissolution of poorly soluble drug molecules (16–23). Medicinal chemists often identify drug candidates with ionizable groups that are amenable to salt formation (24,25). For ionizable compounds, the intrinsic solubility is defined by the unionized form. The pH solubility of an ionizable compound can be estimated by its pH solubility profile defined by the Henderson–Hasselbalch equation. For example, for a slightly soluble weak acid such as phenytoin with single pK_a , the solubility at a particular pH can be expressed using following version of the Henderson–Hasselbalch equation.

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$$Sol_{(total)} = S_0 \left(1 + 10^{(pH - pK_a)} \right)_{(Acid)} \quad (1)$$

where

Sol Total solubility in aqueous

S_0 Intrinsic solubility in aqueous

pK_a Dissociation constant at logarithmic scale

In the process of salt formation, the above relationship only holds true before the pH of maximum solubility (pH_{max}) is reached. At the pH_{max} , the compound forms a salt and the solubility product of the salt (K_{sp}) determines the solubility (24–26). This is typically referred to for an acid as the region of $\text{pH} > \text{pH}_{\text{max}}$ (the pH where the solution is saturated with respect to both the free and salt forms). The relationship between pH_{max} and K_{sp} is described by the following quadratic equation:

$$\text{pH}_{\text{max}} = \text{p}K_{\text{a}} - \log \frac{S_0}{\sqrt{K_{\text{sp}}}} \quad (2)$$

where:

pH_{max} pH of maximum solubility
 K_{sp} Solubility product of the salt

Based on the relationship shown in Eq. 2, the solubility of a monovalent salt in an aqueous environment is governed by its solubility product and the salt solubility can be estimated as $(K_{\text{sp}})^{1/2}$. This estimation provides a theoretical solubility of a monovalent salt. Calculation of a salt's solubility using the K_{sp} provides a means to verify that experimentally determined salt solubility is that of the salt, and not of a salt/free acid mixture that can arise from *in situ* conversion of the salt back to free acid.

The solubility of a salt is pH independent which can lead to supersaturation, especially at pH values where the free acid or base has poor solubility. Supersaturation enabled using salts effectively results in an improved solubility following *in vivo* oral delivery, and may lead to improvements in oral bioavailability (22,26–28). This phenomenon is especially important for compounds with poor solubility and high permeability characteristics (BCS class II). Typically, in the salt selection process, more soluble salt forms are favored as a means to maximize supersaturation and improve oral exposure (27–29). In fact, salt solubility is commonly suggested as a key parameter for salt selection (27–32), and often salts with lower solubility are de-prioritized without adequate evaluation. Despite the importance of salt solubility, *in vivo* oral absorption is a complex dynamic process resultant from the complex interplay between various factors affecting dissolution and intestinal permeation. As such, the overall oral absorption is ultimately determined by the rate limiting step in the entire process and by the factors that govern the rate limiting step. A negative consequence of improving the solubility through the use of salts is that more rapid precipitation can occur as pH changes from the stomach to the small intestine. Therefore, the highest solubility salt may not always provide the highest *in vivo* oral exposure due to the above issue. The *in vivo* impact of solubility of various salts is somewhat dose dependent and compound dependent. Differences in oral exposure due to salt solubility may be less obvious at lower doses compared to higher doses since complete dissolution in the gastric is more likely. Therefore, for a given compound, the salt solubility required for optimal *in vivo* performance is dependent on various factors such as dose, solubility (supersaturation), and permeability, rather than a single solubility measurement (24–26). Thus, a better understanding of optimal salt solubility and dose relationship

are advantageous early in the salt selection process. Despite the obvious need, there is very little research reported in this area mainly due to the complexity of such a system. To our knowledge, no report has been published to guide this important process and salt solubility requirements still remain to be defined.

