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Article

Effects of experimental conditions on solubility measurements for BCS classification in order to improve the biowaiver guidelines

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Among the methods described for determining the solubility, shake-flask is suitable to evaluate the equilibrium solubility according to the BCS. Nevertheless, experimental conditions related to the shake-flask method are not well described. Evaluating the effects of experimental conditions on solubility measurements by shake-flask method is important and contributes in biowaiver decision. For this work, propranolol hydrochloride and nimesulide were used as model compound of high and low solubility, respectively. Equilibrium solubility was evaluated at 37 ºC, 100 rpm during 48 hours in buffer media. Effects of the rotation speed, temperature, substance in excess and aliquot withdrawn were evaluated. Small variations of temperature caused significant differences in the solubility and then this parameter must be controlled. Excess of raw material influenced the results of the nimesulide, then, little excess is recommended. Rotation speed did not cause differences in the equilibrium solubilities, but at 150 rpm the equilibrium was reached faster. Aliquot did not present significant differences, but excessive withdrawn should be avoided. Therefore, the evaluation of equilibrium solubility using shake-flask method must be performed in physiological pH conditions, 37 ± 1 ºC, substance in excess 10% above saturation, 50, 100 or 150 rpm and aliquot withdrawn not more than 10% of the media volume.

Keywords: Solubility. Shake-flask. Effects. Propranolol hydrochloride. Nimesulide.

INTRODUCTION

Solubility is a physical constant and refers to the ability of one substance to dissolve in a specific solvent. In quantitative terms, it is the concentration of solute in a saturated solution at a certain temperature and in qualitative terms, it may be defined as the spontaneous interaction of two or more substances to form a homogeneous molecular dispersion. The extent of substance solubility in a specific solvent is measured as the saturation concentration, when the addition of more solute does not change the concentration (Savjani, Gajjar, Savjani, 2012).

Aqueous solubility (S_w) is dependent of the molecular characteristics of drugs and is also a function of their ability to form hydrogen bonds with the water molecules. In general, aqueous solubility of substances is directly proportional to the number of hydrogen bonds that can be formed with water (Martinez, Amidon, 2002).

Besides the aqueous solubility, it is possible to evaluate the intrinsic solubility of drugs (S_0), i.e., solubility of the uncharged species and the solubility of the ionized species (S_i), a conditional constant, depending on the concentration of ions in solution (Avdeef, 2003).

Drug solubility is one of the parameters used to determine the drug class according to the Biopharmaceutics Classification System (BCS). This

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system is based on drug solubility, its rate of dissolution from the dosage form, and gastrointestinal permeability. These are the fundamental parameters that control the rate and extent of drug absorption. According to these parameters, drugs are grouped into four classes: class I drugs exhibit high solubility and high permeability; class II exhibit low solubility and high permeability; class III, high solubility and low permeability and class IV, low solubility and low permeability (Amidon *et al*., 1995).

The main purpose of this system is to provide regulatory subsidies for the biowaivers, which consist of the substitution of *in vivo* studies, as bioequivalence, by *in vitro* assays that allow conclusions about drugs solubility and permeability. Besides that, the risk associated with a biowaiver should be low. In general, biowaiver is recommended for highly soluble and highly permeable drugs (i.e., class I) and drugs with high solubility and low permeability (i.e., class III) in immediate release solid oral dosage forms that exhibit rapid or very rapid *in vitro* dissolution (EMA, 2010; FDA, 2017).

Among the methods described for determining the solubility it is possible to obtain apparent solubility by potentiometric titration and turbidimetry, but shake-flask method is considered suitable to evaluate the equilibrium solubility (S_{pH}) (Lindenberg, Kopp, Dressman, 2004; EMA, 2010; Brasil, 2011; FDA, 2017).

Potentiometric method is able to create a pH/solubility profile with one single determination, but it is limited to ionizable compounds. Turbidimetry, in turn, includes the use of DMSO, which can increase the solubility to an unknown and unpredictable extent. Besides that, the precipitate may not be the most stable form, which is a problem because polymorphs can differ in their solubilities (Glomme, März, Dressman, 2005).

