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# Treball Final de Grau

**Optimization of dissolution rate measurements of poor-soluble drugs.**

**Optimització de les mesures de velocitat de dissolució de fàrmacs amb baixa solubilitat.**

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A la meva família per recolzar-me en tot moment.



**REPORT**



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# 1. SUMMARY

One of the most important parameters to be determined during the drug development process is the dissolution rate. The *in vitro* study of this property is essential to evaluate the bioavailability of drugs in the drug discovery process.

Traditional methods for dissolution rate determination require high amounts of the active pharmaceutical ingredient. However, it is a problem when drug characterization is carried out in the early stages of the process, when only little amounts of drug are available. For these reasons, miniaturized methods, which use low drug amounts, were developed. The *gastrointestinal dissolution method* is an example of miniaturized method, and it is used in the present work. The fact of extracting part of solution at several times, for *off-line* quantification is not a problem in traditional methods, because it does not affect the dissolution rate profile curve, as the extracted volume is not significant compared to the total one. However, that might not be the case in miniaturized methods, where smaller dissolution volumes are used.

In order to compare the effect of extracting part of solution to quantify off-line the concentration of drug on the profile curve, dissolution rate measurements have been carried out for 120 minutes at constant pH for two different drugs: trazodone and warfarin. First, dissolution profiles have been determined *in situ*, with spectrophotometric detection. Next, experiments have been repeated performing several extractions and quantifying off-line by HPLC.

Results show that dissolution profiles are comparable, although the solubility of the compounds can be a limiting factor.

Determined profiles are similar in the case of trazodone. However, warfarin, with a lower solubility than trazodone, needs support from a cosolvent to reach equivalent concentrations in the HPLC profile.

**Keywords:** Dissolution rate, drug, solubility, active pharmaceutical ingredient, *gastrointestinal dissolution method*, trazodone, warfarin, HPLC



## 2. RESUM

La velocitat de dissolució és un dels paràmetres més importants a determinar durant el procés de desenvolupament dels fàrmacs. L'estudi *in vitro* d'aquesta propietat és essencial per avaluar la biodisponibilitat dels fàrmacs en el seu procés de descobriment.

Per determinar la velocitat de dissolució, els mètodes tradicionals, requereixen quantitats de principi actiu elevades. No obstant això, suposa un problema quan es fa la caracterització de fàrmacs en les primeres etapes del procés, quan només hi ha petites quantitats de fàrmacs disponibles. Per aquests motius, es van desenvolupar mètodes miniaturitzats, els quals utilitzen quantitats baixes de compost. El *gastrointestinal dissolution method* és un exemple de mètode miniaturitzat i ha estat l'utilitzat en aquest treball. El fet d'extreure part de la solució en diverses ocasions, per a la quantificació *off-line* no afecta el perfil de la corba, per tant, no suposa un problema en els mètodes tradicionals, ja que el volum extret no és significatiu en comparació amb el total. Tanmateix, podria no donar-se el cas en els mètodes miniaturitzats, on s'utilitzen volums de dissolució més reduïts.

Per comparar com afecta el perfil de la corba, el fet d'extreure part de la solució per quantificar la concentració de fàrmac *off-line*, s'han dut a terme mesures de velocitat de dissolució durant 120 minuts a pH constant per a dos fàrmacs diferents: la trazodona i la warfarina. Primer, els perfils de dissolució s'han determinat *in situ*, amb detecció espectrofotomètrica. A continuació, s'han repetit els experiments realitzant diverses extraccions i quantificant-les *off-line* per HPLC.

Els resultats obtinguts mostren que els perfils de dissolució són comparables, tot i que la solubilitat dels compostos pot ser un factor limitant.

Els perfils determinats són similars en cas de trazodona. No obstant això, la warfarina, que té una solubilitat més baixa que la trazodona, necessita el suport d'un cosolvent per arribar a concentracions equivalents en el perfil de HPLC.

**Paraules clau:** Velocitat de dissolució, fàrmac, solubilitat, principi actiu, *gastrointestinal dissolution method*, trazodona, warfarina, HPLC



### 3. INTRODUCTION

Many drugs are synthesized by the pharmaceutical industry. The physicochemical characterization in the early stages of the drug development is a very important step in the drug discovery process because all those drugs that do not have adequate parameters will not be further developed. This previous stage allows to save time and money.<sup>1</sup> Solubility is one of the most important parameters because the drug has to be soluble in an aqueous medium<sup>2</sup> or has to be prepared in an appropriate formulation to overcome the limitations of solubility<sup>1</sup> to be absorbed.

The other parameter that is very important for the drug development process is the dissolution rate. This parameter is used to evaluate the stability of drugs and provides information about when the drug starts working inside the organism.<sup>3</sup>

However, while solubility is a static concept, which refers to a state of thermodynamic equilibrium, the dissolution rate responds to a kinetic concept of the process: amount or concentration of solute that dissolves per unit of time.<sup>4</sup> To understand the importance of both parameters it is necessary to relate them to the pharmacokinetic concept.

#### 3.1. PHARMACOKINETICS

Pharmacokinetics is a pharmacology branch dedicated to the study of how a drug affects an organism and determines the fate of substances administered to a living organism.<sup>5</sup> The pharmacokinetic process usually explains the ADME properties of a drug which are Absorption, Distribution, Metabolism, and Excretion. ADME studies provide an estimation of human pharmacokinetic and metabolic profiles<sup>6</sup>.

The absorption stage consists of the transfer of a drug from a place of administration to the blood. The Biopharmaceutics Classification System (BCS) was created based on the acknowledgement of the importance of solubility and permeability on the fraction absorbed of a drug.<sup>7</sup> It has a regulatory purpose whose underlying idea is that in vitro measurement of solubility, dissolution, and permeability of drugs in an aqueous medium can predict their in vivo intestinal absorption.<sup>8</sup>

BCS categorize drugs into four different classes according to the cut-off values for solubility and permeability (Figure 1). The highest therapeutic dose must be completely dissolved in 250 mL of water at pH range 1 to 7.5 to be considered a highly soluble drug.<sup>8</sup> To be highly permeable at least 85% of the dose has to be absorbed.<sup>9</sup>

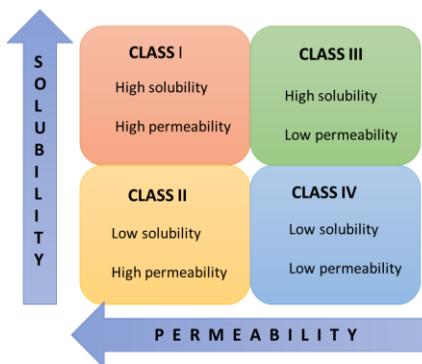


Figure 1. Representation of the Biopharmaceutics Classification System.

### 3.1.1. Solubility

Solubility is the maximum amount of a compound that can be dissolved in a specified volume of a solvent at a given condition of temperature and pH. It plays a key role in oral absorption because all drugs should possess an adequate solubility in the gastrointestinal fluids in order to be able to be absorbed correctly.<sup>10</sup>

The solubility of a drug depends on many factors and some of them are the hydrophobicity of the drug molecule and the melting point because it indicates different interactions with the solvent.<sup>11</sup> The higher the interaction drug-drug the lower the solubility and dissolution rate of the compound. However, the pH of the medium is a very important parameter because it affects ionizable drugs charge state.<sup>11</sup>

#### 3.1.1.1. pH-Solubility profile

Changes in pH affect the extent of ionization for an ionizable molecule and the relative proportion of the ionized and unionized drug in solution. Ionizable drugs demonstrate pH dependence via the Henderson-Hasselbalch solubility profile.<sup>11</sup>

The profile changes depending on the acid-base character of the compound. However, in both cases, the lowest solubility is shown when the drug is fully unionized and it is called intrinsic solubility ( $S_0$ ).<sup>11</sup>

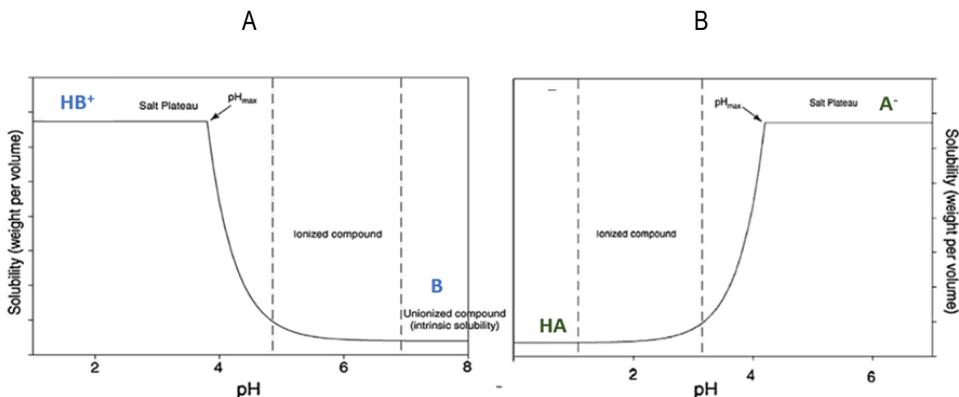


Figure 2. pH solubility profile for a (A) weak base and (B) weak acid.

