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Treball Final de Grau

Solubility determination of compounds of pharmaceutical interest.
Determinació de la solubilitat de compostos d'interès farmacèutic.

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Agrair a tot el grup de recerca de PhysChem el bon ambient de treball al laboratori, concretament a Diego amb qui més he compartit el dia a dia. També a Clara i Xavi per l'ajuda constant durant tot aquest temps.

I en especial, a la meva família i amics que han estat al meu costat al llarg de tot el grau fent-me passar els millors moments.

REPORT

CONTENTS

1. SUMMARY

Solubility determination is one of the most important parameters to study when developing a new drug. Solubility gives information about the tendency of the compound to be dissolved when having a particular liquid media surrounding it. Ensuring the drug solubility in body fluids enhances bioavailability which is completely necessary to have the desired effect on cells.

A significant factor that must be taken into consideration while determining solubilities is the possibility of most compounds of pharmaceutical interest to have ionisable groups. The presence of acid and/or basic groups in the molecule would imply a variation of the solubility with the pH. Therefore, depending of the pH of the media and the pK_a of the molecule, it will be ionised or in its neutral form and as a consequence, its solubility will vary.

In this work, two ionisable drugs relatively insoluble have been studied: pioglitazone which is an amphoteric substance and glimepiride which is an acidic compound.

In order to determine solubility, shake-flask method has been used. Although there are other recognized procedures, shake-flask is the reference method to carry out solubility determinations. It consists of reaching the thermodynamic equilibrium between a saturated solution and the precipitated solid at different pH. Then, the supernatant is analysed through HPLC to determine solubility and, as the solubility is measured for the solid in equilibrium with the saturated solution, the solid is analysed by X-Ray diffraction to know which compound and in which polymorphic form is being studied. As a result, the solubility-pH profile of the compound is obtained.

Keywords: Solubility, drug, ionisable group, shake-flask, X-Ray diffraction, solubility-pH profile.

2. RESUM

La solubilitat és un dels paràmetres més importants que cal estudiar quan es s'investiga el desenvolupament d'un nou fàrmac, dóna informació sobre la tendència del compost a dissoldre's en presència d'un medi líquid concret. Garantint la solubilitat del principi actiu en els fluids corporals es potencia la biodisponibilitat que és completament necessària per tenir els efectes desitjats en les cèl·lules.

Un factor rellevant que s'ha de tenir en compte quan es determinen solubilitats és l'alta possibilitat dels compostos d'interès farmacèutic de tenir grups ionitzables. La presència de grups àcids i/o bàsics a la molècula implicaria una variació de la solubilitat amb el pH. Per tant, depenent del pH del medi i el pK_a de la molècula, aquesta estarà ionitzada o en la seva forma neutra i com a conseqüència, la seva solubilitat variarà.

En aquest treball, s'han estudiat dos fàrmacs ionitzables relativament insolubles: la pioglitazona, un compost amfòter, i la glimepirida, un compost àcid.

Per la determinació de solubilitat, s'ha seguit el mètode shake-flask. Encara que hi ha altres procediments reconeguts, el shake-flask és el mètode de referència per dur a terme determinacions de solubilitat. El mètode consisteix en establir l'equilibri termodinàmic entre una solució saturada i el precipitat sòlid a diferents pH. A continuació, el sobrenedant és analitzat mitjançant HPLC per tal de determinar la solubilitat i, com que la solubilitat que es mesura és la del sòlid en equilibri amb la solució saturada, el sòlid s'analitza mitjançant difracció de raig X per conèixer quin compost i en quina forma polimòrfica està sent estudiat. Com a resultat s'obté el perfil de solubilitat del compost.

Paraules clau: Solubilitat, fàrmac, grup ionitzable, shake-flask, difracció de raig X, perfil de solubilitat.

3. INTRODUCTION

Solubility determination of drug candidates is important in drugs research, both in discovery and development stages. Physicochemical characterization (not only solubility) in early stages is a key point for saving money and time and avoiding the development of a not viable drug. Despite the wide variety of physicochemical parameters, solubility is one of the most important features to be determined so that drug solubility in aqueous media can be ensured and consequently, its proper oral absorption. As the pH of the human body changes in its different parts, solubility has to be determined in a wide range of pH to be able to compare it to each pH of the digestive system and know where the active pharmaceutical ingredient (API) is more likely to be dissolved in [1,2].

Not only does the determination of solubility consist of measuring the compound in a saturated solution but also of analysing the solid in equilibrium with the solution to know the solubility of which compound and in which form is being measured.

In case the drug is not enough soluble, an effective and recurrent way to increase drug solubility and dissolution rate is salt formation. In general, salts of basic and acidic drugs have a higher solubility than their corresponding base forms. However, paying careful attention to identify and develop an optimal salt form is essential to ensure the desired solubility raise [3,4].

3.1. SOLUBILITY DEFINITIONS

Solubility is the property of solute to dissolve in a solvent and it depends on the solute chemical parameters and other external ones such as pH or temperature. In case of drug solubility, it is as important to determine it in aqueous media (gastrointestinal fluids and blood) as in lipid based media (cell membranes). In this study, only aqueous solubility will be taken into account. The solubility in water, S_w, is defined by the saturation concentration of the substance in water [5,6].

Other relevant solubility definitions are kinetic and equilibrium solubilities. Kinetic solubility, S_k, is defined as the concentration of a compound at the time when an induced precipitate first appears in the solution. However, when the compound is in a saturated solution with excess of

solid precipitated, then the equilibrium solubility is reached. When working with ionisable substances, talking about intrinsic solubility and solubility at a determined pH has sense. Intrinsic solubility, S_0, refers to the equilibrium solubility of the free acid or base form of an ionisable compound at a pH where it is fully un-ionised. For the same kind of compounds, solubility at a certain pH, S_{pH}, is defined as the sum of the concentrations of all compounds species dissolved in the aqueous solution.

Solubility is generally expressed in mg/mL, moles/L or log*S* (decimal logarithm of solubility expressed as moles/L) [1].

3.2. SOLUBILITY-PH PROFILES

The theoretical solubility-pH profile of a compound can be obtained through the Henderson-Hasselbalch (HH) equations just using the pK_as and the intrinsic solubility of the studied compound [2].

3.2.1. Solubility of acidic substances

In the case of an acidic compound, the equilibriums that would take place in an aqueous solution are represented in figure 1 where HA represents the neutral form of the acid and A^- the deprotonated one.

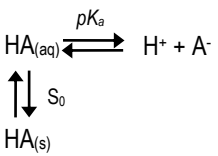

Figure 1. Acid-base and solubility equilibriums of an acid substance HA.