Physiologically based pharmacokinetic (PBPK) models provide a means to dynamically integrate the complex interplay of the processes determining oral absorption (33,34). In recent years, there has been an increase in the use of PBPK models to offer a means to identify the rate limiting factors influencing the overall absorption (33–44). The objective of the current study is to illustrate the use of PBPK modeling as a means to evaluate the optimal solubility of salts using phenytoin and its salts as model compounds. Phenytoin is a well-characterized BCS class II drug with low solubility and high permeability (32). Using an oral PBPK model, we establish the relationship between solubility and % drug absorbed. Based on our analysis, a theoretical salt solubility requirement was determined and a solubility cut-off was established. We show that beyond a certain solubility cut-off, any further increases in solubility created by forming different salts has no effect on *in vivo* performance for a given dose. Finally, we demonstrate a pioneering approach of integrating the use of PBPK modeling in the salt selection process.

MATERIALS AND METHODS

HPLC-grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI), reagent grade formic acid was obtained from EM Science (Gibbstown, NJ). Both phenytoin and sodium phenytoin were purchased from Sigma-Aldrich (St. Louis, MO). Other crystalline phenytoin salts were identified from preliminary salt selection work and made in-house. The salts used in this study that were made in-house were ethylenediamine, ethanolamine, piperazine, and piperidine. The water purification system used was a Millipore Milli-Q system.

PXRD and FT-Raman

In order to verify the physical form of samples of phenytoin free acid and its various salts, PXRD and FT-Raman were performed. PXRD patterns were recorded at room temperature with a Rigaku (TX, USA) MiniFlex II Desktop X-ray Powder Diffractometer. Radiation of $\text{Cu K}\alpha$ at 30 KV –15 mA was used with 2θ increment rate of $3^\circ/\text{min}$. The scans run over a range of $2\text{--}40^\circ 2\theta$ with a step size of 0.02° and a step time of 2 s. The powder samples were placed on a flat Silicon Zero Background sample holder.

For the FT-Raman, a Bruker FT-Raman system (MultiRAM, Bruker, Billerica, MA) was used. Sample spectrum (32 scans/spectrum with a resolution of 4 cm^{-1}) was recorded using a 500 mW diode pumped Nd:YAG laser operating at 1,064 nm as the excitation source and 302 mW as the operating laser power. Data was collected from $200\text{--}3,600\text{ cm}^{-1}$. A liquid nitrogen-cooled germanium detector was used to collect backscattered radiation. Data was processed using OPUS® software (version 6.5, Bruker Optics) Bruker (Billerica, MA, USA).

Evaluation of Water Solubility of Phenytoin Salts

The purpose of this study was to determine the solubility of various phenytoin salts and select one salt with a solubility estimate closest to 0.3 mg/mL for *in vivo* evaluation along with the sodium phenytoin salt. Based on PBPK modeling, 0.3 mg/mL was a threshold solubility beyond which we anticipate to observe no further improvements in oral exposure with increases in solubility. The sodium phenytoin salt is known to have high solubility, and therefore we wanted to select a salt as close to the 0.3 mg/mL threshold as possible in order to cover the largest range of solubility. Briefly, an excess amount (approximately 100 mg) of individual phenytoin salts (ethylenediamine, ethanolamine, piperazine, and piperidine) that were made in small scale was added into separate vials with 1 mL of water and equilibrated at room temperature for 30 min. Solubility measurement on the sodium salt was performed on a scale of 500 mg since very high solubility was expected. The resulting mixtures were filtered by a 0.22 μ M filter and supernatant pH was checked by pH meter and HPLC for concentration. Filtrate was dried in a vacuum oven with house vacuum overnight and then analyzed by PXRD and FT-Raman to obtain solid form information.