In Figure 2 the dependence that exists between the solubility and pH can be observed. The solubility of a weak base (Figure 2A) gets higher when pH decreases. The lowest solubility appears when the drug is unionized, and the pH is higher than its  $pK_a$ . Instead, it is observed that solubility of a weak acid (Figure 2B) gets higher when the pH increases, and the lowest solubility appears when pH is lower than its  $pK_a$ .<sup>12</sup>

There is a steep increase in the solubility of the molecule as it reaches its  $pK_a$  and keeps rising until it reaches  $pH_{max}$  which is the point where the ionizing portion of the curve reaches the maximum solubility point. This is called a salt plateau. It is the region where the salt form (ionized molecule associated with a counterion) is in equilibrium with its solid state.<sup>11</sup>

The counterion effect is significant to determine the solubility constant ( $K_{ps}$ ). The counterion associated in the salt form has an opposite charge and it is attracted by coulombic interactions. The interaction strength determines the delimiting solubility.<sup>11</sup>

### 3.1.2. Intrinsic Dissolution Rate

The intrinsic dissolution rate is defined as the rate of dissolution of an API under the constant condition of surface area, pH, temperature and ionic strength.<sup>13</sup> The determination of this parameter helps us understand the solution behaviour of drugs under biophysiological conditions.

The dissolution rate can be expressed by the Noyes-Whitney equation, which fits a first order exponential Equation 1. From this expression the dissolution rate and an extrapolated solubility value can be obtained.<sup>14</sup>

$$[X]_t = S (1 - e^{-K_d(t-t_0)}) \quad (1)$$

$[X]_t$  is the weight of the drug in solution in the experiment time (min);  $S$  (g) is the extrapolated solubility of the drug;  $K_d$  is the rate constant for dissolution ( $\text{min}^{-1}$ ), and  $t_0$  (min) is the measurement start time.<sup>14</sup>

Through a refinement process based on the least square adjustment the  $S$ ,  $K_d$  and  $t_0$  values are obtained. The dissolution rate is given by the product  $K_d \cdot S$  ( $\text{g} \cdot \text{min}^{-1}$ ).<sup>14</sup> However, the intrinsic dissolution rate (IDR) is the product of normalizing the dissolution rate by surface area.

### 3.1.2.1. Methods of IDR measurement

To evaluate the bioavailability of Active Pharmaceutical Ingredients (APIs) it is important to carry out dissolution rate tests. Three different methods exist to perform the dissolution rate (DR) measurements, mainly differentiated by the way the drug is added to the dissolution vial. DR is typically measured using a rotating or static disc of compacted powder (disc method) where the drug powder is compacted on a disc which has a defined surface area. In addition, disc method is usually used for drugs with an apparent solubility  $S_{\text{app}} > 1 \text{ mg/mL}$ .<sup>9</sup> Other not so common methods are the coarse powder, where the solid compound is added directly to the vial, and the suspensions method, where a controlled particle size of powder drug is dispersed. In contrast to disc method, coarse powder and suspensions method are more commonly used for drugs with a lower apparent solubility ( $S_{\text{app}} < 100 \text{ } \mu\text{g/mL}$ )<sup>9</sup> because the surface area which is in contact with the dissolution medium is higher and it speeds up the dissolution of the drug. However, apparent solubility is not considered a limiting parameter to decide which method has to be used.

Pharmacopoeias list two types of traditional apparatus to determine the dissolution rate; A rotating disc system which uses United States Pharmacopeia (USP) Wood Apparatus (Wood method) (Figure 3A), where drug dissolution is carried out by a rotating force system on spindle which is fitted to the tablet die (Figure 3A). API is compressed in a steel punch and die (Figure 3A), and the obtained tablet must be smooth and free of visible defects with a  $0.5 \text{ cm}^2$  to  $1.3 \text{ cm}^2$  of surface area.<sup>8</sup> The compound disc is then fitted to a rotational assembly in a dissolution bath as shown in the Figure 3A.<sup>13</sup> The other traditional apparatus is the fixed-disc system (Figure 3B) where as Wood method, the compacted drug is inside a pellet and die assembly (Figure 3B), however, in this system, the assembly is fixed, and the dissolution is achieved by moving the volume of dissolution medium with a paddle apparatus over the disc (Figure 3B).<sup>13</sup>

In both systems, 150-700 mg of API are used, and they have a cavity for inserting the drug with a 0.5 to 1.3 surface area to produce a tablet without visible surface damages.<sup>13</sup>

All assemblies are lowered into the dissolution vessel whose temperature is controlled and it contains 900 mL of dissolution medium volume.<sup>9</sup> Dissolution profile is performed by taking little samples from the vessel at different times and determining drug concentration *ex situ* with other techniques such as HPLC or UV-Spectrophotometry.<sup>9</sup>

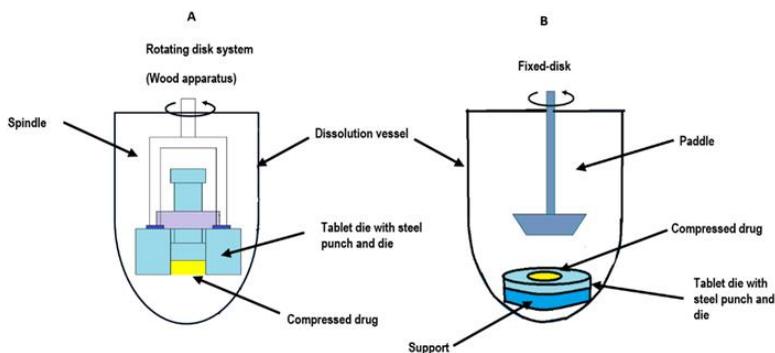


Figure 3. (A) Rotating disk system (Wood apparatus). (B) Fixed-disk system.

These methods usually are applied in drug development, but they require a high amounts of API, limited to subsequent stages of development where available sample quantities are higher.<sup>14</sup>

For these reasons, miniaturized dissolution rate measurements, that allow the characterization of drugs earlier in their development cycle, have been developed. These other methods allow to measure the dissolution rate using a smaller amount of powder in the discs and results have been obtained with good correlation with traditional methods.<sup>14</sup>

### 3.1.2.2. Miniaturized dissolution methods

Miniaturized disk methods usually compact around 5 mg of the compound on a 0.071 cm<sup>2</sup> surface miniaturized disc. Usually only 10-15 mL of dissolution medium are employed. In contrast to traditional methods, the concentration is measured by an *in-situ* optic fibre UV.<sup>8</sup>

The method used in the present work is the *gastrointestinal (GI) dissolution method*, a low volume *in vitro* technique using as instrument, the GLpKa (Figure 4A), which has an automatic titration system designed for the measurement of physicochemical properties as solubility or pK<sub>a</sub>, among others.<sup>14</sup>

The *GI dissolution method* allows to investigate the behaviour of drugs simulating the gastrointestinal tract (GIT). This consists of the study of the dissolution in four pH zones; 1.9, 3.8, 5.3 and 7.2.<sup>14</sup> Furthermore, the method allows compressing less amount of API (4-10 mg) into a static disc and carry out the dissolution in a low medium volume (15 mL). As in the fixed-disc system, the tablet is fixed in a static disc and the dissolution is achieved by moving the volume of dissolution medium with an agitator (Figure 4B) over the disc.

In contrast to traditional methods, dissolution rate is controlled *in situ* by UV absorbance spectroscopy *via* a fixed optic dip probe with a diode array spectrophotometer<sup>14</sup> (Figure 4B) recording in continuous the absorbance spectra.

The drug concentration at each time point is calculated by Lambert-Beer law from the spectroscopic data using the previously determined molar extinction coefficient.<sup>14</sup>

However, sometimes drugs analysed have interferences which do not allow a good quantification. An example are co-crystals; as they are formed by two different compounds (the API and a cofomer), absorbance spectrum may be the sum of two contributions, which difficult the quantification of the API. For these reasons, the sample should be analysed *ex-situ* by other techniques such as HPLC. Whereas this is not a problem in the traditional methods, the fact of doing extractions when the total volume is much lower can be a handicap. For this reason, the present work will evaluate if the *GI dissolution method* provides equivalent results, depending on whether quantification is done *in situ* or *off-line*.

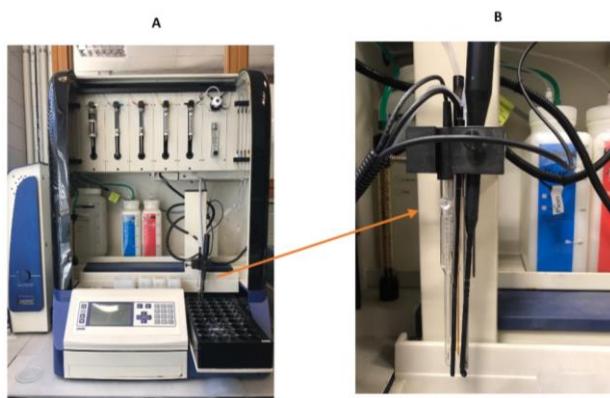


Figure 4. (A) GLpKa (Sirius Analytical Instruments, East Sussex, UK) (B) pH electrode, spectrophotometric probe, capillaries, and agitator.

