FAST DETERMINATION OF DRUG SOLUBILITY OF SINGLE SOLUTIONS AND BINARY MIXTURES BASED ON 96-WELL PLATE SURFACE TENSION MEASUREMENTS

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First Master of drug development

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<td>ADME</td>
<td>adsorption, distribution, metabolism, excretion</td>
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<td>API</td>
<td>active pharmaceutical ingredient</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>CMC</td>
<td>critical micelle concentration</td>
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1. INTRODUCTION

1.1. GENERAL TERMS

The aqueous solubility of an API (active pharmaceutical ingredient) is very important in the formulation of a drug. In fact, low solubility can lead to low bioavailability, which limits the number of possible drug administration routes and pharmaceutical forms. Most recently developed API’s however, are increasingly water-insoluble, and therefore, methods to determine the solubility have an important role in the preformulation process.

In this study a new approach for the solubility determination of poorly soluble drug substances, based on 96-well plate surface tension measurements, is used. Heikkilä et al. (2008) applied this technique for the first time to determine the solubility of ibuprofen and indomethacin. In this study the applicability of the technique for other API’s and binary mixtures is investigated.

1.2. SOLUBILITY

1.2.1. Definition and importance

Solubility, in a broad sense, can be defined as the molecules of a solute (e.g. drug) that remain in solution in a given volume of solvent at a given temperature and pressure under equilibrium conditions.

The dissolution of a solid occurs in three stages (fig 1.1). First, a solute molecule has to be removed out of its crystal lattice. In most cases this is an energy-consuming process. Secondly, a cavity is created in the solvent: the molecules of the solvent move apart to make place for the dissolving molecule. This also costs energy. Finally, the solute molecule moves into the cavity, which is called solvation. In this last step energy is released. The solute will dissolve if the energy released in the last step is higher than the energy consumed in the first two stages (spontaneous process).
Solubility measurement is increasingly important in research and development. API’s have to be adequately soluble if we want to execute structure-activity relationship screenings. Also, ADME (adsorption, distribution, metabolism and excretion) parameters can be determined by using solubility information. (Bhattachar et al., 2006)

1.2.2. Intrinsic factors influencing solubility

The molecular structure of the solute plays a major role in solubility. The properties of the substituents (e.g. -OH, -COOH) determine the polarity of the compound and, in this way, the solubility. Not only the nature of the substituent, also its position may influence the solubility of the parent molecule. Furthermore, smaller particles are generally more soluble than larger ones.

Secondly, a substance can show polymorphism. Polymorphs are defined as different crystalline forms of the same substance. They differ in Gibbs free energy, because the position of the molecules in the crystal lattice is different. The lower the Gibbs free energy of a crystal, the easier it is to remove a molecule from its lattice, and thus less energy is needed for dissolution (as explained in 1.2.1). Pudipeddi & Serajuddin (2004) examined the polymorphs of 55 compounds. Different solubility values were found for all the polymorphs of the same compound.
Finally, solubility also increases when a weak acid or a weak base is converted into a salt. This leads to a higher dissociation degree of the substance when it dissolves in water. Consequently, the interaction of the compound with the water molecules is increased and the solubility will rise. (Florence & Attwood, 2006)

1.2.3. External factors influencing solubility

The general rule in case of temperature influence is that solubility increases with increasing temperature. Higher temperature means more energy, which will ease the breaking of intermolecular bonds in the solvent as well as the solid. In this way, the overall energy needed for the solid to dissolve, decreases, and solubility increases. Exceptions are compounds that dissolve through an exothermic reaction instead of the common endothermic reaction (e.g. Ca(OH)$_2$). (Aulton, 2002)

The choice of the solvent is very important. The general rule is: like dissolves like. This means that the polarity, and therefore the dielectric constant, of the solvent used, determines whether a molecule will dissolve or not. For example: polar solutes will preferably be dissolved in polar solvents, with high dielectric constant values. This explains the use of cosolvents to increase solubility of a poorly water-soluble molecule. By adding a cosolvent, the dielectric constant of the solvent is lowered, hereby bringing its polarity closer to the polarity of the solute. In this way, dissolution occurs easier. Important is that the cosolvent is miscible with the solvent. Peña et al. (2006) showed that the use of ethanol enhances the solubility of poorly water-soluble drugs. Another study that examined cosolvent effects was accomplished by Khalil et al. (2000). They proved that the solubility of diclofenac sodium increases in the presence of cosolvents. (Aulton, 2002)