The mathematical equations for each equilibrium are:

$$HA_{(s)} \rightleftharpoons HA_{(aq)} \qquad S_0=[HA_{(aq)}] \qquad (1)$$

$$HA_{(aq)} \rightleftharpoons H^+ + A^- \qquad K_a=[A^-][H^+]/[HA] \qquad (2)$$

At a determined pH, the solubility is described as follows:

$$S_{pH}=[HA]+[A^-] \qquad (3)$$

Finally, combining all the 3 equations above, the HH equation for acid compounds is obtained.

$$\log S=\log S_0+\log(1+10^{pH-pKa}) \qquad (4)$$

If logS_0 and pK_a are known, the theoretical solubility-pH profile of an acid substance can be represented using equation (4). Figure 2 shows the solubility-pH profile for a hypothetical acid compound of logS_0=-3 and pK_a=7.

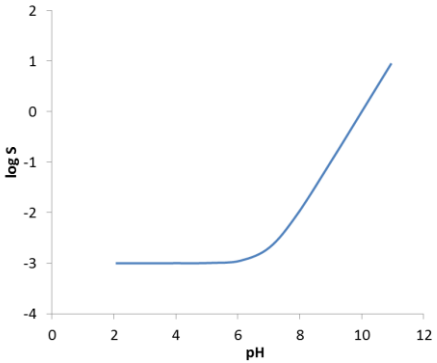

Figure 2. Solubility-pH profile for a hypothetical acid compound of logS_0=-3 and pK_a=7.

As figure 2 depicts, the minimum solubility of an acidic compound is found under its pK_a where the log$S$ is constant and corresponds to logS_0. When pH is higher than its pK_a the solubility increases performing a line of slope +1 as the pH rises.

3.2.2. Solubility of amphoteric substances

As it has been previously explained for acidic compounds, the HH equations can also be deduced for amphoteric compounds.

In this case, the equilibriums that an amphoteric molecule would suffer in an aqueous solution are represented in figure 3 where H_2X^+ represents the protonated molecule, HX the neutral form of the amphoteric substance and X^- the deprotonated one.

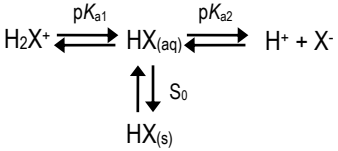

Figure 3. Acid-base and solubility equilibriums of an amphoteric substance HX.

The mathematical equations for each equilibrium are:

$$HX_{(s)} \rightleftharpoons HX_{(aq)} \qquad S_0=[HX_{(aq)}] \qquad (5)$$

$$H_2X^+ \rightleftharpoons HX_{(aq)} + H^+ \qquad K_{a1}=[HX][H^+]/[H_2X^+] \qquad (6)$$

$$HX_{(aq)} \rightleftharpoons H^+ + X^- \qquad K_{a2}=[X^-][H^+]/[HX] \qquad (7)$$

At a determined pH, the solubility is described as follows:

$$S_{pH}=[HX]+[X^-]+[H_2X^+] \qquad (8)$$

Finally, combining all the equations above the HH equation for amphoteric compound is obtained.

$$\log S=\log S_0+\log(1+10^{pK_{a1}-pH}+10^{pH-pK_{a2}}) \qquad (9)$$

If $\log S_0$, pK_{a1} and pK_{a2} are known, as a result of using equation (9), the theoretical curve for solubility-pH profile of an amphoteric substance is deduced, figure 4. It shows the solubility-pH profile for a hypothetical amphoteric compound of $\log S_0$=-3, pK_{a1}=4 and pK_{a2}=9.

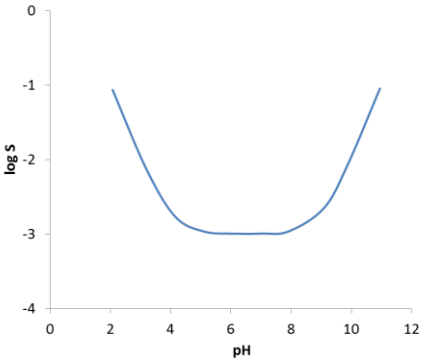

Figure 4. Solubility-pH profile for a hypothetical amphoteric compound of $\log S_0$=-3, pK_{a1}=4 and pK_{a2}=9.

As figure 4 deploys, the minimum solubility of an amphoteric compound is found between its two pK_a, where the $\log S$ is constant and corresponds to $\log S_0$. Therefore, if there is enough difference of pH between the two pK_as of the compound, the $\log S_0$ can be deduced. When pH is lower than its pK_{a1}, the solubility increases performing a line of slope -1 as the pH decreases. The same way, when pH is higher than its pK_{a2}, the solubility increases performing a line of slope +1 as the pH rises.

3.3. METHODS TO MEASURE SOLUBILITY

3.3.1. Shake-flask method

Shake-flask is the method used in this study to determine solubility. In fact, it is the reference one. It consists of two important but different parts; first of all samples are vigorously agitated in different pH buffers in presence of solid phase and then, they sediment until they reach the equilibrium. Once the procedure has finished, the liquid and solid phase are separated and they are analysed independently. There are some variables that must be considered because they might have an effect on the reliability of results [1].

3.1.1.1. Buffer solution

Solubility of ionisable drugs depends on pH, therefore a buffer solution is required to maintain the pH stable when studying ionisable drugs at an specific pH value.

There are multiple buffers solutions which have a buffer effect on a wide range of pH such as Britton-Robinson buffer, Sörensen buffer or the minimalist universal buffer (MUB). Nevertheless, depending on the chosen buffer the solubility determination might be affected by the variation of ionic strength of the buffer with the pH. A proper option is to use the MUB whose ionic strength keeps constant in all its range of buffer capacity as it can be observed in figure 5 [5].

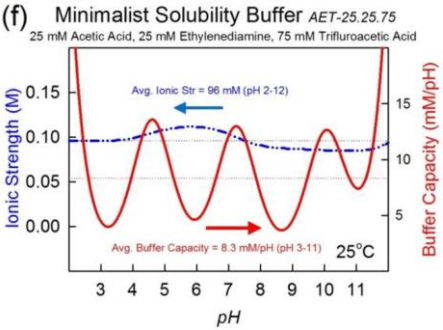

Figure 5. Buffer capacity and ionic strength of minimalist universal buffer as function of pH. [5]

3.3.1.2 Amount of solid excess

In shake-flask method, an amount of solid excess is required to reach equilibrium with the dissolved solid. Studies that have been carried out show that the amount of solid excess does

not affect to the final solubility result whereas it might influence the solubility rate. The advice is to weight small amount of solid (5-10mg/5ml) in order to avoid difficulties in sampling [1].