## 4. OBJECTIVES

Traditional methods for measuring dissolution rate of drugs use high sample amounts and volumes, for this reason the fact of extracting part of the solution to determine the concentration of drug does not affect the curve of the dissolution rate. In contrast, in the *GI dissolution method* the volumes are very small, and extracting a quantity of solution may affect more in the obtained dissolution rate.

The objective is to study how doing extractions in a low volume dissolution apparatus affects the dissolution rate and check if there is any difference between the dissolution rate curve analysed by the *GI dissolution method* or by HPLC.

Also, another objective is to determine to which extent drug solubility limits the quantification *off-line* by HPLC.

## 5. EXPERIMENTAL SECTION

### 5.1. REAGENTS AND MATERIALS

#### 5.1.1. GI dissolution method reagents

In the preparation of the pH 1.6 buffer for the *GI dissolution method* the following chemicals were used: sodium acetate anhydrous (99,6%) from J.T. Backer (Deventer, Holland), potassium dihydrogen phosphate (>99,5%) and 0.5 M hydrochloric acid solution from Merck (Darmstadt, Germany). pH was measured with pH meter Crison GLP22 (Italy). Water was purified by a Mili-Q plus system from Millipore (Bedford, MA, USA) with a resistivity of 18.2 M $\Omega$  · cm.

In the *GI dissolution method*, the following chemicals were used: 0.15 M potassium chloride (99.5%) (ISA water), DMSO (99,8%) and Titrisol® solutions of 0.5 M potassium hydroxide and 0.5 M hydrochloric acid all from Merck.

#### 5.1.2. HPLC reagents

The methanol (HPLC grade) used for the HPLC mobile phase was from Thermo Fisher Scientific U.K.

In the preparation of the buffers for the HPLC mobile phase, the following chemicals were used: formic acid (98%), sodium hydroxide (>99%) both from Merck, formic acid (85%) from Carlo Erba and acetic acid (99-100%) from J.T. Backer.

### 5.1.1. Studied drugs

The drugs used were trazodone hydrochloride (>99%) and warfarin (>98%) both from Sigma Aldrich, Germany.

## 5.2. INSTRUMENTATION

### 5.2.1. GI dissolution method

The apparatus used to measure the molar extinction coefficients, pK<sub>as</sub> and the dissolution rate was GLpKa (Sirius Analytical Instruments, East Sussex, UK) equipped with a fibre optic dip probe with a diode array spectrometer D-PAS (Dip Probe Absorption Spectroscopy). The apparatus was controlled from a computer running the RefinementPro2 software and the data refinement was completed with Sirius T3 Refine.

All measurements were performed at room temperature in 0.15 M KCl solution (ISA Water) using standardized 0.5 M KOH and 0.5 M HCl solutions. The measured pH range was from 1.8 to 12.2.

### 5.2.2. Liquid Chromatography measurements

A Shimadzu liquid chromatograph equipped with two Shimadzu LC-10AD VP pumps and a Shimadzu SPD-10AV VP UV-VIS detector was used for the quantification of samples.

The apparatus was controlled from a computer running the LC solutions software from Shimadzu.

## 5.3. PROCEDURES

### 5.3.1. Molar extinction coefficients (MEC) and pK<sub>as</sub> determination

The molar extinction coefficients for neutral and ionized form of drugs and pK<sub>as</sub> were determined by UV- metric titration using the instrument GLpKa (Figure 4) in aqueous medium.

10 mM stock solution of sample was prepared in DMSO to obtain a peak UV absorbance of approximately 1 absorbance unit.<sup>14</sup>

50  $\mu$ L of sample stock solution and 250  $\mu$ L of buffer (potassium dihydrogen phosphate 15 mM) were added in a vial. The instrument next added 10 mL of ISA water. To analyse trazodone hydrochloride the titration was from low pH to high pH, the pH of the sample solution was adjusted to 2 with 0.5 M HCl before starting the titration. After that, the titration was completed using 0.5 M KOH up to pH 12.20. However, to analyse warfarin the titration was from high pH to low pH, first pH adjustment was 12.2 with 0.5 M KOH and then, the titration was done using 0.5 M HCl up to pH 2.

To make the reference spectrum, the instrument uses a reference vial where buffer (250  $\mu$ L) and solvent without sample (50  $\mu$ L of solvent DMSO) are added.

The instrument collected each titration addition by a fiber optic dip-probe and recorded an UV absorption spectrum which is refined through the RefinementPro software. MEC and  $pK_a$  values are obtained by Target Factor Analysis.

### 5.3.2. Tablet production

4-10 mg of API were weighted and compressed inside a tablet disc (Figure 5A) (3 mm diameter) using a manual hydraulic tablet press (Specac Ltd, Orpington, UK) and a compression rod (Figure 5A). The tablets were produced applying a force of 100 N for 120 seconds and the obtained tablet was checked to discard any surface damage. Total exposed surface area was 0.5  $cm^2$ .<sup>14</sup>

Once the tablet was made, the tablet disc was placed in a tablet disc holder (Figure 5A), which rises 7 mm the tablet over the vial base when it was inserted into an appropriate-sized vial (Figure 5B). It allowed the introduction of up to 2 mL of buffer into the vial without wetting the tablet.<sup>14</sup>

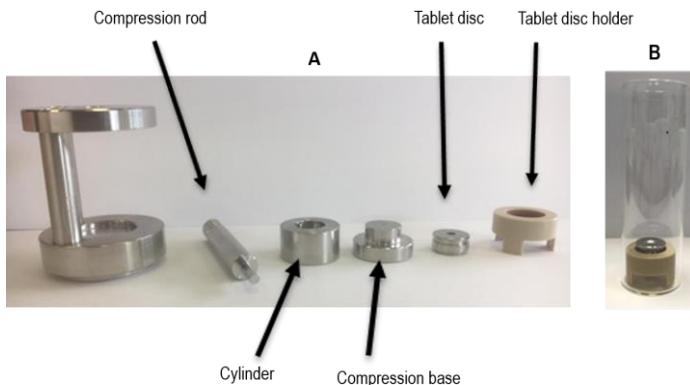


Figure 5. (A) Apparatus required for GI *dissolution* method (B) Tablet disc with tablet disc holder inside the vial.

### 5.3.3. Dissolution rate measurements

Usually, to measure the dissolution rate by the GI *dissolution* method two cells were used; the vial with the sample (Figure 5B), and the reference one (without sample).

1.5 ml buffer pH 1.6 was added to each cell. The instrument automatically added 13,5 mL of ISA water. The initial pH work was adjusted automatically by the instrument to the wished pH with 0.5 M KOH or 0.5 M HCl solutions. Then, the reference spectrum is taken in the reference vial. Afterwards, the medium in the sample cell is stirred at a constant rate during the GI *dissolution* experiment, and the pH is constantly readjusted every 30 seconds (point collected) for 2 hours.

For spectrophotometrical quantifications the UV absorption spectra was recorded between 190 and 700 nm through a fibre optic dip probe with a diode array spectrometer D-PAS. The collected data were refined with Sirius T3 Refine software.

### 5.3.4. Quantification by liquid chromatography

#### 5.3.4.1. Extractions

During the GI *dissolution* experiment, 10 sample extracts were taken at times 4,6,8,12, 22, 32,47,62, 92 and 120 min.

A sample of 50  $\mu\text{L}$  was extracted, initially in very small intervals of time to study in detail the evolution of the dissolution rate. However, when the dissolution experiment evolved, the time intervals were larger because there were fewer changes in solution concentration.

In case that drug solubility was extremely low, the samples extracts were dissolved in 50  $\mu\text{L}$  of methanol to keep all the drug in solution and avoid precipitation.

#### 5.3.4.2. HPLC

First, the calibration curves for each drug were carried out. The solvent used to prepare the standard solutions was the same as the mobile phase used in the chromatographic conditions.

The study of all the drugs was carried out in isocratic conditions using Symmetry® C8 4.6x50mm, 5 $\mu\text{m}$  column without precolumn and at the maximum absorption wavelength in order to obtain the maximum signal. Two examples of chromatograms are shown in Appendix 1 and two examples of calibration curves are shown in Appendix 2.

The chromatographic conditions for each drug are described in Table 1.

Table 1. Chromatographic conditions for each studied drug.