Determination of pK_a , pH Solubility Profile, K_{sp} , and pH_{max}

The pH solubility profile of phenytoin was determined by measuring the saturated solubility of phenytoin in buffers with pH values ranging from 5 to 11. For this study, excess amounts of the phenytoin free acid was added into separate vials with 1 mL of buffer and equilibrated at room temperature for 48 h. The resulting mixture was filtered by a 0.22 μ M filter and the supernatant pH was checked by pH meter. PXRD and FT-Raman was run on each filtrate. The concentration of the supernatant was determined by HPLC. The Henderson–Hasselbalch equation was used to determine the pK_a of phenytoin and its theoretical pH solubility curve. For the K_{sp} experiment, the piperazine salt was equilibrated with 0.1 N piperazine aqueous solution (titrated to pH 9.2 with HCL) for a period of 24 h on a shaker. The mixture was filtered by a 0.22 μ M filter and supernatant pH was checked by pH meter and HPLC for drug concentration. Filtrate was dried in a vacuum oven with house vacuum overnight and then analyzed by PXRD and FT-Raman to obtain solid form information. The supersaturation ratio was obtained by dividing the solubility calculated using the K_{sp} against the solubility of phenytoin at pH 6.5 (physiologically relevant pH).

Physiologically Based Pharmacokinetic Modeling

The rat (fasted) model in GastroPlus (Simulations Plus, Inc., Lancaster, CA) was used for oral PBPK modeling. Estimates of phenytoin systemic clearance ($CL=1.50$ L/h/kg), inter-compartmental clearance ($CL_d=1.17$ L/h/kg), and volume of distribution of the central ($V_c=1.17$ L/kg) and peripheral ($V_p=1.17$ L/kg) compartments were obtained by fitting a two compartment model to phenytoin plasma concentration–time data from a separate intravenous rat study (14). The following parameters were used as inputs

for the simulations of the oral pharmacokinetics of phenytoin in rat: solubility=45 μ g/mL at 37°C (pH 6.8), effective permeability (P_{eff})=1.86 $\times 10^{-4}$ cm/s (program simulated from Caco2 of 34.3 $\times 10^{-6}$ cm/s (32)), $\log P=2.5$, $pK_a=8.4$. Stomach transit time was adjusted to reflect the well documented influence of oil on stomach transit in rats (transit time ~ 2 h) (45–47). Despite this change in stomach transit time, oil appears to have no impact on the overall exposure of orally administered phenytoin (48). Our assumption was that the small intestine was the main absorption site for phenytoin based on literature (49). In the simulation of oral administration of phenytoin salts, all parameters were kept constant with the exception of solubility which was replaced with the respective salt solubility that was determined experimentally. It is worth mentioning that originally, *in vitro* intrinsic dissolution rate experiment was conducted to compare the sodium and piperazine salts which represented salts with the largest difference in solubility. The dissolution of both salts were found to be similar, being almost instantaneous, which is consistent with literature (55). As we did not anticipate other factors governing dissolution would be different between the various salts tested, as mentioned, we altered only solubility when performing simulations for other salts.

Formulation Preparation

In order to avoid *ex vivo* salt conversion, a non-aqueous vehicle was used for formulation preparation (25,26). Phenytoin free acid, phenytoin Na salt, and phenytoin piperazine salt were suspended in Miglyol 810. The total concentration and supernatant concentration of each formulation was confirmed by HPLC and solid state was checked by PXRD and FT-Raman. Water or 2% low viscosity hydroxypropylmethyl cellulose (HPMC) in water was used as chaser to study the effect of potential *in vivo* precipitation (as free acid) of the various salts. The 2% low viscosity HPMC included in the chaser served as a precipitation inhibitor.

Pharmacokinetic Study in Rats

Male Sprague–Dawley rats, obtained from Charles Rivers Laboratories (Wilmington, MA), were housed in a room with an ambient temperature of 22 $\pm$ 1°C on a 12 h light/dark cycle. Animals were allowed 7 days to acclimate and given *ad libitum* access to standard rat chow (0.5% NaCl) (Baxter Healthcare, Deerfield, IL) and tap water until the initiation of the experiment (14). The current study was conducted in accordance with the institutional guidelines for humane treatment of animals and was approved by the IACUC of Genentech. At the initiation of the study, the rats weighed from 297 to 329 g. Briefly, five groups of three male Sprague–Dawley rats were given a 100 mg/kg (free acid equivalent) oral dose of phenytoin or phenytoin salts followed by an aqueous chaser (with or without precipitation inhibitor (*i.e.*, 2% HPMC)). Blood samples (~ 0.2 mL per sample) were collected from each animal *via* jugular vein cannulae at the following time points: predose, 5, 15, and 30 min post dose, and 1, 2, 4, 8, and 24 h post dose. All samples were collected into tubes containing potassium ethylenediaminetetraacetic acid as an anticoagulant. Blood