Shake-flask method can be used for all compounds and a wide variety of media. It is precise and reproducible when compared to potentiometric titration and turbidimetry. It has been widely recommended because it enables to obtain the dose/solubility ratio (D/S), which is the ratio between highest dose of the drug, marketed or administered at one time, and active substance solubility. According to the BCS, the drug is considered highly soluble when it has a D/S ratio less than or equal to 250 mL, in a pH range of 1.2 to 6.8 at 37 ± 1 ºC (Lindenberg, Kopp, Dressman, 2004; Glomme, März, Dressman, 2005; EMA, 2010; Brasil, 2011; FDA, 2017).

However, despite the recommendation, there is no standardization of experimental conditions related to shake-flask method for BCS solubility classification. Thus, the purpose of this work was to evaluate the effects of the rotation speed, temperature, substance in excess, and aliquot withdrawn on solubility measurements for BCS classification in order to improve the biowaiver guidelines. The standardization of these experimental conditions in shake-flask method can contribute to obtain reliable results for drugs solubility and consequently will allow a significant reduction of the inherent risks about biowaiver decision.

With the purpose of evaluating the effects of experimental conditions on solubility measurements by shake-flask method, propranolol hydrochloride and nimesulide were defined as model compounds of high solubility and low solubility, respectively. Propranolol hydrochloride is an antihypertensive drug of the β-blocker class classified as BCS class I (Vogelpoel *et al*., 2004), while nimesulide is an anti-inflammatory drug, COX-2 selective inhibitor, classified as class II according to the BCS (Dellis, Giaginis, Tsantili-Kakoulidou, 2007).

MATERIAL AND METHODS

Material

Nimesulide and propranolol hydrochloride, SQR, were obtained from the Brazilian Pharmacopoeia/Fiocruz/INCQS (Rio de Janeiro, Brazil).

Nimesulide, raw material, was purchased from DEG (São Paulo, Brazil), lot 01.9295, content of 100.5%. Propranolol hydrochloride, raw material, was purchased from All Chemistry (São Paulo, Brazil), lot M111011, content of 100.24%.

Hydrochloric acid and sodium chloride were purchased from Proquimios (Rio de Janeiro, Brazil) and Neon (São Paulo, Brazil), respectively. Glacial acetic acid, sodium acetate, monobasic potassium phosphate, sodium hydroxide, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was obtained from a Millipore purification system (Darmstadt, Germany). All other chemicals were of analytical grade or higher.

Buffer media

Simulated Gastric fluid without enzymes (pH 1.2): 2.0 g of sodium chloride, 7.0 mL of hydrochloric acid and enough water to complete 1000.0 mL. Acetate buffer (pH 4.5): 2.99 g of sodium acetate trihydrate, 14.0

mL of acetic acid 2.0 N and enough water to complete 1000.0 mL. Simulated Intestinal fluid without enzymes (pH 6.8): 250.0 mL of monobasic potassium phosphate 0.2 M, 112.0 mL of sodium hydroxide 0.2 M and enough water to complete 1000.0 mL.

pH values were adjusted using HCl 0.1 M and NaOH 0.1 M solutions. All buffer solutions were prepared according to the United States Pharmacopeia 37th edition (United States Pharmacopeia, 2014).

The buffer compositions described are in accordance with those recommended in FDA (2017); EMA (2010); ANVISA (Brasil, 2011) and WHO (2015) guidelines about biowaiver studies.

Evaluation of the equilibrium solubility of propranolol hydrochloride using the shake-flask method

Equilibrium solubility was evaluated by adding propranolol hydrochloride in each buffer media until a saturated solution was obtained, characterized by the presence of substance excess in flasks. 300 mg of propranolol hydrochloride were added in 10.0 mL of simulated gastric fluid without enzymes (pH 1.2), acetate buffer (pH 4.5) and simulated intestinal fluid without enzymes (pH 6.8). The flasks were placed in an incubator with orbital agitation platform (IKA® KS 4000i control, Staufen, Germany) using 100 rpm as rotation speed for 48 hours at 37 ± 1 ºC. Aliquots of 0.3 mL were colleted at predetermined times (0, 1, 2, 4, 5, 10, 15, 22, 28, 33, 43, 48 hours), and replacement was made with the same media volume, in the same conditions. Each sample was filtered through 0.45 µm filtering units (Millex-HV Filter, 0.45 µm, Darmstadt, Germany), diluted with the respective buffer media and immediately quantified by chromatographic method. To confirm the equilibrium between the phases, the solubility must be constant in determinations carried out in consecutive times. The pH of all media was measured at the beginning and at the end of the experiment.