	<b>Trazodone hydrochloride</b>	<b>Warfarin</b>
		$\text{CH}_3\text{COOH}$ 17 mM;
Aqueous phase; pH	$\text{HCOOH}$ 10 mM; 3	3.4
Organic phase	MeOH	MeOH
% Organic Phase	25	60
Injection volume [ $\mu\text{L}$ ]	10	10
$\lambda$ [nm]	254	280
Total run time [min]	5	6
Calibration range [mg/L]	100-600	0.02-1
Flow rate [mL/min]	1	1
Retention Time [min]	2.3	4.2

## 6. TRAZODONE HYDROCHLORIDE

Trazodone is an antidepressant derivative of triazolopyridine which works as an inhibitor of serotonin uptake. It has absolute therapeutic efficacy in depressive illness<sup>15</sup>. Figure 6 shows trazodone hydrochloride structure.

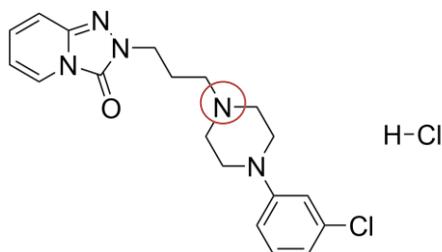


Figure 6. Trazodone hydrochloride structure.

From the chemical point of view, trazodone hydrochloride (408.32g/mol) is a weak base with a  $pK_a$  of  $6.7 \pm 0.4$ . The ionizable atom is the red nitrogen shown in Figure 6. A solubility-pH profile like the one in Figure 2A is expected. It has an intrinsic solubility (M) of  $\log S_0 = -4.54$  (0.01 mg/mL) in water which means an intermediate solubility.

### 6.1. WORKING CONDITIONS OPTIMIZATION

#### 6.1.1. Determination of the molar extinction coefficient of trazodone hydrochloride.

Figure 7 shows the molar extinction coefficient (MEC) profile of trazodone obtained in aqueous medium using a solution with a known concentration of trazodone hydrochloride. It displays the molar absorptivities of  $BH^+$  (blue line) and  $B$  (red line) in the wavelength range of interest.

Depending on the pH of the dissolution rate of the experiment and the  $pK_a$  of the drug, different percentages of each species are present in solution, and the quantification is done using their MEC accordingly.

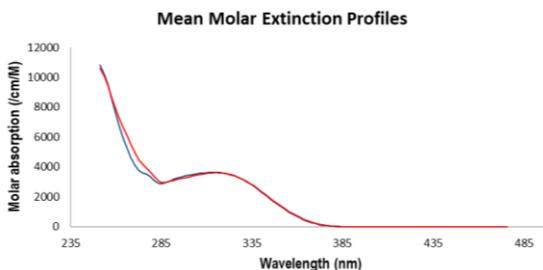


Figure 7. Molar absorption profile of trazodone hydrochloride.

### 6.1.2. pH

Before evaluating the dissolution rate profile, working conditions must be optimised. In case of acid-base compound, one of the most important parameters to control in the dissolution rate measurements is pH. Figure 8 presents a typical plot of drug weight versus time at pH 10 (drug weight was 6 mg). At pH 10 trazodone is in its neutral form in the solution. Therefore, at this pH the solubility of trazodone is the lowest one,  $S_0$ . Thus, the solution became soon saturated, it reached supersaturation stage and precipitate was observed such as Figure 8 indicates.

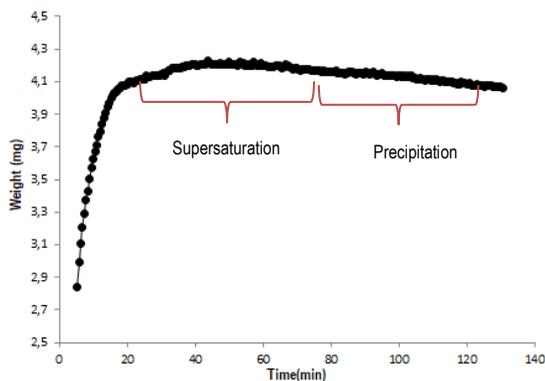


Figure 8. Dissolution rate of a sample at pH 10

A way to improve solubility is to work at a pH closer to the  $pK_a$  value. Therefore, pH 7.4 was tried. Figure 9 shows the observed behaviour at this pH value. Solution did not seem to become supersaturated and a precipitate was not observed, as indicates the constant amount of drug in solution. As after dissolution rate experiment, the tablet was not completely dissolved, the maximum weight value observed could be a solubility value corresponding to the saturated solution.

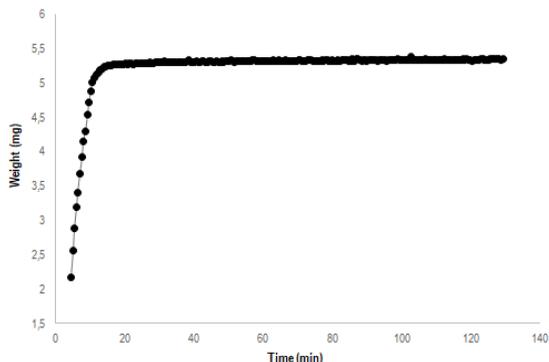


Figure 9. Dissolution rate of samples at pH 7.4.

### 6.1.3. Drug weight

Dissolution rate does not depend on the tablet weight. When dissolution rate reaches the saturation concentration, the drug starts to precipitate and a previously supersaturation stage can, sometimes, be observed. When the solution is supersaturated, trazodone concentration in solution is higher than its solubility. Therefore, it is a no-equilibrium system. In these cases, curves like the orange or the blue one which appear in Figure 10 are obtained. In each one it is possible to see that the concentration is higher than the plateau over a period of time, and then there is a drop which indicates that the precipitation has just started.

The presence of precipitate is not a problem if the first part of the curve is well defined when we need to measure the dissolution rate *in situ*. But it makes it difficult to do a quantification afterwards through an off-line technique such as HPLC. Extracting particles which are in suspension implies a previous filtration before the quantification and the extracted sample volume is too small to filter (50  $\mu\text{L}$ ). In addition, in case that the solution is supersaturated, the precipitation can be induced by the extraction process.

Tablets of different weights were tested in order to see in which conditions precipitation was avoided. Figure 10 shows three samples with different weight of trazodone hydrochloride. It can be observed that the tablet made with 6 mg of compound does not reach the supersaturation and consequently, a precipitation is not observed, so this amount of trazodone is correct for our purposes.

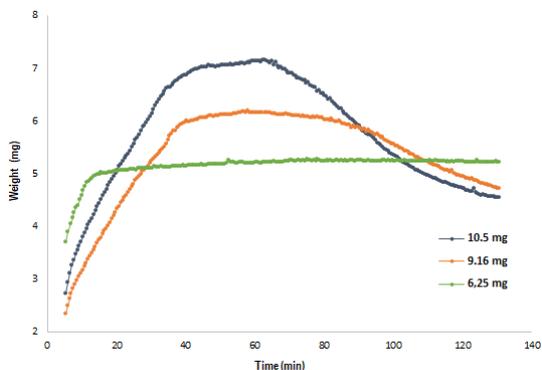


Figure 10. Dissolution rate of three samples with different weights.

## 6.2. DISSOLUTION RATE MEASUREMENTS

### 6.2.1. Dissolution rate determined spectrophotometrically without extractions.

Figure 11 shows a typical plot of weight versus time for the dissolution rate determined spectrophotometrically of three replicates without carrying out sample extractions. Phosphate/acetate buffer was added before starting the dissolution to keep a constant pH all time, and pH was adjusted to 7.4 by the titration system.

Trazodone dissolution rate behaviour is described in Figure 11, whose curves reach a constant concentration value after 15 minutes of starting the dissolution. After 120 minutes, the tablets were not completely dissolved. For this reason, solubility at pH 7.4 can be estimated from the plateau. Anyway, this value is not an equilibrium one, so it can differ from the one provided by equilibrium measurements.

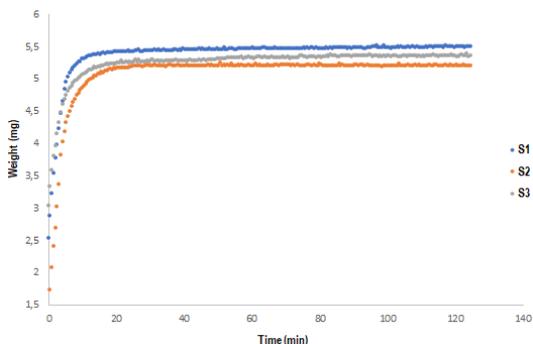


Figure 11. Spectrophotometric experimental values of three samples without extractions.

The first order Equation 1 was adjusted to the experimental values obtained spectrophotometrically, in order to obtain the  $S$ ,  $K_d$  and  $t_0$  parameters. DR was calculated as the product of  $S$  and  $K_d$ . Table 2 shows the parameters of  $S$ ,  $K_d$  and DR with the corresponding standard deviations in brackets.

In all tables, standard deviations associated to the parameters are provided by the fit of Equation 1, and standard deviations of the arithmetic mean have been calculated through the different parameters values.

Table 2. Solubility,  $K_d$  and DR obtained spectrophotometrically values with their SD of three samples without extractions.