samples were centrifuged within 30 min of collection and plasma was harvested. Plasma samples were stored at $\sim 70^{\circ}\text{C}$ until analysis for phenytoin concentrations by a liquid chromatography–tandem mass spectrometric (LC/MS/MS) assay method.

LC/MS/MS Analysis

Phenytoin plasma concentrations were quantified by using LC/MS/MS. The LC/MS/MS system consisted of a Nexera UPLC (Shimadzu, Kyoto, Japan) coupled with a Shimadzu SIL-30AD solvent delivery system, and a Sciex API5500 QTrap equipped with a TurboIonSpray source. The TurboIonSpray source was operated under multiple reaction monitoring (MRM) mode for the quantitation of the compounds. Phenytoin and its internal standard, labetalol, were detected using negative ionization mode. The mass spectrometer was operated at unit mass resolution for both Q1 and Q3 quadrupoles. The chromatography separation was achieved on a Phenomenex Kinetex C18 (50×2.1 mm, $2.7\ \mu\text{m}$) column with gradient elution using mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% formic acid in acetonitrile. The LC flow rate was 0.7 mL/min and the sample injection volume was 10 μL . The column temperature was set at 30°C . The MRM (parent/daughter) transition for phenytoin was 250.9/102.0. The lower limit of quantitation (LLOQ) was 1.02 ng/mL in plasma.

Pharmacokinetic Analysis

All pharmacokinetic parameters were calculated by non-compartmental methods as described in Gibaldi and Perrier (50) using WinNonlin[®] version 5.0 (Pharsight Corporation; Mountain View, CA, USA). Parameters are presented as a mean $\pm$ standard deviation. Oral bioavailability (%F) was determined by dividing the dose normalized AUC following oral dosing by the dose normalized AUC following IV dosing (data not shown) multiplied by 100%. Analysis of variance with a Fisher's least significant difference post-hoc test was performed using IBM SPSS[®] Statistics Software (IBM, Armonk, NY, USA) in order to compare pharmacokinetic parameters of different phenytoin salt treatment groups to the phenytoin free acid treatment group.

RESULTS

Evaluation of Water Solubility of Phenytoin Salts

The objective of this experiment was to select two phenytoin salts covering a large solubility range for *in vivo* testing for scale up. Aqueous solubility of crystalline salts of phenytoin (sodium, ethylenediamine, ethanolamine, piperazine, and piperidine salts) was estimated in aqueous media (Table I). The solubility of the salts tested ranged from 1.2 mg/mL for the piperazine salt to 73.4 mg/mL for the sodium salt. The main component of the residual solid sample recovered from the solubility test was determined by PXRD and FT-Raman. The free acid was found to be the main component of the solid sample for all salts with the exception of the sodium and piperazine salts which were primarily in the salt form (Supplementary Materials Figure 1 and Figure 2).

The distinct Raman shift from $1,775\ \text{cm}^{-1}$ (free acid) and $1,680\ \text{cm}^{-1}$ (salt) was used to confirm the form of the residual solid samples. The described results suggest that aside from the sodium and piperazine salts, all other phenytoin salts experienced conversion back to the free acid form during solubility testing. Furthermore, this suggests that the true solubility of the ethylenediamine, ethanolamine, and piperidine salts could be higher than the measured values since it is unlikely that the samples of these salts were at conditions where solubility is determined by the K_{sp} for the entire duration of the solubility test (25,26). Since we wanted to test salts with the largest range of solubility, the sodium and piperazine salts were selected for further evaluation.