Evaluation of the equilibrium solubility of nimesulide using the shake-flask method

Equilibrium solubility was evaluated by adding nimesulide in each buffer media until a saturated solution was obtained, characterized by the presence of substance excess in flasks. 3 mg of nimesulide were added in 50.0 mL of simulated gastric fluid without enzymes (pH 1.2), acetate buffer (pH 4.5) and simulated intestinal fluid without enzymes (pH 6.8). The flasks were placed in an incubator with orbital agitation platform (IKA® KS 4000i control, Staufen, Germany) using 100 rpm as rotation speed for 48 hours at 37 ± 1 ºC. Aliquots of 5 mL were colleted at predetermined times (0, 1, 3, 5, 7, 9, 10, 24, 26, 28, 30, 33, 48 hours), and replacement was made with the same media volume in the same conditions. Each sample was filtered through 0.45 µm filtering units (Millex-HV Filter, 0.45 µm, Darmstadt, Germany) and immediately quantified by chromatographic method. To confirm the equilibrium between the phases, the solubility must be constant in determinations carried out in consecutive times. The pH of all media was measured at the beginning and at the end of the experiment.

Analysis of the parameters that can affect the equilibrium solubility of the model compounds

After evaluating the drugs solubility using the parameters shown above, the experimental conditions that could influence the equilibrium solubility of nimesulide and propranolol hydrochloride were analyzed. In order to do this, the critical steps of the shake-flask method were evaluated by varying one experimental parameter at a time.

The conditions evaluated were selected because they are not specified in the biowaiver guidelines and there are reports in the literature citing that these conditions could influence the results of drug solubility (Baka, Comer, Takacs-Novak, 2008).

Table I shows the parameters evaluated concerning their influence on the process of determining the equilibrium solubility by the shake-flask method.

Chromatographic conditions

Quantification methods of nimesulide and propranolol hydrochloride were developed and validated using HPLC system (Waters Alliance® e2695, Milford, MA, USA) coupled with ultraviolet (Waters 2489, Milford, MA, USA) and fluorescence (Waters 2475, Milford, MA, USA) detectors, respectively, and the chromatographic conditions described in Table II.

The quantification method of nimesulide in equilibrium solubility studies was linear in the range of 0.5-10.0 µg/mL for simulated gastric fluid without enzymes (pH 1.2), 1.0-25.0 µg/mL for acetate buffer

TABLE I – Experimental conditions used for evaluation of parameters that can affect the equilibrium solubility of the drugs in buffer media. Each parameter was varied individually

Parameter	Negative variation	Nominal value	Positive variation
Rotation speed (rpm)	50	100	150
Temperature (ºC)	34 ± 1	37 ± 1	40 ± 1
Propranolol hydrochloride			
Substance added in excess (mg)	-	300	400
Aliquot (mL)	0.1	0.3	0.5
Nimesulide			
Substance added in excess (mg)	-	3	20
Aliquot (mL)	2.0	5.0	7.0

TABLE II – Chromatographic conditions used for quantification of nimesulide and propranolol hydrochloride in equilibrium solubility studies

Chromatographic conditions	Nimesulide	Propranolol hydrochloride
Detector	Ultraviolet	Fluorescence
Wavelength (nm)	300	Excitation: 290 Emission: 358
Mobile phase	Water, acetonitrile and acetic acid (45:55:1)	Acetonitrile and phosphate buffer 40 mM (28:72) pH 3.5
Column	C18 (150 x 4.6 mm; 3.5 µm) Waters SunFire®	C18 (50 x 4.6 mm; 5 µm) Varian
Temperature (ºC)		
Injection	25	25
volume (µL)	20	8
Flow (mL/minute)	1.2	1.0

(pH 4.5), and 1.0-40.0 µg/mL for simulated intestinal fluid without enzymes (pH 6.8), with respective limits of quantification equal to 0.32, 0.70 and 0.15 µg/mL

Regarding propranolol hydrochloride, the quantification method was linear in the range of 40.0-160.0 µg/mL for simulated gastric fluid without enzymes (pH 1.2), acetate buffer (pH 4.5) and simulated intestinal fluid without enzymes (pH 6.8), with respective limits of quantification equal to 0.99, 0.76 and 0.93 µg/mL.