<b>Sample</b>	<b>S [mg]</b>	<b><math>K_d</math> [<math>\text{min}^{-1}</math>]</b>	<b>DR [<math>\text{mg}\cdot\text{min}^{-1}</math>]</b>
<b>1</b>	5.5 (0.002)	0.30 (0.003)	1.65 (0.02)
<b>2</b>	5.2 (0.002)	0.26 (0.002)	1.36 (0.01)
<b>3</b>	5.3 (0.003)	0.23 (0.004)	1.24 (0.02)
<b>Arithmetic mean</b>	5.3 (0.1)	0.26 (0,03)	1.4 (0.2)

### 6.2.2. Dissolution rate determined spectrophotometrically with extractions.

Figure 12 shows a typical plot of weight versus time corresponding to the dissolution rate determined spectrophotometrically for five samples. 10 sample extractions of 50  $\mu\text{L}$  at different times and ex-situ HPLC analysis have been done to the samples.

The results are similar as it happened in the DR determined spectrophotometrically without extractions. The curves reach the same constant concentration value after 15 minutes of starting. Once the quantification was finished, the tablet was not completely dissolved. Therefore, it could be considered that saturation was reached.

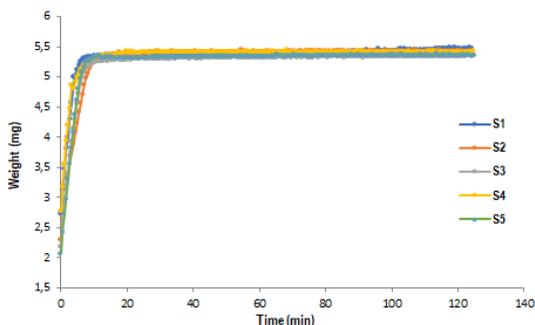


Figure 12. Spectrophotometrically experimental values of five samples with extractions.

Table 3 shows the obtained parameter of  $S$ ,  $K_d$  and DR with its standard deviations.

Table 3. Solubility,  $K_d$  and DR obtained spectrophotometrically values with their SD of five samples with extractions.

Sample	$S$ [mg]	$K_d$ [ $\text{min}^{-1}$ ]	DR [ $\text{mg}\cdot\text{min}^{-1}$ ]
1	5.41 (0.003)	0.40 (0.007)	2.17 (0.04)
2	5.42 (0.003)	0.24 (0.003)	1.28 (0.02)
3	5.33 (0.003)	0.31 (0.004)	1.64 (0.02)
4	5.41 (0.002)	0.39 (0.004)	2.09 (0.02)
5	5.35 (0.004)	0.32 (0.006)	1.72 (0.03)
<b>Arithmetic mean</b>	5.38 (0.04)	0.31 (0.07)	1.7 (0.4)

Comparing DR value doing extractions  $1.7 (0.4) \text{ mg}\cdot\text{min}^{-1}$  (Table 3) and without doing extractions  $1.4 (0.2) \text{ mg}\cdot\text{min}^{-1}$  (Table 2), there are no significant differences between them. In order to compare if both procedures are equivalent to each other, Figure 13 shows the average curve profile doing and not doing extractions. The orange profile represents the average experimental points of dissolution rate with the standard deviations as error bars when extractions are done. The blue one represents the experimental values analysed spectrophotometrically without carrying out extractions also with the error bars. Figure 13 proves the fact that doing 10 extractions of  $50\mu\text{L}$  does not affect considerably the curve of dissolution rate, as both curves overlap.

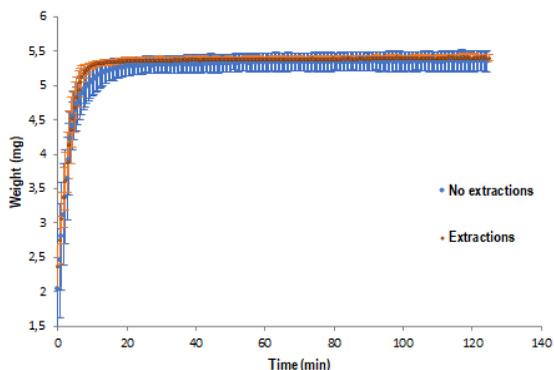


Figure 13. Comparison of average curves with their respective errors.

### 6.2.3. Dissolution rate profiles obtained by off-line quantification.

The 10 extracts (50  $\mu\text{L}$ ) of the sample are chromatographically analysed at times 4,6,8,12, 22, 32,47,62, 92 and 120 min. in order to compare the obtained values to those obtained previously with GLpKa.

The calibration curves obtained which have been used to quantify most of the samples had a  $r^2$  value of 0.999 (calibration curve in Appendix 2). As chromatographic peaks areas of the extracts were not higher than the most concentrated standard dilution was not performed. The extracts were quantified the same day of the extraction.

Figure 14 shows the comparison of two samples, quantified spectrophotometrically *in situ*, and *off-line* by HPLC. A correlation between GLpKa values (grey points) and chromatographic values (orange points) can be observed because both curves values overlap.

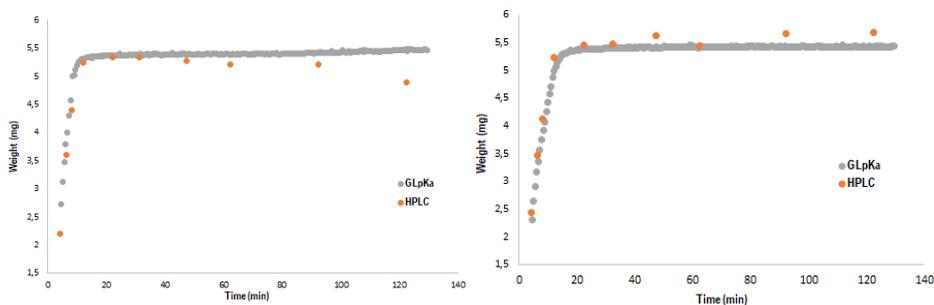


Figure 14. GLpKa (grey) and chromatographic (orange) experimental values of two samples with extractions.

The first order Equation 1 was adjusted to the experimental chromatographic points, in order to obtain the  $S$ ,  $K_d$  and  $t_0$  parameters. Results are shown in Table 4. Figure 15 shows how Equation 1 fits well the experimental points for one of the samples.

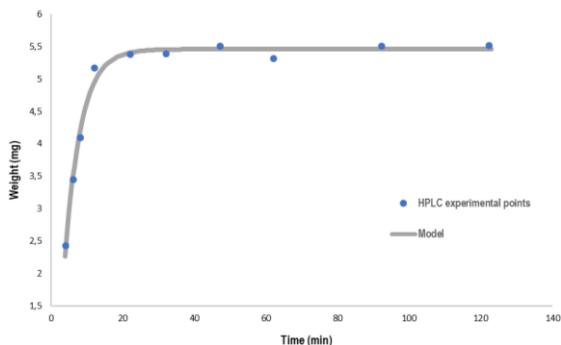


Figure 15. Experimental values of HPLC and predicted values by the model of a sample.

Table 4. Solubility,  $K_d$ , and DR obtained values with their SD of four samples HPLC quantified.

Sample	$S$ [mg]	$K_d$ [ $\text{min}^{-1}$ ]	DR [ $\text{mg}\cdot\text{min}^{-1}$ ]
1	5.3 (0.1)	0.18 (0.02)	1.0 (0.1)
2	5.46 (0.05)	0.23 (0.02)	1.2 (0.1)
3	5.24 (0.05)	0.43 (0.05)	2.3 (0.3)
4	5.26 (0.09)	0.26 (0.03)	1.4 (0.2)
<b>Arithmetic mean</b>	5.3 (0.1)	0.3 (0.1)	1.3 (0.6)

In-situ (doing extractions) and off-line DR values have been  $1.7 (0.4) \text{ mg}\cdot\text{min}^{-1}$  (Table 3) and  $1.3 (0.6) \text{ mg}\cdot\text{min}^{-1}$  (Table 4) respectively. They do not present significant differences because arithmetic mean in both quantifications are similar and they are within the error range.

In order to affirm that there are no differences between doing the quantification *in-situ* or *off-line*, Figure 16 compares the average curves profile (with their standard deviation such as error bars) based on GI dissolution method (spectrophotometric *in situ* study) to the results of the chromatographic technique (*off-line*). The blue curve represents the average experimental spectrophotometric points and overlap with the chromatographic points can be observed. To sum up, it has been demonstrated that GLpKa and HPLC are two equivalent techniques to quantify

trazodone hydrochloride dissolution rate. Moreover, both techniques will probably be valid for those drugs which present an intrinsic solubility(M) higher than  $\log S_0 = -4.54$ .