Determination of pK_{a} , pH solubility profile, K_{sp} , and pH_{max}

The pH solubility profile of phenytoin free acid was determined by measuring the saturated solubility of phenytoin in buffers with pH values ranging from 4 to 11. The intrinsic solubility of phenytoin in pH 6.5 buffer was found to be approximately 25 $\mu\text{g/mL}$ at 25°C , and approximately 45 $\mu\text{g/mL}$ at 37°C . An approximate pK_{a} of 8.4 was estimated from fitting Henderson–Hasselbalch equation to the pH solubility curve of phenytoin (Fig. 1). Since the solid sample of the piperazine salt was found to remain in the salt form, we verified the measured *in situ* aqueous solubility using the K_{sp} method to calculate the piperazine salt solubility. The K_{sp} of piperazine salt was estimated by the counter-ion method to be around $3.17 \times 10^{-5}\ \text{M}^2$. pH_{max} was determined to be approximately 9.7 based on the quadratic equation (Eq. 2). Using the K_{sp} method, the solubility of piperazine salt was estimated to be 1.4 mg/mL which is consistent with the measured value of 1.2 mg/mL (Table I).

Physiologically Based Pharmacokinetic Modeling

PBPK modeling was performed to predict the phenytoin free acid concentration–time profile in rats. Figure 2 is a concentration–time plot of observed and predicted concentration–time profiles of phenytoin free acid administered orally at 100 mg/kg. As shown in Fig. 2, there is good agreement between observed and PBPK model predicted phenytoin concentrations. This “base” case for the free acid was used in subsequent sensitivity analyses examining the effect of changes in solubility and permeability on the percent fraction of the dose absorbed (F_{a}). Figure 3 is a plot of changes in solubility and its effect on % F_{a} . The effect of solubility was assessed from 0.005 to 100 mg/mL. As shown in Fig. 3, increases in solubility are predicted to improve fraction absorbed up to approximately 0.3 mg/mL. Beyond this solubility, no additional improvement was anticipated as the F_{a} is approximately 100%. A second sensitivity analysis was performed to evaluate the influence of alterations of permeability (P_{eff}) on F_{a} . Permeability was varied from 0.6 to $5.5 \times 10^{-4}\ \text{cm/s}$. This upper and lower limit of this range represents an approximate threefold decrease and threefold increase of the permeability estimate we used in our base simulations. The result of the sensitivity analysis is shown in Fig. 4. Overall, a change in permeability is not anticipated to impact F_{a} greatly over the range tested.

Fig. 1. A pH solubility curve of phenytoin and calculated solubility of the piperazine salt (K_{sp}) and sodium salt (assuming pH_{max} reached)

Pharmacokinetic Study in Rats

The objective of the pharmacokinetic study in rats was to confirm the results of our sensitivity analysis. The concentration–time profiles following varying dosing of phenytoin free acid and the sodium and piperazine salts given at orally at 100 mg/kg (free base equivalent) is presented in Fig. 5. Associated pharmacokinetic parameters are presented in Table II. Rats given either the sodium or piperazine salts showed significantly higher phenytoin exposures (AUC and C_{max}) in plasma ($p < 0.05$) compared to the free base. No differences were

found when comparing C_{max} and AUC between salt groups with or without precipitation inhibitor.

DISCUSSION

Salts of ionizable compounds with dissolution/solubility limited absorption offer a means to improve the oral exposure of the compound. They act to improve oral exposure *via* several mechanisms. First, salts provide a means to increase the solubility of poorly soluble compounds. In addition, salts induce pH changes and act as their own buffer facilitating dissolution and oral absorption by retarding precipitation (25,26). Due to the familiarity of using salts and the obvious benefit, they are commonly tested as the first option for improving poor bioavailability in pharmaceutical industry. The primary goal in the salt selection process is to improve solubility. However, salt selection based on solubility alone with little or no regard to its *in vivo* properties and dose is not ideal (21,25,26). Salts with better solubility may precipitate more rapidly as pH in the gastrointestinal tract changes (25,26). Therefore, maintenance of a supersaturation state and permeation through the intestinal wall are, in general, two key events for improving overall oral bioavailability. Finally, solubility requirements for a salt will vary from compound to compound and is dependent on the size of the intended dose. Based on the factors discussed, a higher solubility salts may not provide appreciable gains in oral bioavailability. This has been illustrated in several studies that have reported no significant differences in oral bioavailability despite large differences in the solubility of different salt forms (52–54).