3.3.1.3 Temperature

The dependence of solubility on temperature is well known due to the fact that temperature has an effect on equilibrium constants. This fact implies the necessity of a controlled and constant temperature during the shake-flask procedure. In general, drugs have an endothermic dissolution process and consequently solubility increases when there is a temperature rise. [5]

3.3.1.4 Time of stirring

Time of stirring has to be controlled because it is necessary to ensure enough time to dissolve the maximum possible quantity of compound.

Figure 6 shows an increase of solubility until 6 hours of stirring however, from 6 to 48 hours there are no significant differences. As a consequence, the minimum stirring time is six hours although a longer time will not have a negative impact on the results [1].

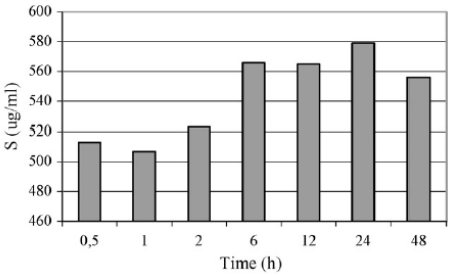

Figure 6. Variation of solubility of hydrochlorothiazide with stirring time. [3]

3.3.1.5 Time of sedimentation

After the stirring process, a supersaturated solution is obtained where the solute concentration exceeds the concentration of the real thermodynamic equilibrium. In order to obtain the real solubility and not a higher one, time of sedimentation has also to be controlled to ensure enough time to reach the equilibrium [5].

In figure 7, it can be observed how the solubility decreases as the equilibration time increases due to the fact that equilibrium is not reached before 24 hours of sedimentation. Therefore, the samples need to sediment at least for 24 hours [1].

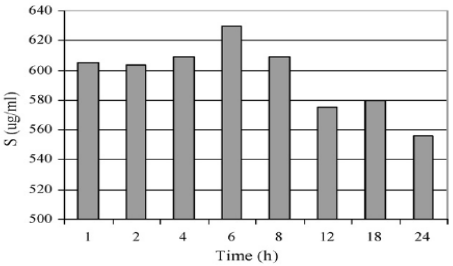

Figure 7. Variation of solubility of hydrochlorothiazide with sedimentation time [1].

3.3.1.6 Separation of solid and liquid phases

When separating the solid and liquid phases after the sedimentation time, it is important to ensure that aliquots taken are completely transparent and free of solid particles which might lead to a higher solubility result than real.

Two separation techniques are compared in studies: centrifugation and filtration. The obtained results showed that when using filtration the solubility results were further to reality than with centrifugation. With filtration unreal results could be obtained: a higher solubility caused by particles that are still suspended in the solution which are smaller than the porus size and cannot be separated and also a lower solubility because of a possible favourable interaction between the filter and the molecule. Therefore, centrifugation seems a clear better option to separate phases in shake-flask method [1,7].

3.4. X-RAY CRYSTALLOGRAPHY

X-ray crystallography is a technique used to determine the atomic and molecular structure of a crystal. The incidental X-rays are diffracted by the electrons surrounding the atoms because their atomic radius are of the same order of magnitude as the incidental wavelength. The X-ray beam emerging after this interaction contains information about the position and type of atoms found along the ray path. By measuring the angles and intensities of these diffracted beams, a three dimensional picture of the density of the electrons in the crystal can be deduced. Consequently, from this electron density, the main positions of the atoms in the crystal can be determined, as well as their chemical bonds.

3.5. STUDIED DRUGS

The most significant characteristics of the pharmaceutical drugs that have been studied are explained below. Pioglitazone and glimepiride are used to treat diabetes mellitus type 2 (T2DM) which is a type of diabetes caused by cells that fail to respond to insulin properly although the pancreas does produce insulin effectively. Both drugs act increasing the activity of intracellular insulin receptors in order to decrease blood sugar levels [8].

3.5.1. Pioglitazone

Pioglitazone is an amphoteric molecule whose structure is shown in figure 8. It has a basic centre (N, marked in green) with a pK_a of 5.65 and an acid centre (NH, marked in red) with a pK_a of 6.62. As it is an amphoteric substance, its solubility-pH profile is expected to be like figure 4 with its minimum solubility around pH 6 [9].

Its molecular weight is 356.44 g/mol.

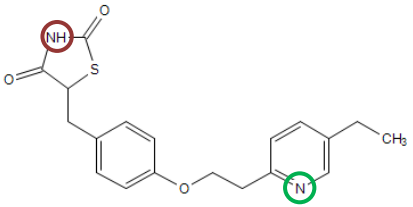

Figure 8. Pioglitazone structure.

Its hydrochloride salt is also studied. Its solubility-pH profile is expected to be the same as for pioglitazone; it would only change if the solid in the saturated solution was different from pioglitazone or a different polymorphic form.

3.5.2. Glimepiride

Glimepiride is an acidic molecule whose structure is shown in figure 9. It has an acid centre with a pK_a of 5.22 (N, marked in red). As it is an acidic substance, its solubility-pH profile is expected to be like figure 2 [9].

Its molecular weight is 490.62 g/mol.

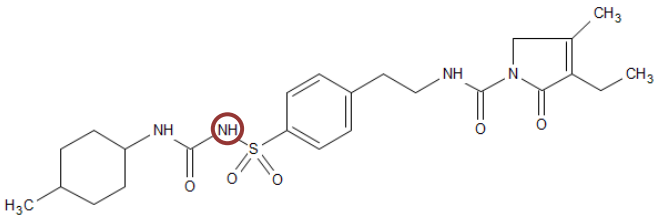

Figure 9. Glimepiride stucture.

4. OBJECTIVES

The main objective of this study is to determine the solubility-pH profiles of molecules of pharmaceutical interest, specifically pioglitazone and glimepiride. As they are active ingredients of drugs, ensuring the solubility in body fluids enhances bioavailability which is completely necessary to have the desired effect on cells. Both pioglitazone and glimepiride are ionisable substances therefore, their solubility vary with pH. Solubility will be determined at a wide range of pH so that a relationship between the solubility-pH profile and the pH at the different body parts can be established.

5. EXPERIMENTAL SECTION

5.1. REAGENTS AND MATERIALS

5.1.1. Reagents

Water used was purified by a Milli-Q gradient system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩ·cm.

The buffer used in the shake-flask method was prepared using acetic acid (HAc) (99.0-100%), ethylendiamine (en) (98.8%) from J.T.Baker (Deventer, Holland) and trifluoroacetic acid (TFA) (>99%) from Merck (Darmstadt, Germany). The pH adjustment was done with 0.5 M sodium hydroxide solution from Merck.

Two different buffers were used for HPLC mobile phase. One of them was the formate buffer prepared with formic acid (99.5%) from J.T.Baker and sodium hydroxide (>98%) from Sigma-Aldrich (St. Louis, Missouri, USA) and the other one was the ammonium buffer prepared with ammonia 25% and chlorhydric acid 30% both from Merck.