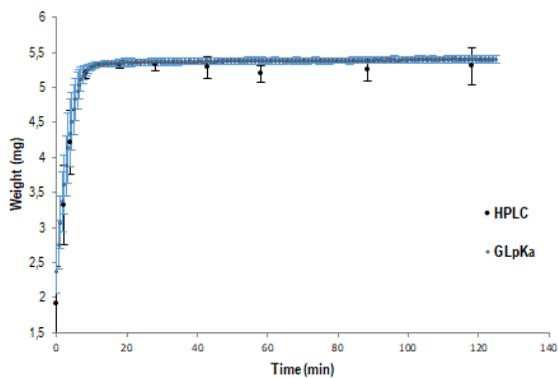


Figure 16. Comparison of *in-situ* (GLpKa) and *off-line* (HPLC) averages curves with their corresponding SD as error bars.

## 7. WARFARIN

Warfarin is an anticoagulant which works as an inhibitor of the vitamin K production, reducing the ability of the blood to clot.<sup>16</sup> Figure 17 shows warfarin structure.

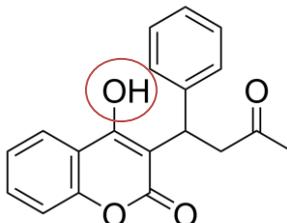


Figure 17. Warfarin structure

From the chemical point of view, warfarin (308.34 g/mol) is a weak acid with a  $pK_a$  of  $4.9 \pm 0.4$ . The ionizable atom is the red oxygen shown in Figure 17. A solubility-pH profile like the one Figure 2B is expected. It has an intrinsic solubility (M) of  $\log S_0 = -4.33$  (6  $\mu\text{g/mL}$ ) in water which means a poor solubility.

### 7.1. WORKING CONDITIONS OPTIMIZATION

#### 7.1.1. Determination of the molar extinction coefficient of Warfarin.

Figure 18 shows the molar extinction coefficient profile of warfarin obtained in aqueous medium using a solution with a known concentration of warfarin. It displays the molar absorptivities of HA (blue line) and  $A^-$  (red line).

Depending on the pH of the dissolution rate experiment and the  $pK_a$  of the drug, different percentages of each species are present in solution, and the quantification is done using their MEC accordingly.

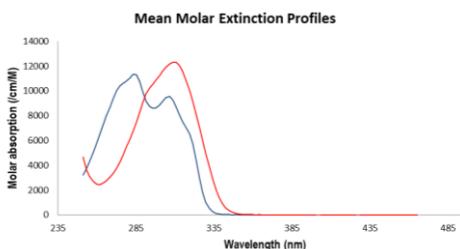


Figure 18. UV absorption spectra of warfarin with molar absorption profile.

### 7.1.2. pH and drug weight

In contrast to trazodone, warfarin is poorly soluble in water in its neutral form. A first attempt of dissolution experiment was tried at pH 2, using 7 mg of warfarin to do the tablet. Figure 19 shows that, in contrast to trazodone, after 120 minutes of experiment warfarin does not reach a constant concentration value. Once the measurements were finished, the tablet had hardly dissolved. The highest weight value in Figure 19, which shows the highest concentration, is 27.56  $\mu\text{g}$  (1.84  $\mu\text{g}/\text{mL}$ ), far below solubility (6  $\mu\text{g}/\text{mL}$ ). So, warfarin does not reach saturation after two hours of experiment.

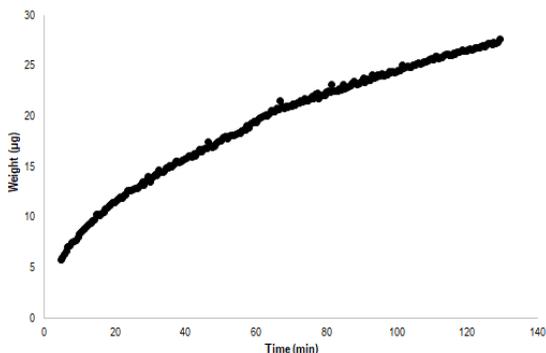


Figure 19. Dissolution rate corresponding to 2 hours experiment at pH 2.

### 7.1.3. Measurement time

Due to the fact that after 120 minutes of experiment the solution did not reach saturation, Figure 20 shows the dissolution rate profile of warfarin during 8 hours of dissolution. As in Figure 20, the highest concentration is 58.24  $\mu\text{g}/\text{mL}$  (3.88  $\mu\text{g}/\text{mL}$ ), almost half the value of solubility (6  $\mu\text{g}$ ), so it does not reach saturation after 8 hours of experiment either. Once the measurements were finished, the tablet had hardly dissolved. Our conclusion is that warfarin is hardly soluble and that its dissolution rate is very low in an aqueous medium, as it has not reached saturation after 8 hours.

Figure 20 shows two different profiles related to the dissolution of warfarin. There is a first slope from 0 to 20 minutes and then, slope decreases, and a second dissolution profile is observed. This could be due to two phenomena:

- Warfarin is difficult to press as a solid. Usually, some particles of powder remained attached to the disc around the tablet and it was difficult to remove them. Its dissolution

- would present a higher DR than the tablet DR, because the superficial area of the particles is higher, and consequently, so is the dissolution rate (powder coarse method).
- The crystalline structure of the tablet might change during the dissolution process, which would result in two different DR, the first one would be caused by the initial warfarin's solid, and the second one to the change of crystalline structure in the tablet. In order to prove this, an x-ray analysis of warfarin powder and solid tablet after DR measurement should be performed.

Figure 20 shows a fit of Equation (1) to the experimental points corresponding to the first 20 minutes of dissolution (green). The obtained dissolution rate is  $3.1 (0.2) \mu\text{g}\cdot\text{min}^{-1}$ . The blue curve belongs to the fit of the second profile, which gives a dissolution rate of  $0.302 (0.002) \mu\text{g}\cdot\text{min}^{-1}$ .

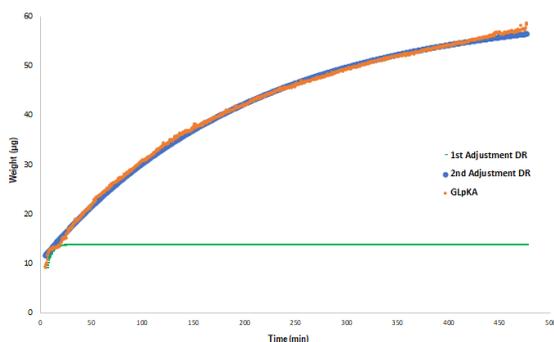


Figure 20. Dissolution rate corresponding to 8 hours experiment at pH 2. Orange: Experimental points  
Green: fit of the first 20 minutes of dissolution. Blue: fit of the rest of the curve.

After these preliminary experiments, tablets of 6-7 mg were done for the rest of experimental, and pH 2 was selected as an appropriate pH, as our objective was to work with a compound with much lower solubility than trazodone.

Dissolution rate experiments were 120 minutes long and in case that two profiles were observed, the whole curve was fitted to Equation 1.

## 7.2. DISSOLUTION RATE MEASUREMENTS

Phosphate/acetate buffer was added before starting the dissolution to keep a constant pH all time, and pH was adjusted to 2 by the titration system. The experiments were carried in two different ways: DR determined spectrophotometrically (*in-situ*) without doing exactions and with doing it. The extracts were analysed *off-line* by HPLC in order to compare the results.

### 7.2.1. Dissolution rate determined spectrophotometrically without extractions.

Figure 21 shows the dissolution rate determined spectrophotometrically *in-situ* of three replicates without carrying out sample extractions.

In all cases it is observed that warfarin does not reach a constant concentration value after two hours of experiment, and when GI method finished, the tablet was not completely dissolved. However, as concentration working range involve low concentrations ( $\mu\text{g}$ ), the more dispersion between curves is seen in the graphic (Figure 21).

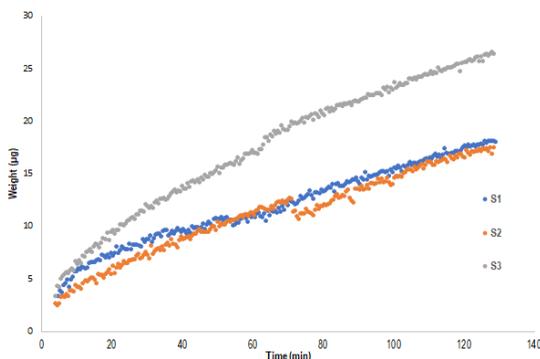


Figure 21. GLpKa experimental values of three samples without extractions.

The first order Equation 1 was adjusted to the experimental values obtained spectrophotometrically, in order to obtain the  $S$ ,  $K_d$  and  $t_0$  parameters. Table 5 shows the results of obtained parameters of  $S$ ,  $K_d$  and DR with the corresponding standard deviation in brackets.

In all tables, standard deviations associated to the parameters are provided by the fit of Equation 1, and standard deviations of the arithmetic mean have been calculated through the different parameters values.

The solubility value obtained is extrapolated to the amount drug dissolved in two hours of experiment because now, the solubility cannot be estimated from the plateau for the reason that warfarin does not reach it. It must be noticed that there are some factors we cannot control such as possible temperature changes or little differences during the tablet press which can affect the DR measurement because, as we are working at small scale, the minimum change is reflected in the parameters. In this case, warfarin did not reach the plateau, for this reason the obtained solubility value is extrapolated, and it has a significant SD.