The exact mechanism by which salt formation increases *in vivo* oral bioavailability is poorly understood. However, maintenance of *in vivo* supersaturation and the avoidance of precipitation from this supersaturation state are of high importance and can be viewed from the perspective of

Fig. 2. Plot of observed vs. predicted concentration–time profile of phenytoin free acid dosed at 100 mg/kg in rats ($n=3$). Simulations were performed using an oral rat PBPK model (See supplemental Fig. 3 for more information on simulation)

Table I. Phenytoin Salt Solubility Evaluation

Salt	Solubility from water (mg/mL)	Main component of salt sample recovered from solubility testing determined by PXRD and FT-Raman	Supersaturation ratio (S_x)
Ethylenediamine	9.5	Free acid	475
Ethanolamine	10.3	Free acid	515
Sodium	73.4	Salt (major component)+Free acid	3670
Piperazine	1.2	Salt	60
Piperidine	8.5	Free acid	425

changes in Gibbs free energy. Changes in Gibbs free energy from a supersaturated solution to equilibrium can be expressed as the following equation:

$$\Delta G = -R_g T \ln S_x; \quad (3)$$

where R_g is the gas constant, T is the absolute temperature, and S_x is the supersaturation ratio which is concentration dependent (25,26). Increasing S_x leads to increases in negative free energy ΔG which results in a greater potential for precipitation. Therefore, the highest solubility salt may not necessarily provide the highest *in vivo* oral exposure due to an increased rate of precipitation. The *in vivo* impact of solubility of various salts is somewhat dose dependent. Differences in oral exposure due to salt solubility may be less obvious at lower doses compared to higher doses since complete dissolution in the gastric is more likely. Thus, for a given compound, the salt solubility required for optimal *in vivo* performance is dependent on various factors such as dose, solubility (supersaturation), and permeability, rather than a single solubility measurement (24–26).

Recently, physiologically based pharmacokinetic modeling has been used as a tool in pharmaceutical industry to understand the complex interactions between the various processes involved in oral absorption (36–45). PBPK models offer a means to assess how changes in various compound properties will affect overall absorption. A parameter sensitivity analysis using PBPK models can be used to identify rate limiting steps in

the absorption process and provide useful guidance in formulation strategies. An improvement in solubility is an established strategy for improving oral bioavailability. However, the magnitude of solubility increase required for an optimal increase in oral bioavailability is less certain. Furthermore, desired dose should be considered. A need to increase our understanding of solubility requirements and dose for salt selection has been discussed in the literature (51–56).

Our current study illustrates how information from classical *in vitro* and *in vivo* experimental investigations can be coupled with PBPK modeling in order to determine “solubility needs” for salt in a much more efficient manner. Sensitivity analyses are performed as a means to see how variations in a particular parameter can impact an outcome of interest. The sensitivity analysis described in this manuscript using a PBPK model serves as a means to provide *in vivo* context to the impact of solubility improvements on the fraction of the dose absorbed, and helps to define threshold solubility requirements for salt selection. According to the analysis, solubility improvements beyond ~0.3 mg/mL are not anticipated to improve the oral bioavailability of a 100 mg/kg (free acid equivalent) dose of phenytoin. In order to test the prediction from our sensitivity analysis, we performed a salt screen with phenytoin salts. Ethylenediamine, ethanolamine, piperazine, piperidine and sodium salts of phenytoin were evaluated. The highest and lowest solubility salts were the sodium (73.4 mg/mL) and the piperazine (1.2 mg/mL) salts,

Fig. 3. A parameter sensitive analysis showing the relationship between solubility and the percent of a 100 mg/kg phenytoin dose that is absorbed

Fig. 4. A parameter sensitive analysis showing the relationship between P_{eff} and the percent of a 100 mg/kg phenytoin dose that is absorbed