5.1.2. Studied drugs

The pharmaceutical studied drugs, pioglitazone and glimepiride, were supplied by the Unitat de Polimorfisme dels Centres Científics i Tecnològics de la UB, Barcelona, Spain.

5.2. INSTRUMENTATION

Two rotational stirrers Movil-ROD from Selecta (Abrera, Spain) were used to shake the samples. In order to measure pH, a pH electrode connected to a potentiometer GLP 22 from Crison (Alella, Spain) was employed. The centrifugation of the samples were carried out using a Rotanta 460RS from Hettich (Tuttlingen, Germany).

Regarding the quantification of the pioglitazone supernatant in the shake-flask method, a liquid chromatograph from Shimadzu (Kyoto, Spain) was used. The chromatograph consists of two LC-30AD pumps which include a degasser, a SPD-M20A UV-Vis diode array detector, a

CTO-10ASVP column oven which controls the temperature at 25 ºC and a SIL-30AC automatic injector. The column, which is connected to a pre-column, is an Acquity BEH C18 reversed phase column from Waters (Mildford, MA, USA), its particle size is 1,7 µm and its dimensions 2,1x50 mm.

With reference to the quantification of the glimepiride supernatant in the shake-flask method, a different liquid chromatograph from Shimadzu was used. The chromatograph consists of two LC-20AD pumps which include a degasser, a SPD-10AV UV-Vis detector, a CTO-10ASVP column oven which controls the temperature at 25 ºC and a SIL-20AC HT autosampler. The column is a Symmetry C18 reverse phase column from Waters, its particle size is 5 µm and its dimensions 4,6x150 mm.

For X-Ray diffraction, the instrument used was a PANalytical X'Pert PRO MPD 0/0 power diffractometer of 240 mm of radius equipped with a PIXcel detector from PANalytical B.V. (Almelo, Netherlands).

5.3. PROCEDURES

5.3.1. Shake-flask method

The MUB was used to control the pH during the shake-flask method. First of all, a solution of 50 mM HAc, 50 mM en and 150 mM TFA was prepared. Then, an aliquot of 25 mL was taken and adjusted at the desired pH using the necessary quantity of NaOH in each case. Finally, it was brought to a final volume of 50 mL reaching a concentration of 25 mM HAc, 25 mM en and 75 mM TFA and an ionic strength of 0.125 M. As a result, different buffers at different pH were obtained.

To start with the shake-flask procedure itself, between 5 mg and 20 mg of solid were weighed. There has to be enough solid to saturate the solution, therefore 3 times the expected solubility has to be weighed. An aliquot of 3 mL of the buffer at the desired pH was added to the solid. The shake-flask experiment was repeated 3 times for each pH. Right after, the samples were stirred during 24 hours at 25 ºC. Four hours later, the pH was measured and adjusted, if necessary, to the initial pH. When the stirring time had ended, the samples rested during another 24 hours also at 25 ºC until the equilibrium was reached. Afterwards, the pH was measured so that the final conditions which the solubility will be determined in were known and the samples were centrifuged during 30 minutes at 25 ºC and 3500 rpm in order to separate

completely the solid from the saturated solution. Finally, the supernatant was separated from the solid with Pasteur pipettes. While the supernatant was analysed by liquid chromatography, the solid was filtered and dried to be analysed by X-ray diffraction.

Table 1 summarizes the shake-flask method conditions used, which have been previously optimized by the same research laboratory team.

Table 1. Conditions used in the shake-flask method.

Parameter	Conditions
Buffer	MUB (0.125 M)
Amount of solid	5-20 mg
Temperature	25 ºC
Stirring time	24 h
pH adjustment	After 4 h started stirring
Sedimentation time	24 h
Phase separation	Centrifugation

5.3.2. Quantification by liquid chromatography

For the liquid chromatography assays, a calibration curve for each drug was prepared. Standards were prepared with methanol:aqueous solution in the same percentage as the mobile phase used in the chromatographic conditions. The separation of substances was carried out in isocratic mode and the chromatogram was registered at the maximum absorption wavelength in order to obtain the maximum signal for each compound. The parameters of chromatographic conditions are described in table 2.

Table 2. Chromatographic conditions for each studied drug.

	Pioglitazone and its hydrochloride	Glimepiride
Aqueous phase; pH	HCOOH / $COOH^-$; 3.75	NH_4^+ / NH_3; 9
Organic phase	MeOH	MeOH
Separation mode	Isocratic	Isocratic
% Organic phase	45	60
Total time [min]	5	10
Calibration curve [mg/L]	0.05-100	0.1-100
λ [nm]	267	225
Injection volume [µL]	10	10

5.3.3. Ray-X diffraction

The solid remaining after removal of the supernatant with the Pasteur pipette is filtered and dried.

The XRDs were performed in the CCiTUB by external staff.

In this study, the diffractograms of the samples obtained in the different pHs were compared with a standard sample of high purity to determine which solid is in equilibrium with the solution at each pH.

6. RESULTS AND DISCUSSION

6.1. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR PIOGLITAZONE DETERMINATION

In order to establish the chromatographic conditions, a standard of 50 mg pioglitazone/L is prepared. Several trials with different flows and mobile phase compositions are carried out. Finally, the chosen method has the following conditions: a flow of 0.5 mL/min, a composition of the mobile phase of MeOH:HCOOH/NaCOOH (45:55) pH=3.75. Using this method, the pioglitazone peak appears at 1.1 minutes which is enough separated from the dead time peak and does not imply a long time of analysis, 2 minutes are enough. The working wavelength is 267 nm where the pioglitazone has a maximum of absorption in its spectrum.

Whereas at first 2 minutes of analysis seem to be enough, when the firsts samples are injected in the chromatograph, other peaks appear between 3 and 4 minutes, so the total time of analysis has to be 5 minutes.

With reference to the working pH, it is 3.75 where the pioglitazone will be positively charged and consequently, its solubility will be considerably high and the retention time will be shortened.

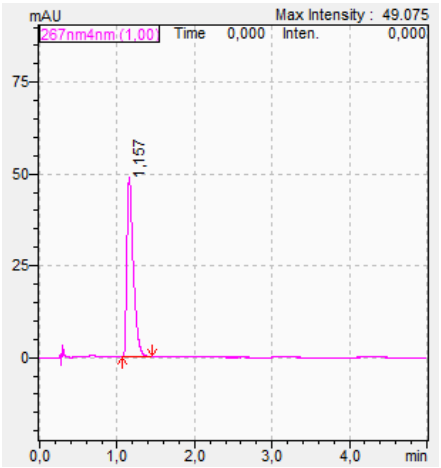

Figure 10. Chromatogram of standard 50 mg/L pioglitazone using the established chromatographic conditions.