Table 5. Solubility,  $K_d$  and DR values with their SD of three samples without extractions.

Sample	S [ $\mu\text{g}$ ]	$K_d$ [ $\text{min}^{-1}$ ]	DR [ $\mu\text{g}\cdot\text{min}^{-1}$ ]
1	25,5 (0.2)	0,0127 (0.0003)	0,32 (0.01)
2	29,2 (0,4)	0,00833 (0.0002)	0,24 (0.01)
3	33,2 (0.3)	0,0108 (0.0003)	0,36 (0.01)
<b>Arithmetic mean</b>	28 (3)	0.010 (0.002)	0.29 (0.08)

### 7.2.2. Dissolution rate determined spectrophotometrically with extractions.

Figure 22 shows the experimental points corresponding to the dissolution rate determined spectrophotometrically from five samples. Ten extractions of 50  $\mu\text{L}$  at different times and *off-line* analysis have been done to those sample through the HPLC.

As before, the curves do not reach the plateau after 120 minutes of experiment. The results are similar to the ones shown previously without doing extractions. Once the quantification was finished, the tablet was not completely dissolved.

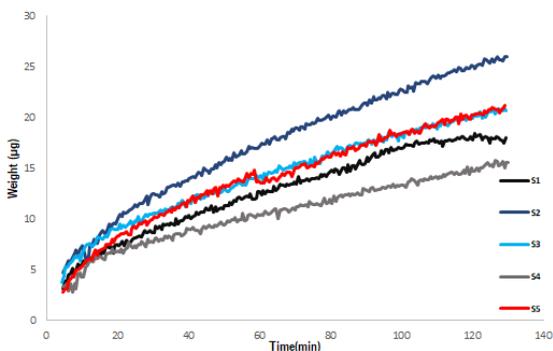


Figure 22. GLpKa experimental values of five samples with extractions.

Table 6 shows the parameters of S,  $K_d$  and DR with its standard deviations.

Table 6. Solubility,  $K_d$  and DR obtained spectrophotometrically values with their SD of five samples with extractions.

Sample	S [ $\mu\text{g}$ ]	$K_d$ [ $\text{min}^{-1}$ ]	DR [ $\mu\text{g}\cdot\text{min}^{-1}$ ]
1	23.5 (0.2)	0.0164 (0.0004)	0.38 (0.01)
2	38.4 (0.7)	0.0103 (0.0004)	0.39 (0.02)
3	29.0 (0.6)	0.0082 (0.0003)	0.23 (0.01)
4	24.8 (0.4)	0.0099 (0.0004)	0.25 (0.02)
5	33.2 (0.2)	0.0118 (0.0002)	0.392 (0.008)
<b>Arithmetic mean</b>	29 (13)	0.012 (0.003)	0.3 (0.2)

Comparing DR value doing extractions  $0.3 (0.1) \mu\text{g}\cdot\text{min}^{-1}$  (Table 6) and without doing extractions  $0.29 (0.08) \mu\text{g}\cdot\text{min}^{-1}$  (Table 5) it is observed that there are no significant differences between them. In order to compare if both procedures are equivalent to each other, Figure 23 shows the average curve profile doing and not doing extractions. The orange profile represents the experimental points (doing extractions) of dissolution rate, with the standard deviations as error bars. The blue one represents the experimental values analysed spectrophotometrically without carrying out extractions also with the error bars. Figure 23 proves the fact that doing 10 extractions of  $50\mu\text{L}$  does not affect considerably the curve of dissolution rate, as both curves overlap.

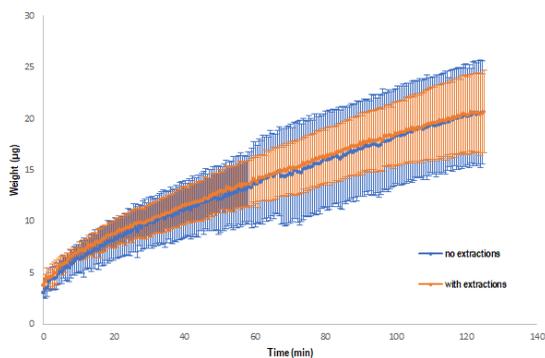


Figure 23. Comparison of average curves with their respective errors.

### 7.2.3. Dissolution rate profiles obtained by off-line quantification.

The 10 extracts (50  $\mu\text{L}$ ) of the sample are chromatographically analysed in order to compare the obtained values to those obtained previously with GLpKa.

The calibration curves obtained which have been used to quantify most of the samples had a  $r^2$  value of 0.999 (calibration curve in Appendix 2). As chromatographic peaks areas of the extracts were not considerably higher than the most concentrated standard, dilution was not performed. The extracts were quantified the same day of the extraction.

Figure 24A shows the techniques (HPLC and spectroscopic values) comparison for one sample. Chromatographic values are below those obtained spectrophotometrically. This might be due to the appearance of a precipitate which could be caused by the manipulation of the solution during the extraction process. To prove this, the samples, immediately after each extraction, were diluted in methanol (warfarin is moderately soluble in methanol) in order to re-dissolve all the possible particles in suspension. Figure 24B shows chromatographic values of samples diluted in methanol. As we expected, they have increased with respect to the undiluted ones. Performing extractions may affect the amount of drug in solution for those drugs with a poor solubility in water, which difficult the chromatographic quantification *off-line*.

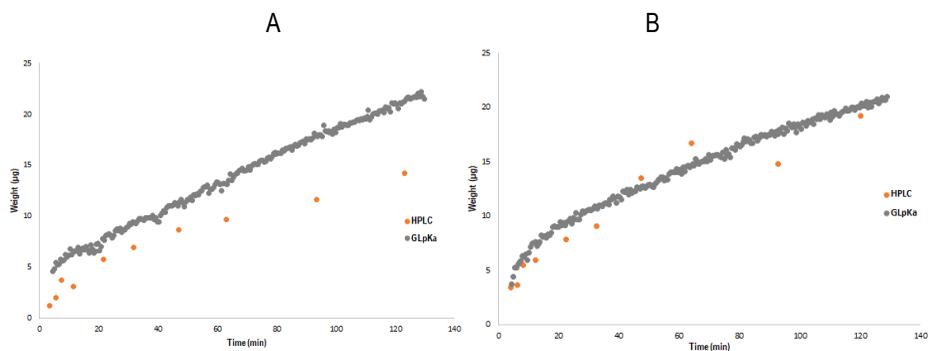


Figure 24. GLpKa (grey) and chromatographic (orange) experimental values of (A) sample with extractions without dilution (B) sample with extraction and dilution with methanol.

As the fact of diluting the extracts with methanol raised the chromatographic quantification to the one performed spectrophotometrically, all extracts were dissolved in methanol immediately

after each extraction. The first order Equation 1 was adjusted to the experimental values. Figure 25 shows how Equation 1 fits well the experimental points for one of the samples.

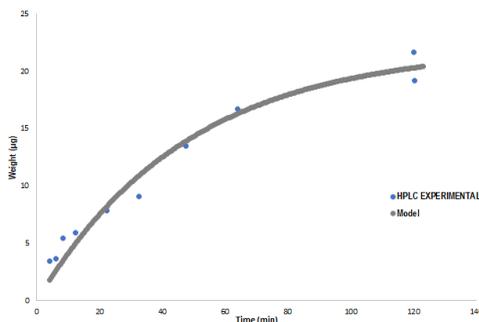


Figure 25. Experimental values of HPLC and predicted values by the model of a sample.

Table 7 shows the parameters values of  $S$ ,  $K_d$  and  $DR$  with the corresponding standard deviations in brackets. As before, the solubility obtained value is extrapolated because warfarin does not reach the plateau. As mentioned previously, there are uncontrollable factors which can produce little changes in the  $DR$  measurements. As we are working in a small scale, these changes can be reflected in the parameters values.

Table 7. Solubility,  $K_d$ , and  $DR$  obtained values with their SD of three samples HPLC quantified.

Sample	$S$ [ $\mu\text{g}$ ]	$K_d$ [ $\text{min}^{-1}$ ]	$DR$ [ $\mu\text{g}\cdot\text{min}^{-1}$ ]
1	22 (2)	0.02 (0.004)	0.5 (0.1)
2	23 (2)	0.021 (0.003)	0.5 (0.1)
3	14 (5)	0.02 (0.01)	0.2 (0.03)
<b>Arithmetic mean</b>	19 (5)	0.02 (0.002)	0.4 (0.1)

In-situ (doing extractions) and off-line  $DR$  values have been  $0.3$  ( $0.2$ )  $\mu\text{g}\cdot\text{min}^{-1}$  (Table 6) and  $0.4$  ( $0.1$ )  $\mu\text{g}\cdot\text{min}^{-1}$  (Table 7) respectively. They do not present significant differences because arithmetic mean in both quantifications are similar and they are within the error range. The obtained  $DR$  value is smaller than the value of trazodone. If we look at the dissolution rate value it can be concluded that warfarin has a low dissolution rate, which is usually associated with a low solubility.