Precision and accuracy were satisfactory with relative standard deviation less than 5% and recovery between 98-102%. In addition, the analytical methods were selective and indicative of stability.

Propranolol hydrochloride and nimesulide solutions, in all buffer media, showed stability for 50 hours at 37 ºC, with absence of degradation products and decrease of the drug concentration by less than 5%.

Statistical analysis

Statistical comparison of shake-flask method parameters was performed using t-Student test ($p < 0.05$ was considered as statistically significant).

RESULTS AND DISCUSSION

Evaluation of the equilibrium solubility of propranolol hydrochloride and nimesulide using the shake-flask method

Propranolol hydrochloride presented high solubility in all buffer media, however a higher solubility was observed in acid pH (pH 1.2). Nimesulide, on the other hand, presented low solubility in the three buffer media, with a greater solubility in simulated intestinal fluid without enzymes (pH 6.8). Data regarding the equilibrium solubility of the drugs and the respective times to achieve equilibrium are shown in Table III.

The equilibrium between the drug solution and nimesulide excess was reached with a longer time, 10 hours, for pH 6.8, and 7 hours for pH 1.2 and pH 4.5. For all media, these times are considered reduced and allow several analysis of drug solubility in a short time.

Nimesulide is a weak acid having a pK_a of 6.4 (Dellis, Giaginis, Tsantili-Kakoulidou, 2007). Therefore, in media with high or neutral pH, the solubility of nimesulide is higher than in acid media (equilibrium solubility of nimesulide at pH 6.8 was approximately 9-fold higher than at pH 1.2). This is due to the presence

TABLE III – Parameters obtained from the evaluation of the equilibrium solubility of propranolol hydrochloride and nimesulide. The values shown indicate the mean of three replicates

Drug (dose)	pH	Solubility (mg/mL)	Time (hours)	LogS[a]	D/S[b] (mL)
Propranolol hydrochloride (80 mg)	1.2	210.45	7	-0.14	0.38
	4.5	161.23	7	-0.26	0.49
	6.8	92.98	10	-0.50	0.86
Nimesulide (100 mg)	1.2	0.0034	5	-4.95	29411.17
	4.5	0.0064	5	-4.68	15625.00
	6.8	0.0301	5	-4.01	3322.25

[a]Logarithm of substance concentration in mol/L
[b]D/S: dose/solubility ratio

of the weakly acid methanesulfonamide group in drug structure (Dellis, Giaginis, Tsantili-Kakoulidou, 2007). In basic media, the methanesulfonamide is deprotonated, and there are intermolecular ion-dipole interactions and hydrogen bonds between the ionized drug and the water keeping the drug solubilized in alkaline aqueous media (Gonsalves *et al*., 2013).

Propranolol hydrochloride, in turn, is a weak base having a pK_a of 9.05 (Vogelpoel *et al*., 2004) and according to the Henderson-Hasselbach equation at acid pH (< 2.5) almost 100% of the drug will be in the ionized form. In addition, the amine present in the drug structure will be protonated, forming the amine salt. The salt formed will allow interaction of the substance with water (ion-dipole), besides the hydrogen bonds already existing, which explains the higher drug solubility at acid pH (Gonsalves *et al*., 2013; Avdeef, Berger, Brownell, 2000; Wang, Zou, Wan, 2013)

Equilibrium between the drug solution and propranolol hydrochloride excess was reached in 5 hours in the three analyzed media. The times mentioned are considered reduced and, as for nimesulide, make it possible to carry out several analyses of drug solubility in a short time.

D/S values of propranolol hydrochloride were lower than 250 mL, indicating the high solubility of the drug in the media and, in contrast, the D/S results of nimesulide were higher than 250 mL, demonstrating its low solubility in the buffer media.

After drug addition, no changes were observed in the initial pH of the media, and therefore it was not necessary to adjust the pH after addition of the drug. At the end of the experiment no differences were observed between the initial and final pH of the solution containing the drug.

It is known that there must not exist variation greater than 0.1 unit between the pH measured at the beginning and at the end of the test (Brasil, 2017). Some drugs, such as rosuvastatin and promethazine hydrochloride, change the pH of the media due to the capacity of the buffer media and the ionization characteristics, amount and solubility of the drug (Avdeef *et al*., 2016). However, this phenomenon has not been observed for propranolol hydrochloride nor nimesulide.