6.1.1. Calibration curve

Once the chromatographic conditions have been established, standards between 1-100 mg/L dissolved in mobile phase are injected in the chromatograph. Figure 11 (right) shows the calibration curve obtained which has been used to quantify most of the samples. However, the areas of the chromatographic peaks of the samples with pH between 4 and 8 are considerably lower than the area of the less concentrated standard (1 mg/L). This fact suggests that the lower concentration range of the calibration curve would be better defined if more diluted standards (0.01-2.5 mg/L) were considered. It should be pointed out that all standards used must be above the limit of quantification. For this reason, the limits of detection and quantification are calculated.

The limits of detection and quantification of the instruments are calculated following the next steps. First of all, different volumes (1 µL, 2.5 µL, 5 µL and 10 µL) of a standard 0.01 mg/L are injected. When 1 µL is injected, which corresponds to a concentration of 0.001 mg/L when considering a reference injection volume of 10 µL, it is hard to differentiate the peak from the noise signal of the base line whereas injecting 2.5 µL (0.0025 mg/L) the peak starts to become visible. Once this information is already known, the following volumes of the lowest standard (0.01 mg/L) are injected 10 times each: 2.5 µL (0.0025 mg/L), 5 µL (0.005 mg/L) and 10 µL (0.01 mg/L). Also 10 µL of the second standard (0.05 mg/L) is injected 10 times. With all of the obtained chromatograms, the average intensity (I_{noise}) and its associated standard deviation (s_{noise}) of the baseline next to the pioglitazone peak is calculated. As a result, the intensity for the limit of detection (I_{LOD}) and quantification (I_{LOQ}) of each peak can be calculated applying the equations (10) and (11) respectively.

$$I_{LOD}=I_{noise} + 3s_{noise} \qquad (10)$$

$$I_{LOQ}=I_{noise} + 10s_{noise} \qquad (11)$$

Then, if the intensity of a peak is higher than its corresponding I_{LOD}, the peak can be detected. At the same time, if the intensity of a peak is higher than its corresponding I_{LOQ}, the peak can be quantified. After treating the chromatograms, the conclusions obtained are: only the concentration of 0.05 mg/L is quantifiable nevertheless, from concentration 0.0025 mg/L all of them are detectable. Therefore, $I_{0.001mg/L} < I_{LOD} < I_{0.0025mg/L}$ which means that the concentration of the limit of detection is the concentration whose intensity corresponds to peaks of concentration between 0.001 mg/L and 0.0025 mg/L. The same way, $I_{0.01mg/L} < I_{LOQ} < I_{0.05mg/L}$

which means that the concentration of the limit of quantification is the concentration whose intensity corresponds to peaks of concentration between 0.01 mg/L and 0.05 mg/L.

Finally, a calibration curve in the lower concentration range from 0.05 mg/L to 5 mg/L is prepared (figure 11, left) and used to quantify the samples between pH 4 and 8.

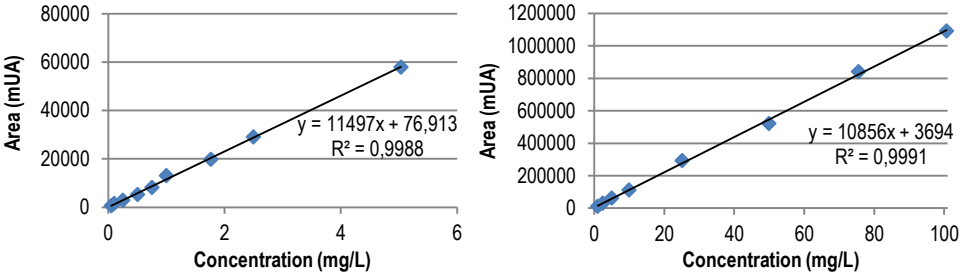

Figure 11. Calibration curve for pioglitazone 0.05-5 mg/L (left) and 1-100 mg/L (right).

6.2. PIOGLITAZONE SOLUBILITY-PH PROFILE DETERMINATION

The solubilities of pioglitazone and its hydrochloride salt have been determined through shake-flask method at different pH values between 2 and 11, three determinations have been done at each pH. As the pH has a great influence on the solubility, if the variation of the final pH in these repetitions is lower than 0.1 units of pH, the average has been calculated for all the repeated determinations and if it is higher, then, the repetitions have been treated as individual experiments.

Tables 3 and 4 show the obtained results including all pH variations during all shake-flask procedure for pioglitazone and its hydrochloride salt. These tables summarize different values of pH in consecutive columns: initial pH (pH_0), pH after 4 hours of stirring the samples (pH_{4h}) and pH at the end of the procedure (pH_f). They also show the log*S* and the solid state of the solid in equilibrium with the solution according to the XRD data.

Table 3. Results of shake-flask method for pioglitazone (standard deviations in brackets).

pH_0	pH_{4h}	pH_f	logS	Solid State
2.075	No data	2.167	-3.306	Neutral form
2.075	No data	2.035	-3.336	Neutral form
2.075	No data	1.979	-3.425	Neutral form

3.130	3.32(0.02)	3.2(0.1)	-4.06(0.09)	Neutral form
4.092	4.0(0.3)	4.22(0.01)	-5.2(0.2)	Neutral form
4.092	4.080	4.080	-4.959	Neutral form
4.950	4.9(0.1)	5.11(0.04)	-5.7(0.1)	Neutral form
6.110	5.9(0.2)	6.08(0.07)	-6.15(0.09)	Neutral form
6.980	6.862(0.021)	7.028(0.025)	-5.82(0.01)	Neutral form
7.995	7.80(0.01)	7.92(0.02)	-5.18(0.07)	Neutral form
9.120	8.948	9.254	-4.163	Neutral form
9.120	9.050(0.004)	9.09(0.04)	-4.62(0.04)	Neutral form
9.966	9.746(0.007)	9.87(0.01)	-3.79(0.04)	Neutral form
11.100	10.89(0.05)	10.96(0.05)	-2.74(0.06)	Neutral form

Table 4. Results of shake-flask method for pioglitazone hydrochloride (standard deviations in brackets).

pH_0	pH_{4h}	pH_f	log*S*	Solid State
2.055	2.213	2.360	-3.134	Neutral form
2.055	2.088	2.036	-3.157	Neutral form
2.055	2.103	2.058	-2.935	Neutral form
3.130	2.254	3.563	-4.773	Neutral form
3.130	2.337	3.771	-4.885	Neutral form
3.130	2.144	2.959	-4.311	Neutral form
3.977	3.60(0.08)	4.04(0.07)	-5.13(0.03)	Neutral form
4.950	4.66(0.02)	5.01(0.02)	-6.43(0.07)	Neutral form
5.995	5.56(0.02)	6.02(0.08)	-6.71(0.03)	Neutral form
6.980	6.56(0.01)	7.01(0.02)	-6.32(0.07)	Neutral form