Fig. 5. Concentration–time profiles of phenytoin administered as the free acid (*open diamond*), sodium salt without precipitation inhibitor (*open square*), sodium salt with precipitation inhibitor (*filled square*), piperazine salt without precipitation inhibitor (*open upright triangle*), and piperazine salt with precipitation inhibitor (*filled upright triangle*)

respectively. The aqueous solubility of phenytoin Na salt is 73.4 mg/mL, thus the complete dose (100 mg/kg) is likely completely dissolved in the animal stomach when 1 mL of chaser was given. In contrast, the majority of the piperazine salt should be in solid form in the stomach at the same dose (with chaser) since the basal volume of gastric fluid in fasted animals is very low (26). Despite this difference, *in vivo*, both the sodium salt and piperazine salt provided improvements on oral bioavailability compared to the free acid. These improvements were consistent with the increase in solubility provided by both salts (Table I) and were predicted based on simulations performed with the PBPK model (Fig. 1). Phenytoin sodium salt has been reported to undergo *in vivo* conversion from a salt to the free acid (54,55). Therefore, the sodium and piperazine salts were tested in the rat with and without concurrent administration of precipitation inhibitor (2% HPMC) in the chaser in order to evaluate the effect of salt precipitation *in vivo*. Based on comparable exposures

with and without precipitation inhibitor, we concluded that *in vivo* precipitation of the salt was not a factor for our study with phenytoin (Table II and Fig. 5). Of significance, there appeared to be no difference in the *in vivo* performance of the sodium and piperazine salt despite an approximately 60-fold difference in solubility. The parameter sensitivity analysis presented in Fig. 3, suggests that there would be no further improvements in oral phenytoin exposure at salt solubility of >0.3 mg/mL. As both our measured (1.2 mg/mL) and calculated (1.4 mg/mL) estimates of solubility for the piperazine phenytoin salt were greater than 0.3 mg/mL, the *in vivo* results were in line with our sensitivity analysis performed using the PBPK model. A similar exercise using an equation published by other researcher (58) found a very similar solubility cut-off for complete absorption of phenytoin in rats. An absorbable dose (AD)/solubility/permeability plot and relative absorbable dose of different phenytoin forms

Table II. *In Vivo* Pharmacokinetics of Phenytoin Following Administration of a 100 mg/kg Oral Dose to Rats in the Form of the Free Acid or Salts ($n=3$ per Dose Group)

Group	Form dosed	Precipitation inhibitor	AUC ($\mu\text{M}\times\text{h}$)	C_{max} (μM)	%F
1	Free acid	No	155 $\pm$ 61	18 $\pm$ 1	34 $\pm$ 8
2	Na salt	No	444 $\pm$ 96*	36 $\pm$ 6*	97 $\pm$ 22*
4	Piperazine salt	No	493 $\pm$ 159*	36 $\pm$ 12*	107 $\pm$ 40*
3	Na salt	Yes	405 $\pm$ 168*	35 $\pm$ 15*	88 $\pm$ 36*
5	Piperazine salt	Yes	405 $\pm$ 74*	30 $\pm$ 4*	88 $\pm$ 19*

AUC area under the concentration–time profile, C_{max} maximum observed concentration

* $p<0.05$; significantly different than group 1 using ANOVA followed by the least significant difference (LSD) post-hoc test

Absorbable dose (AD)/Solubility/Permeability

Fig. 6. Absorbable dose (AD)/solubility/permeability plot and relative AD to different forms of phenytoin (phenytoin free acid (white circle), phenytoin piperazine salt (black circle), phenytoin Na (gray circle))

is presented as Fig. 6 by using the following equation and parameters for rats (58).

$$AD = S \times Pe \times (A/V) \times SIV \times SITT$$

Where

AD	Absorbable dose (in milligram)
S	Solubility (in milligram per milliliter)
Pe	Caco2 permeability
A/V	10 cm ⁻¹ (rat)
SIV	20 mL and
SITT	270 min

The plot shown in Fig. 6, clearly shows that the piperazine salt has sufficient solubility to deliver a 100 mg/kg dose (AD of 25 mg for a 0.25 kg rat).