During the evaluation of the equilibrium solubility of nimesulide and propranolol hydrochloride, each collected sample was filtered through hydrophilic PVDF filters with 0.45 μm pore size. About 20% of the total volume collected was discarded in order to saturate the filter. Remaining sample was filtered directly into vials and taken for quantification by a previously validated chromatographic method.

There are several works reported in the literature that use filtration process for phase separation. Face to an extensive literature search, Avdeef (2015) concluded that filtration can be recommended as a phase separation process in equilibrium solubility studies by shake-flask method, using hydrophilic PVDF (polyvinylidene fluoride) and PES (polyether sulfone) filters with 0.22 or 0.45 μm pore size. In addition, it is useful to discard the first 10-25% filtered solution to allow filters and surfaces to be saturated with adsorbed compound (Avdeef, 2015).

As the work presents a proposal to evaluate the effects of experimental conditions on equilibrium solubility measurements by shake-flask method, the filtration process was used throughout all the experiments, presenting high reproducibility, being therefore the selected process for the phase separation.

Analysis of the parameters that can affect the equilibrium solubility of the model compounds

Shake-flask method has been widely used and recommended to evaluate drugs solubility. However, precise values of equilibrium solubility are difficult to obtain because the results are affected by many experimental factors such as temperature and rotation speed (Volgyi *et al*., 2011).

Therefore, the parameters rotation speed, temperature, substance added in excess and aliquot collected were evaluated for their influence in the determination of the equilibrium solubility by the shake-flask method. The results showing the value of the equilibrium solubility, the time to reach equilibrium and the p-value indicating the presence of statistically significant differences are listed in Table IV.

Influence of rotation speed

Influence of rotation speed was evaluated at three levels, considering the experimental solubility (50, 100 and 150 rpm). The speeds selected were the most used in studies to evaluate substances' solubility using shake-flask method (Baka, Comer, Takacs-Novak, 2008; Glomme, März, Dressman, 2005; Heikkila *et al*., 2011; Takács-Novák *et al*., 2013). However, there is no consensus about what speed to use, nor studies evaluating this parameter for the shake-flask method.

For the evaluation of the nimesulide solubility by varying the rotation speed, statistically significant differences were not obtained between the means of the equilibrium solubilities ($p > 0.05$). However, as it can be observed in Table IV, the reach time of the equilibrium solubility at 150 rpm was 5 hours, while in the experimental solubility, at 100 rpm, the equilibrium was reached in 7 hours for pH 1.2 and pH 4.5. At pH 6.8 the equilibrium was reached in 7 hours and the experimental solubility, at 100 rpm, was reached in 10 hours.

Thus, as obtained to nimesulide, the experiments performed with propranolol hydrochloride at 150 rpm resulted in a reduction of time for equilibrium to be achieved in all buffer media, and there were no statistically significant differences between means of experimental solubilities and tests ($p > 0.05$).

The influence of rotation speed in equilibrium solubility studies was also evaluated by Avdeef (2015), which afirms that high rotation speed during the experiment can accelerate the dissolution rate, allowing for equilibrium to be reached more quickly. This observation agrees with the data obtained to nimesulide and propranolol hydrochloride (Avdeef, 2015).

Then, variation of rotation speed did not show statistically significant differences between the means of the equilibrium solubilities for the drugs in question, being necessary a shorter time to reach the equilibrium with 150 rpm. An advantage for studies with drugs that require long periods of time to reach equilibrium.

Influence of temperature

Temperature variation to a lower level (34 ± 1 ºC) caused a decrease in the equilibrium solubility values for nimesulide and propranolol hydrochloride when compared to the values obtained in the experimental solubility. The decrease occurred in all media and was significant ($p < 0.05$), demonstrating influence of temperature in equilibrium solubility measures.

On the other hand, increase the temperature to 40 ± 1 ºC promoted an increase in the equilibrium solubility of both nimesulide and propranolol hydrochloride in all buffered media. The difference was also significant for both drugs in all media ($p < 0.05$).

The influence of temperature on solubility is widely discussed and can be understood from the Le Chatelier principle. Considering a solution saturated in equilibrium, when heat is supplied, the equilibrium will move in the direction of absorption of heat. Thus, if the dissolution process is endothermic, the absorption of heat will cause displacement to increase the mass of solute in the aqueous phase. Therefore, there will be increased solubility due to the increase in temperature (Treptow, 1984).