7.968	7.67(0.03)	7.94(0.01)	-5.52(0.05)	Neutral form
9.120	8.24(0.05)	9.12(0.04)	-4.42(0.04)	Neutral form
9.991	9.71(0.01)	9.93(0.03)	-3.56(0.05)	Neutral form
11.100	10.54(0.03)	11.08(0.06)	-2.52(0.01)	Neutral form

As the solubility of ionisable molecules depends on the pH, it is important to control this parameter in order to maintain it as stable as possible. This is why after 4 hours of stirring pH is measured and readjusted if necessary. In the case of pioglitazone, pH does not change significantly. Therefore, experimentally it has been observed that pioglitazone does not break the buffering capacity of the buffer. However, table 4 shows how pHs of pioglitazone hydrochloride samples are more likely to decrease. This fact is caused by the presence of an hydrochloride molecule in the salt which is soluble and as it gets dissolved it has an effect on the media by decreasing the pH and breaking the buffering capacity of the buffer.

With regard to the solid state in the shake-flask method, X-Ray diffraction of the solid collected at different pH values has been performed in order to elucidate if the solid has its neutral form structure or a salt structure. The diffractograms obtained have been compared to the ones of the raw materials. Figure 12 shows the diffractograms of pioglitazone and pioglitazone hydrochloride raw materials. The most significant differences can be observed in the regions between the angles 9 and 16 and the angles 24 and 26. In the first region, pioglitazone shows four peaks with medium intensity (9.2; 10.3; 15.1 and 16.3) whereas pioglitazone hydrochloride only shows two peaks with medium intensity (9.0 and 12.8). In the second region, pioglitazone presents several peaks with different intensities whereas no peak is observed for pioglitazone hydrochloride. The diffractograms of all the samples tested, independently of the raw material used (pioglitazone or pioglitazone hydrochloride), are in agreement with the pioglitazone reference diffractogram so the determination of pioglitazone solubility can be ensured in both cases. That means that, in the case of pioglitazone hydrochloride, as much hydrochloride as the sample has, it is all dissolved leaving only pure pioglitazone as a solid. As a result of having the same solid in both cases, the expected solubility-pH profiles are the same for pioglitazone and its hydrochloride salt.

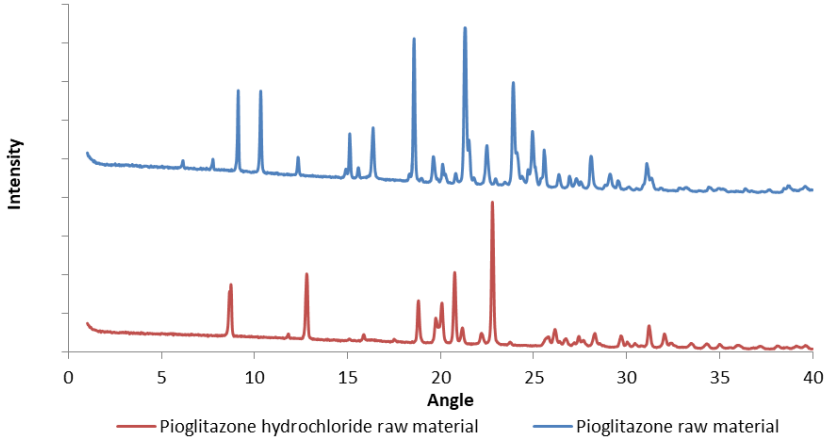

Figure 12. X-Ray diffractogram for pioglitazone (blue) and pioglitazone hydrochloride (red) raw materials.

The solubility-pH profile for each substance is represented in figure 13. The represented pH is the final one where the equilibrium has been reached. As expected after comparing the X-Ray diffractograms, both profiles follow the same tendency and similar to the one predicted by HH model for amphoteric substances (figure 4): the minimum solubility is reached between the two pK_as and it increases as the pH gets more acid or more basic. If pioglitazone solubility-pH profile and its hydrochloride salt profile are compared, they perfectly match from pH 2 to 4 and from pH 9 to 11. However, in the centre of the pH scale, from 5 to 8, they differ slightly probably due to the fact that this is where the concentration is lower and where there have been more difficulties in quantification.

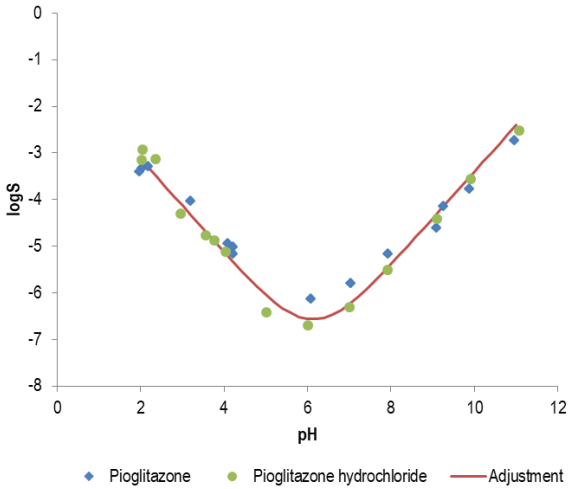

Figure 13. Solubility-pH profile of pioglitazone and its hydrochloride salt.

Once the experimental solubility-pH profiles are known, the intrinsic solubility can be deduced following the HH model for amphoteric substances, equation (9), and using the pK_{a1} and pK_{a2}, 5.65 and 6.62 respectively, previously determined by the research group [9]. LogS_0 is determined by direct adjust of experimental points to equation (9) through Excel software using supplementary optimization Excel Macro [10], and the obtained result is -6.78(0.04). The adjustment, which is showed in figure 13, fits all experimental data quite well despite having fixed the pK_a values. As a result, it is deduced that the equilibriums involved in the determination of solubility are only acid-base equilibriums. Finally, using equation 9, as pK_as and logS_0 are now known, solubility at every pH can be estimated.

6.3. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR GLIMEPIRIDE DETERMINATION

The first trials for glimepiride quantification are carried out with the same UHPLC chromatograph than for pioglitazone. Once the chromatographic method has been developed and the quantification of samples starts, it is found that the limit of detection of this drug is substantially high and it does not allow the quantification of glimepiride samples with pH lower than 7. Finally, the determinations have been carried out with a HPLC instrument which provides two improvements in quantification. On the one hand, as an HPLC column is going to be used, which have bigger dimensions than UHPLC columns, the injection volume will be higher and that will improve detection and quantification. On the other hand, the new chromatograph option has a single wavelength UV-Vis detector which might detect lower concentrations of glimepiride.