When an electrolyte (a substance that dissociates into ions in solution) in solid state is added to a drug solution, the electrolyte will become hydrated. This means that a certain amount of water molecules is no longer available for solvation of the drug, which will lead to precipitation of the drug. This phenomenon is more commonly known as salting out.
Another observable fact is the common ion effect. This can be explained by using the example of sodium chloride (NaCl) and hydrogen chloride (HCl). When brought in water, NaCl crystals dissolve according to the following reaction: \( \text{NaCl} \rightleftharpoons \text{Na}^+ + \text{Cl}^- \). If HCl is added to this solution, the concentration of chloride ions in solution will increase. This disturbs the equilibrium of the previously mentioned reaction. According to the principle of Le Chatelier (a system in equilibrium will oppose the change that causes a disturbance of this equilibrium), the equilibrium shifts back to the left, in an attempt to lower the chloride ion concentrations by lowering the amount of NaCl dissolved, thus causing it to precipitate. (Aulton, 2002; Florence & Attwood, 2006)

Another external influence is the addition of a compound that forms a complex with the drug. The solubility of the complex is different from the solubility of the individual compound.

Surfactants can also change solubility. They consist of a hydrophilic head and hydrophobic tail. When their concentration reaches a certain value, called the critical micelle concentration, or CMC, they form micelles (fig 1.2). If this takes place in aqueous media, the hydrophilic heads are oriented towards the aqueous exterior, whereas the lipid tails group together and form a hydrophobic cavity. By enclosing organic solutes, that are poorly soluble in water, in this hydrophobic cavity, their aqueous solubility will increase drastically. Surfactants can also be used as wetting agents. This causes a decrease of the interfacial tension and the contact angle (becomes lower than 90°) between the solute and the solvent. (Aulton, 2002; Florence & Attwood, 2006)

\[ \text{FIGURE 1.2: MICELLE STRUCTURE} \]

Finally, the pH of the solution is extremely important (this will be discussed in 1.2.4).
1.2.4. Influence of pH

Although new drugs are more and more non-ionic, the pH of a solution still plays a very important role in the solubility of a substance. The relationship between the pH of the solution at equilibrium and the pKₐ of the drug is expressed by the Henderson-Hasselbach equation (eq 1.1 & eq 1.2).

\[
S = S_0 \left[ 1 + 10^{(pK_a - pH)} \right] 
\]  
(1.1)

for a monobasic compound

\[
S = S_0 \left[ 1 + 10^{(pH - pK_a)} \right] 
\]  
(1.2)

for a monoacidic compound

Where:  
S₀: solubility of unionized compound  
S: solubility at given pH

Different regions of solubility can be considered (fig 1.3). These regions are investigated by using the measurement of a weakly basic drug (pKₐ 5) at different pH values.

**FIGURE 1.3: REGIONS OF SOLUBILITY ACCORDING TO pH (BHATTACHAR ET AL., 2006)**
The first region is the intrinsic solubility, in this case when pH > 7. In this pH range, the substance is completely unionized and its solubility is very low. Precipitation of the drug is always in the unionized, free form.

The second region starts around the pK$_a$ of the substance, in this case around pH 5. At pH values equal to the pK$_a$ of the substance, 50% of the drug will be ionized, and 50% will be unionized. The amount of ionized compound in solution alters tenfold when the pH is increased by one unit. Precipitation can occur in the free form as well as in the salt form (depending on strength of solid-state interactions).

The third region is the pH$_{\text{max}}$ region and shows the pH value corresponding to the maximum solubility (pH 4 in this example). The equilibrium solid state is now a salt: the drug is entirely ionized and is linked to an oppositely charged counterion. From this point, the solubility of the salt overcomes the solubility of the free form. The last region is known as the salt plateau (pH > 4 in this case). The solubility $S$ is almost constant and depends on the strength of solubility product $K_{sp}$ (eq 1.3).

$$S = \sqrt{K_{sp}}$$

(1.3)

Where: $S$: solubility

$K_{sp}$: solubility product

This product expresses the strength of the solid-state interactions with the counterion. It is defined as the product of ion concentrations and counterion concentrations in solution (eq 1.4).

$$\text{drug-salt } \leftrightarrow \text{ drug ion + salt counterion}$$

$$K_{sp} = [\text{drug ion}][\text{salt counterion}]$$

(1.4)

$K_{sp}$ is always a constant value. This means that the concentration of the drug in a saturated solution depends on the counterion concentration. Hence, less drug molecules are dissolved, if the concentration of the counterion rises (common ion effect: 1.2.3). (Bhattachar et al., 2006)
1.2.5. Measuring