Most of the drugs have the endothermic dissolution process and as discussed, for these substances, increase the temperature caused the increase of solubility (Liu *et al*., 2010).

Baka, Comer, Takacs-Novak (2008) in order to standardize the shake-flask method, using hydrochlorothiazide as a control drug, performed a study. The authors evaluated the influence of temperature in

TABLE IV – Result of the comparison between the experimental solubility and tests with variations of the parameters: rotation speed, temperature, substance added in excess and aliquot for model compounds propranolol hydrochloride and nimesulide. The values shown indicate the mean of three replicates. p-value < 0.05 was considered as statistically significant

Parameter	pH 1.2			pH 4.5			pH 6.8		
	Solubility (mg/mL)	Time (hours)	p-value	Solubility (mg/mL)	Time (hours)	p-value	Solubility (mg/mL)	Time (hours)	p-value
Nimesulide									
Experimental solubility	0.0034	7	-	0.0064	7	-	0.0301	10	-
Rotation speed of 50 rpm	0.0033	7	0.451	0.0065	7	0.724	0.0298	10	0.064
Rotation speed of 150 rpm	0.0030	5	0.063	0.0063	5	0.507	0.0302	7	0.321
Temperature of 34 ± 1 ºC	0.0023	7	0.000	0.0045	7	0.000	0.0253	10	0.000
Temperature of 40 ± 1 ºC	0.0052	7	0.000	0.0071	7	0.008	0.0309	10	0.001
Substance added in excess = 20 mg	0.0050	7	0.000	0.0068	7	0.047	0.0263	10	0.000
Aliquot of 2 mL	0.0036	7	0.467	0.0065	7	1.000	0.0299	10	0.230
Aliquot of 7 mL	0.0032	7	0.073	0.0063	7	0.131	0.0306	10	0.091
Propranolol hydrochloride									
Experimental solubility	210.450	5	-	161.230	5	-	92.980	5	-
Rotation speed of 50 rpm	210.697	5	0.059	161.276	5	0.016	92.769	5	0.075
Rotation speed of 150 rpm	210.735	3	0.101	161.321	3	1.092	92.809	3.5	0.185
Temperature of 34 ± 1 ºC	210.444	5	0.003	161.098	5	0.000	92.587	5	0.000
Temperature of 40 ± 1 ºC	210.865	5	0.000	161.543	5	0.000	92.798	5	0.024
Substance added in excess = 400 mg	210.697	5	0.059	161.347	5	0.429	92.874	5	0.215
Aliquot of 0.1 mL	210.722	5	0.348	161.402	5	0.095	92.845	5	0.087
Aliquot of 0.5 mL	210.712	5	0.101	161.425	5	0.062	92.945	5	0.361

equilibrium solubility studies, using three temperature values: 15 ºC, 25 ºC and 37 ºC. Hydrochlorothiazide solubility was higher (2-fold) at 37 ºC, when compared to the measurement obtained at 25 ºC.

For the authors, the fact that most drugs have higher solubility at 37 ºC than 25 ºC may be an advantage in the development of new drugs because the substance will have higher bioavailability than expected. In addition, this demonstrates the necessity and applicability of the evaluation of drug solubility at body temperature (Baka, Comer, Takacs-Novak, 2008).

According to Avdeef (2015) solubility is a function of temperature, so this parameter must always to be reported. From literature data it is observed that solubility values increase 0.13 log unit, when the temperature changes from 25 ºC to 37 ºC (Avdeef, 2015).

With the results obtained for propranolol hydrochloride and nimesulide, and the data from the literature, it is recommended that the evaluations of the drugs solubility using shake-flask method be carried out with the fully controlled temperature, with maximum variation of ± 1 ºC, as recommended in the biowaiver guidelines of regulatory agencies (EMA, 2010; Brasil, 2011; FDA, 2017).

Influence of substance added in excess

Nimesulide added in excess to each buffer media resulted in statistically different values of equilibrium solubility ($p < 0.05$), thus, drugs of low solubility present greater influences regarding the amount of raw material used in the shake-flask method.