In order to establish the chromatographic conditions in the HPLC, several trials with different flows and mobile phase compositions are carried out. Finally, the chosen method has the following conditions: a flow of 0.7 mL/min, a composition of the mobile phase of MeOH:NH_4^+/NH_3 (60:40) pH=9. Using this method, the glimepiride peak appears at 2.5 minutes which is enough separated from the dead time peak, whereas at first 5 minutes of analysis seem to be enough, when the samples at lower pHs are injected in the chromatograph, other peaks appear until 7 minutes, so the total time of analysis has to be 10 minutes.

Glimepiride has its highest maximum of absorption at 200 nm, however it is not the chosen wavelength because solvents which absorb at this wavelength cause a significant noise in the base line making more difficult the determination of solubility at lower pHs. Therefore, the

working wavelength is 225 nm where the glimepiride also has a maximum of absorption in its spectrum.

With reference to the working pH, it is 9 where the glimepiride will be negatively charged and consequently, its solubility will be the considerably high and the retention time will be shortened.

6.3.1. Calibration curve

After optimizing the chromatographic conditions and the equipment to be used, standards between 0.1-100 mg/L dissolved in mobile phase are injected in the chromatograph. Figure 14 shows the calibration curve obtained which has been used to quantify the samples.

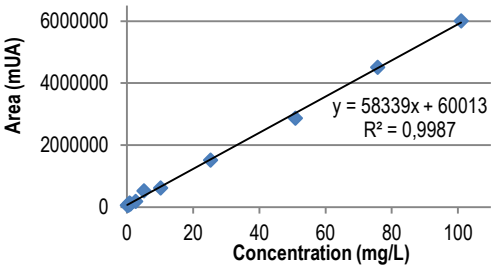

Figure 14. Calibration curve for glimepiride 0.1 mg/L-100 mg/L.

6.4. GLIMEPIRIDE SOLUBILITY-PH PROFILE DETERMINATION

The solubility of glimepiride has been determined through shake-flask method at different pH values between 2 and 11.5, three determinations have been done at each pH. If the variation of the final pH in these repetitions is lower than 0.1 units of pH, the average has been calculated for all the repeated determinations and if it is higher, then, the repetitions have been treated as individual experiments.

Table 5 shows the obtained results including all pH variations during all shake-flask procedure for glimepiride. This table summarize different values of pH in consecutive columns: initial pH (pH_0), pH after 4 hours of stirring the samples (pH_{4h}) and pH at the end of the procedure (pH_f). They also show the log*S* and the solid state of the solid in equilibrium with the solution according to the XRD data.

Table 5. Results of shake-flask method for glimepiride (standard deviations in brackets).

pH_0	pH_{4h}	pH_f	log*S*	Solid State
2.122	2.177	2.256	-4.445	Neutral form
2.122	2.12(0.01)	2.131(0.004)	-4.4(0.1)	Neutral form
2.411	2.418	2.611	-4.453	Neutral form
2.411	2.391	2.391	-4.453	Neutral form
3.204	3.234	3.314	-4.400	Neutral form
3.204	3.208	3.813	-4.408	Neutral form
3.586	3.664	3.670	-4.447	Neutral form
3.586	3.623(0.005)	3.79(0.03)	-4.400(0.004)	Neutral form
4.152	4.155(0.009)	4.19(0.04)	-4.40(0.04)	Neutral form
4.567	4.58(0.02)	4.57(0.02)	-4.439(0.005)	Neutral form
5.061	5.077	5.295	-4.389	Neutral form
5.061	5.10(0.03)	5.16(0.04)	-4.33(0.05)	Neutral form
5.432	5.43(0.01)	5.410(0.009)	-4.41(0.03)	Neutral form
6.170	6.059	6.199	-4.436	Neutral form
6.170	6.042	6.155	-4.429	Neutral form
6.170	6.032	6.264	-4.404	Neutral form
6.555	6.429(0.009)	6.54(0.04)	-4.42(0.01)	Neutral form
7.098	6.93(0.01)	7.03(0.04)	-4.412(0.003)	Neutral form
7.489	7.36(0.01)	7.49(0.03)	-4.37(0.03)	Neutral form
7.857	7.690	8.125	-4.148	Neutral form
7.857	7.72(0.03)	8.025(0.001)	-4.189(0.002)	Neutral form
8.501	8.362	8.364	-4.105	Neutral form
8.501	8.392(0.008)	8.502(0.004)	-4.07(0.09)	Neutral form

9.134	8.981(0.004)	9.080(0.006)	-3.49(0.03)	Neutral form
9.510	9.372(0.008)	9.46(0.02)	-3.24(0.01)	Neutral form
10.044	9.826	10.290	-2.654	Neutral form
10.044	9.84(0.02)	10.02(0.01)	-2.68(0.02)	Neutral form
10.445	10.176(0.006)	10.14(0.02)	-2.667(0.001)	Neutral form + other peaks
11.003	10.23(0.04)	10.91(0.02)	-2.78(0.04)	Neutral form + other peaks
11.461	10.502	11.425	-2.777	Neutral form + other peaks
11.461	10.316	11.290	-2.777	Neutral form + other peaks
11.461	10.319	11.358	-2.803	Neutral form + other peaks

Table 5 shows that for pH lower than 7.5 there are not significant changes in pH. Nevertheless, at pH above 7.5 the variation of pH increases as the pH gets higher. The most significant change occurs at pH 11.5 where the variation is 1.14 units. Therefore, glimepiride is able to break the buffering capacity of the buffer.

With regard to the state of the solid in the shake-flask method, X-Ray diffraction has been performed for gliempiride at different pH values. The reference diffractogram for glimepiride (raw material) is showed in figure 15. The diffractograms of the samples between pH 2 to 10 tested are in agreement with the glimepiride reference diffractogram. However, in diffractograms of samples from pH 10.5 to 11.5 some peaks correspond to glimepiride (5-10%) but there are other peaks which do not correspond to the raw material. The most significant differences of the diffractograms are located between angles 3 and 7 and around angle 22. In the first region, sample at pH 11 has a peak at angle 3.3 while glimepiride raw material does not. Both present a peak in 6.5, which corresponds to glimepiride neutral form structure. The intensity in the sample is much lower than in the raw material indicating that the percentage of glimepiride in the sample only corresponds to 5-10%. In the second region, sample at pH 11 has a peak (angle 21.9) with high intensity which does not appear in glimepiride raw material diffractogram. Finding different solid states in samples from pH 10.5 to 11.5 was expected because when the solids were filtered, they were more crystalline than in the lower pHs. Therefore, determination of solubility from pH 2 to 10 corresponds to neutral form of glimepiride whereas from pH 10.5 to 11.5, the solubility corresponds to a glimepiride derivate.