One thing worth noticing is that despite the fact that the F_a finding for the phenytoin salts is in line with PBPK simulation and our hypothesis, the overall PK curve prediction for the salts is less than optimum. A simulation made by using the same model and piperazine salt solubility is presented as Fig. 7. The modeling suggested that for phenytoin with solubility of piperazine salt (1.2–1.4 mg/mL), complete absorption is expected ($F_a=1$). Although the F_a prediction seems to be correct, the PK curve fit was less than ideal. The C_{max} was off by two-fold and T_{max} were off by almost 5 h (3–4 h for prediction vs. 8 h *in vivo*) while the total F_a is in line with prediction. For example, the C_{max} and T_{max} value were predicted to be 22 μ g/mL and 4 h for the piperazine salt and the *in vivo* values are 9 μ g/mL and 8 h respectively (Fig. 7). We hypothesize this phenomenon maybe attributed to both nonlinear pharmacokinetic (57) and potential free acid precipitation during the transit process (54,55). The potential of *in vivo* precipitation is not a surprise to us since the overall dynamic of the *in vivo* situation is very difficult to mimic, hence, the precipitation time or the degree of precipitation (if any) is very hard to predict and attributed to the deviation. Caution should be made when implementing this approach

PBPK Simulation of The Phenytoin Piperazine Salt and In Vivo Exposure

Fig. 7. PBPK simulation of the phenytoin piperazine salt and *in vivo* exposure (See supplemental Fig. 4 for more information for simulation)

and usage of the results. Similar deviations were observed for all group dosed with salts. Hence, we conclude that no matter what caused the deviation from PBPK to *in vivo*, the degree of impact is the same for both salts. Furthermore, the observed change in T_{max} caused by the different salt formulations is 1 time point difference (4 to 8 h) and this may be attributed by the sampling times included in the PK study. Since the primary focus of our manuscript is to assess changes in overall exposure due to the use of different salts therefore, despite the model not being able to detect some changes in T_{max} , we felt that the model was adequate for our purposes. However, the focus of PBPK modeling should be carefully evaluated before conclusions can be made. In our case, the prediction of the most important factor (F_a) is correct for all cases, therefore, its use is considered appropriate.

Based on our current study, we propose that solubility requirement for salt screening should be carefully evaluated. The current practice in pharmaceutical industry considers solubility to be of prime importance during the salt selection process. Decisions on salt selection are made based on solubility information in isolation, and therefore salts with lower aqueous solubility are typically de-prioritized without further testing. The salts utilized in our current study are approximately 60-fold different in solubility. Despite the large difference in solubility our current study illustrates that a lower solubility salt, which would typically be de-prioritized (*i.e.* phenytoin piperazine salt; 1.2 mg/mL), can perform equally well *in vivo* compared to a much higher solubility salt (*i.e.*, sodium salt; 73.4 mg/mL). In fact, ruling out salts with lower solubility too early will impact the development options of a drug candidate. In particular, careful evaluation can be important for compounds where multiple salts are available and salts with higher solubility may not have the best solid state properties for development. A solubility requirement for salt “go or no go” decision should be established by combining PBPK modeling followed by assessment of *in vivo* performance. PBPK modeling would serve to context the solubility improvement of each salt form in relation to other properties of the drug. Such an evaluation

would incorporate elements such as anticipated dose and a more defined solubility requirement. Again, as illustrated in our case study, higher solubility salts may sometimes not provide any improvements in oral bioavailability in the *in vivo* environment. Proper comparisons beyond just *in vitro* solubility measurements must be made during the salt selection process. In some instances, a lower solubility salt form may have equivalent *in vivo* performance compared to a higher solubility salt but may have superior solid state properties. Our example with phenytoin and its salts illustrates the importance of utilizing PBPK models to enable comprehensive evaluations of salts and provide a more rational means of salt selection.

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