Nimesulide, besides being a drug of low solubility, also presents low wettability (Silva, Volpato, 2002). Therefore, drug particles tend to float on the surface, especially when there is a big substance excess, without the use of surfactants. However, for BCS classification, the use of surfactants to evaluate of solubility must be avoided (Brasil, 2011; FDA, 2017).

During the evaluation of solubility, at the time of collecting the aliquots of the drug solutions in the buffer media, withdrawal of small amount of substance added in excess together with solution is considered, which may interfere with the test results.

Baka, Comer, Takacs-Novak (2008) evaluated the equilibrium solubility of hydrochlorothiazide using small and large excesses and did not obtain statistically significant differences between the means for each experiment. Despite this, the authors recommend the use of small excesses to avoid difficulties during sampling.

Regarding propranolol hydrochloride, substance added in excess did not influence the evaluation of the equilibrium solubility, when compared to the means of the experimental solubilities, i.e., no statistically significant differences were demonstrated in any buffer media ($p > 0.05$).

These results were already expected, since the influence of this parameter in the solubility evaluation only affected drugs of low solubility (Kawakami, Miyoshi, Ida, 2005).

Therefore, in spite of the non-influence for drugs of high solubility, it is recommended to use small substance in excess for evaluation of equilibrium solubility by shake-flask method for both highly soluble drugs and those with low solubility. This excess must be equivalent to a maximum of 10% of the amount of substance necessary for the saturation of the media.

In the case of drugs with low wettability it is necessary to evaluate the feasibility of the use of centrifugation when there is a persistent high coefficient of variation between replicates of solubility results.

Influence of aliquot

The influence of the aliquot withdrawn in the determination of the equilibrium solubility was evaluated. For nimesulide, aliquots of 10% (experimental solubility), 4% (2 mL) and 14% (7 mL) in relation to the total volume of buffer media were used, whereas for propranolol hydrochloride aliquots of 3% (experimental solubility), 1% (0.1 mL) and 5% (0.5 mL) in relation to the total volume of buffer media were used. As shown in Table IV, there were no statistically significant diferences.

TABLE V – Recommendations for evaluation drugs of high and low solubility using shake-flask method

Parameter	Standard conditions
Media	Physiological pH conditions (Preferably pH 1.2, 4.5 and 6.8)
Temperature (ºC)	37 ± 1 (strictly controlled)
Substance added in excess (mg)	10% above the media saturation (critical factor for drugs with low solubility)
Rotation speed (rpm)	50, 100 or 150 (evaluate the need)
Aliquot withdrawn	Not more than 10% of the media volume
Phase separation	Filtration
Acceptance criteria	Coefficient of variation maximum 5% (n=3)
Quantification method	Validated stability-indicating method

* Evaluate the pH of the media after sample addition. Changes in the pH media may indicate instability or reaction with the constituents of the buffer.

Therefore, the aliquot withdrawn in equilibrium solubility studies by shake-flask method should be chosen according to the need of each study, but it should not be excessive in relation to the total volume of the media (≤ 10% of the total volume of the media). In addition, it is necessary to replace the removed media with suitable buffer at a temperature of 37 ± 1 ºC. This procedure is necessary to maintain the system hydrodynamics and the amount of drug substance above the saturation point.

In Table V the main recommendations for evaluation of equilibrium solubility of drugs using shake-flask method are compiled. The recommendations were based mainly on the results obtained in this study and also on data from the FDA (2017), EMA (2010), ANVISA (Brasil, 2011) and WHO (2015) guidelines about biowaiver studies.

CONCLUSION

This work stablishes general conditions to apply shake-flask method. It is strongly recommended to perform a pre-test to establish the time that equilibrium between the phases occurs, i.e., where the solubility remains constant. Note that at this time there should be no degradation of the substance in the media used for the test.

For drugs of low and high solubility, small variations of the temperature, above or below the value of 37 ºC caused statistically significant differences between the means of the equilibrium solubilities. The excess amount of raw material also influenced the results of the drug of low solubility, while excess raw material was not a critical step for the drug of high solubility. However, the use of small excess is recommended for both type of drugs.

The variation of the rotation speed of the method (50 to 150 rpm) did not cause differences between the values of the equilibrium solubilities, but at 150 rpm the equilibrium between the phases (saturated and solid solution) was reached faster. To standardize the use of the shake-flask method in each laboratory, the drugs propranolol hydrochloride and nimesulide can be used as standard of high and low solubility, respectively.

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