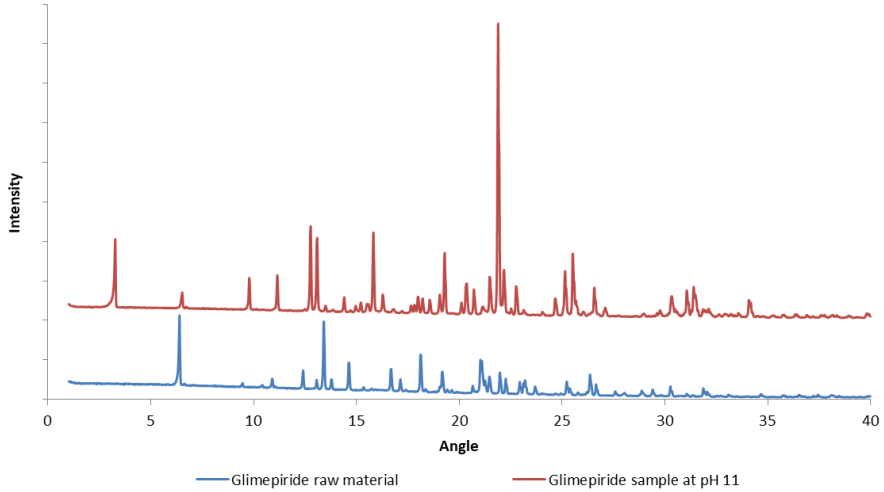

Figure 15. X-Ray diffractogram for glimepiride raw material (blue), and glimepiride sample at pH 11 (red).

The solubility-pH profile for glimepiride is represented in figure 16. The represented pH is the final one where the equilibrium has been reached. Although the profile is similar to the one predicted by HH model for acidic substances (figure 2), there are some differences with the expected profile. On the one hand, it can be observed how from pH 10.5 the solubility-pH profile curve declines. On the other hand, the solubility is constant until pH 8 contradicting what is expected by HH equation (4) that predicts a rise of solubility from pH above the pK_a of the molecule, in this case 5.22.

With regard to the decrease of solubility from pH 10.5 to 11.5, taking into account the presence of two solids (one of them glimepiride) in the samples at these pH values, it suggests the formation of a salt of glimepiride. At these pHs, the transformation of glimepiride into a salt is taking place, this is why there are peaks in different percentages of two structures in the X-Ray diffractograms. When the formation of a salt occurs, there is a parameter named pH_{max} which defines the pH where the solubility is the highest because both species, salt and neutral form, coexist contributing to the total solubility of the drug, in this case it is 10.5 approximately [4].

LogS_0 and pK_a are determined by direct adjust of experimental points to equation (4) through Excel software using a supplementary optimization Excel Macro [10], the adjustment is showed in figure 16. As deduced thanks to X-Ray diffractograms, not all the solids of the samples are pure glimepiride so, only the samples at pH where the solid is just glimepiride are

taken into account to adjust the experimental data. The obtained values of pK_a and logS_0 after the adjustments are 8.34(0.03) and -4.41(0.1) respectively.

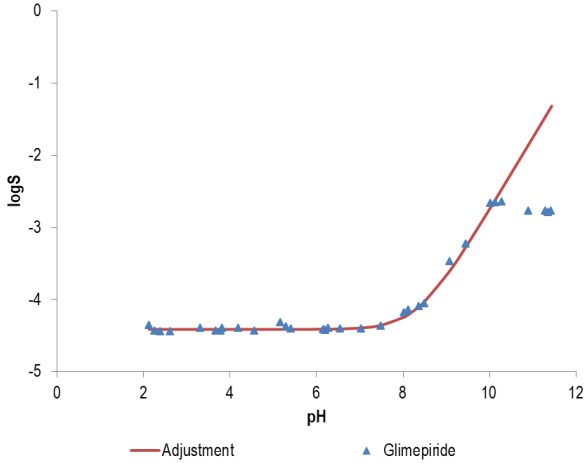

Figure 16. Solubility-pH profile of glimepiride.

The pK_a value obtained in the adjustment of experimental data differs significantly from the one that the research group has previously determined using the reference potentiometric method, there is a difference of 3,12 units of pH. This fact suggests that there are other equilibriums involved in the solubility determination different from acid-base equilibriums already considered by HH equation (4). Therefore, glimepiride solubility-pH profile does not fit with the HH model for acidic substances. These additional equilibriums affect the adjustment of pK_a to the experimental data by obtaining an apparent pK_a quite higher than the real one. Moreover, as the other involved equilibriums affect pK_a they also might have an effect on the logS_0 value and the obtained one through the adjustment could differ from reality. In order to confirm or discard this hypothesis, the planning of new experiments and deeper studies of glimepiride solubility is essential [11].

7. CONCLUSIONS

Solubility of pioglitazone has been determined through shake-flask method using two different raw materials: pioglitazone and its hydrochloride salt. The solubility-pH profiles obtained are really similar between themselves, as it was expected after finding just pioglitazone for all the samples in the X-Ray diffractograms. A slight difference appears between pH 5 and 7 where there have been more difficulties in quantification caused by the low solubility of pioglitazone at these pHs. After adjusting the experimental data to HH model for amphoteric substances, it has been found that the experimental values are in accordance with the predicted model what concludes that the only equilibriums involved are acid-base equilibriums already considered by HH. As pioglitazone is an amphoteric molecule with two close pK_a values, it is quite difficult to calculate the intrinsic solubility just with experimental values, so the adjustment has enabled to deduce it obtaining the following result:-6.78(0.04).

Solubility of glimepiride has also been determined through shake-flask method. It has been found the formation of a salt of glimepiride above pH_{max}, confirmed by the presence of two substances (one of them glimepiride) in X-Ray diffractograms. In the case of glimepiride, the solubility experimental data is not in accordance with HH model for acidic molecules because after adjusting the experimental values, it has been found that the pK_a obtained does not correspond to the pK_a determined by potentiometric technique. This fact implies that there are other equilibriums involved different from acid-base equilibriums already considered by Henderson-Hasselbalch. In order to confirm this hypothesis, further studies are needed.

8. REFERENCES AND NOTES

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9. ACRONYMS

API	Active pharmaceutical ingredient
en	Ethylenediamine
HAc	Acetic acid
HH	Henderson-Hasselbalch
HPLC	High performance liquid chromatography
MUB	Minimalist universal buffer
TFA	Trifluoroacetic acid
UHPLC	Ultra high performance liquid chromatography