In order to compare better if there are differences between doing the quantification in-situ or off-line, Figure 26 shows the average curves profile (with their standard deviation such as error bars) based on *in situ* and *off-line* studies. The orange curve is the average experimental spectrophotometric points, and HPLC experimental values fall within the same concentrations when the standard deviations are considered.

To conclude, it has been demonstrated that in the case of warfarin, doing the quantification in-situ and off-line is equivalent if the extracts have been dissolved previously in methanol in order to re-dissolve all the possible precipitate. However, we might not be dissolving all the precipitate because warfarin is only moderately soluble in methanol. It could be a reason to explain that chromatographic values, even if they are within the error range, are slightly displaced downwards. To verify it, the extracts should be dissolved in other solvent that better solubilizes warfarin such as acetone. Nonetheless, the low concentration range in which we are moving could be also the reason of the observed differences.

Both techniques would probably be valid for those drugs which present an intrinsic solubility(M) higher than  $\log S_0 = -4.33$ . To continue this study, a drug with a lower solubility than warfarin should be tested because maybe we are in the limit to consider quantifications off-line and in-situ equivalent.

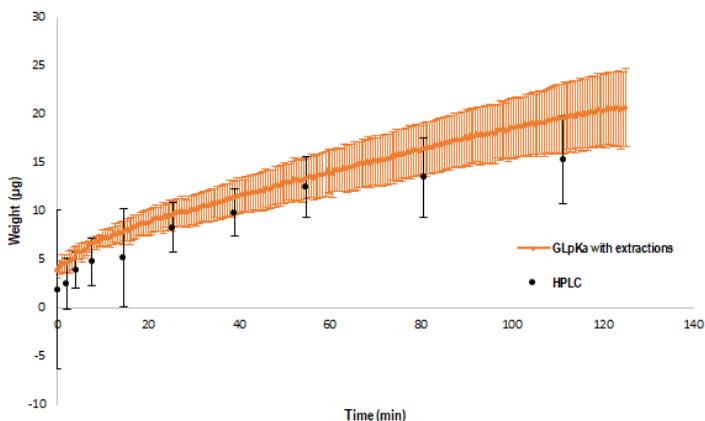


Figure 26. Comparison in-situ (GLpKa) and of-line (HPLC) average curves with their corresponding SD as error bars.



## 10. CONCLUSIONS

The fact of extracting 10 samples of 50  $\mu\text{L}$  at different times for trazodone and warfarin, does not significantly affect the dissolution rate profile obtained through the GI *dissolution* method. In both cases, the calculated DR parameter (doing extractions and without doing it) was within the tolerance range, proving that the results obtained with miniaturized are not different when sample extraction is performed.

The study off-line by HPLC of trazodone's dissolution rate does not differ significantly from in situ-study by GI dissolution method in a low volume. However, warfarin has a lower solubility and differences are observed between the two profiles. The dilution of the extracts with a compatible solvent increases the concentrations obtained by HPLC, making them equivalent to the ones determined spectrophotometrically. This indicates that poor-water solubility of warfarin can limit the quantification off-line by HPLC if a cosolvent is not used.

A dissolution rate study of drug with a higher solubility than warfarin and lower than trazodone should be performed in order to determine the solubility value from which off-line study cannot be performed without using a cosolvent. The study of a drug with lower solubility than warfarin should also be done in order to determine the solubility value which limits completely the quantification off-line by HPLC (even dissolving extracts in a cosolvent).



## 11. REFERENCES AND NOTES

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## 12. ACRONYMS

**ADME** Absorption, Distribution, Metabolism and Excretion

**API** Active Pharmaceutical Ingredient

**BCS** Biopharmaceutics Classification System

**DMSO** Dimethyl sulfoxide

**D-PAS** Dip Probe Absorption Spectroscopy

**DR** Dissolution Rate

**GI** Gastrointestinal

**GIT** Gastrointestinal Tract

**HPLC** High Performance Liquid Chromatography

**IDR** Intrinsic Dissolution Rate

**ISA water** Ionic Strength Adjusted water

**MEC** Molar Extinction Coefficient

**MeOH** Methanol

**SD** Standard Deviation

**USP** United States Pharmacopeia

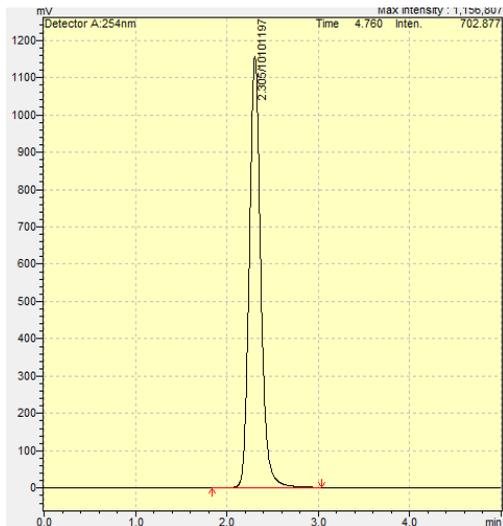
**UV** Ultraviolet



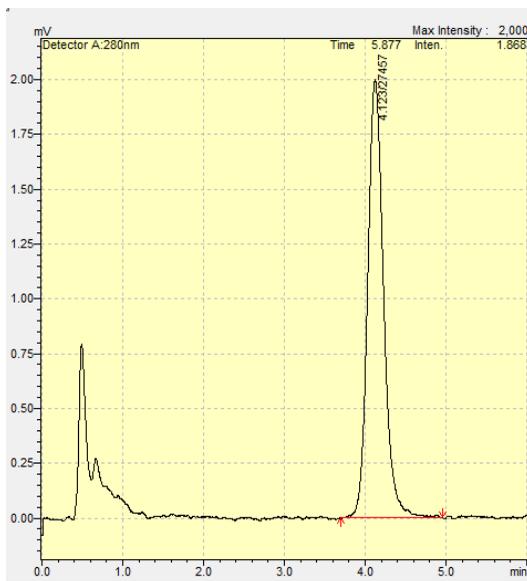
# APPENDICES



# APPENDIX 1: CHROMATOGRAMS

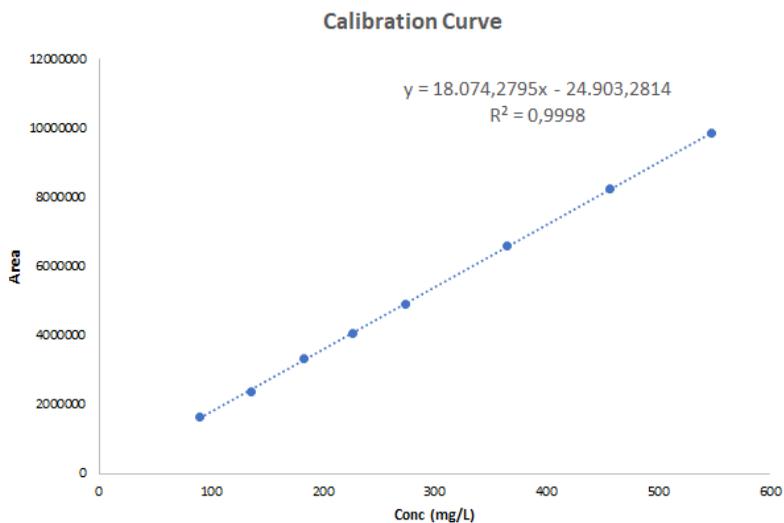


Chromatogram from a 600 mg/L trazodone hydrochloride standard solution.

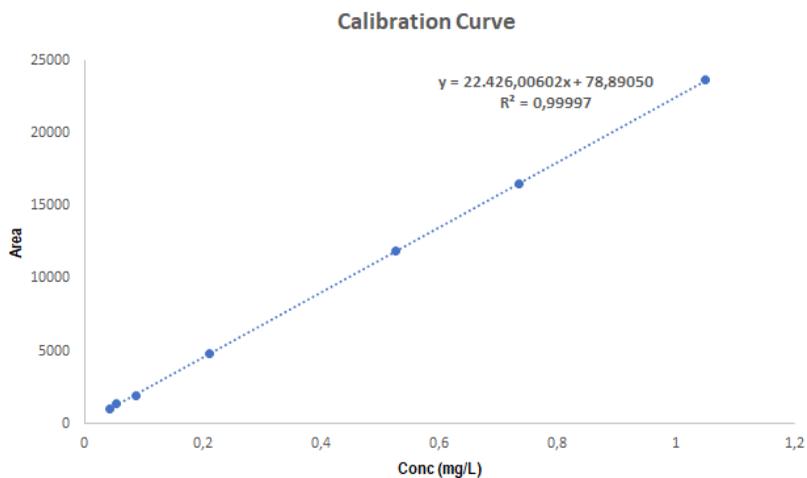


Chromatogram from a 1 mg/L warfarin standard solution.

## APPENDIX 2: CALIBRATION CURVES



Calibration curve of trazodone hydrochloride.



Calibration curve of warfarin.