The traditional measurements are based on the thermodynamic equilibrium: a representation of the saturation solubility of a substance in equilibrium with an excess of undissolved compound, after the dissolution process has ended. This solubility is recognized as the true solubility of a drug and is determined using the shake-flask method. This means that a drug is added to a standard buffer solution until there is saturation. The pH is adjusted, if necessary, with dilute HCl or NaOH. The flask is shaken for at least 24 hours. Afterwards, the solubility is determined by filtering and analyzing the supernatant using an analytical method, for instance gravimetric analysis, UV spectrophotometry or HPLC. (Alsenz & Kansy, 2007; Avdeef et al., 1999)

As can be noticed, thermodynamic equilibrium measurements are very time-consuming and have a maximum throughput (amount of substances analyzed) of 200 compounds per week. Therefore a new high-throughput kinetic measurement is developed: the kinetic solubility. This term however is misleading, because it measures precipitation rather than solubility. This technique allows the analysis of more than 600 compounds per week. The technique was introduced for the first time by Lipinski (1997). Compounds are dissolved in DMSO (stock solutions were made) instead of aqueous media. Then they are diluted in series into a 96-well plate. DMSO lowers the dielectric constant of the solvent and consequently allows more lipophilic substances to dissolve. Thus, as mentioned earlier (1.2.3), DMSO can be defined as a cosolvent. Research conducted by Taub et al. (2002) showed that the increase in solubility by DMSO is highly compound specific and that other cosolvents, for instance ethanol or methanol, could be used. (Alsenz & Kansy, 2007; Bhattachar et al., 2006)

The results obtained with kinetic solubility measurements are not always the same as those found with the shake-flask method. This is due to the fact that both methods are based on 2 different physical phenomena. In kinetic solubility experiments, the drug is fully dissolved at the start of the analysis. Precipitation occurs during or after the measurement. This implies that there is a risk of supersaturation (higher dissolved concentration than the saturation value) during the time of measuring. This is especially the case when the drug has several polymorphic forms or if it exists in an amorphous form. The shake-flask methods on
the other hand, start with the solid, undissolved drug and therefore the risk of supersaturation is very low. This supersaturation is the reason why higher solubility values are reached with kinetic solubility methods, compared to the values that are found with thermodynamic solubility measurements. As discussed in the previous paragraph, DMSO also raises the solubility. Bard et al. (2008), however, proved that the higher solubility values are mainly caused by the supersaturation effects. (Bhattachar et al., 2006)

In the 96-well plate methods known today, the determination of the kinetic solubility is done using turbidity, nephelometry or UV absorption. Both turbidity and nephelometry utilize light that passes through a medium with dispersed particles. The difference is that in turbidity the intensity of the unscattered light is measured, whereas in nephelometry a determination of the scattered light intensity takes place. These methods however show some problems. Bevan & Lloyd (2000) demonstrated that the limit of detection is 54 µM for turbidimetric and nephelometric methods. If the concentrations would be lower, there would not be enough particles in solution for the determination of turbidity or scattered light. So measurements of substances with very low aqueous solubility are impossible with these methods. On the other hand, high concentrations can cause quenching (intensity decrease) of the scattered signal, as indicated by Pan et al. (2000), who also described some problems with UV absorption, like for instance a low absorption if the substance is poorly soluble in water.

In this study a new microtensiometric technique is used. This technique is based on measurements of the surface tension of drug solutions in 96-well plates and can be utilized for high throughput screening (HTS) of drug solubility characteristics.

1.3. SURFACE TENSION

1.3.1. Definition

The surface tension $\gamma$ can be defined as the work $dW$ needed to increase the surface with an area $dA$. The value is expressed as the force (N) per unit length (m).

The molecules in the bulk of a liquid are surrounded by similar molecules; consequently each molecule is pulled equally towards every direction by its neighbouring
molecules. The result is a zero net force. The molecules at the surface on the other hand, are exposed to imbalanced intermolecular attractive forces. The intermolecular attractive forces are imbalanced, because the attraction between similar molecules (e.g. other solvent molecules: represented by the black arrows in fig 1.4) is larger than between different molecules (e.g. solvent molecules and air molecules: represented by the grey arrows in fig 1.4). This causes a surplus of free energy (J) per unit area \( (m^2) \).

A system in equilibrium always strives for the lowest total free energy content. The surface free energy contributes to this total free energy and therefore the surface free energy is minimized. This can be realized by minimizing the surface area of the liquid, since the surface free energy and the surface area are directly proportional. The molecules at the surface will experience an inward force. *(Thiessen & Man, 1999)*

**FIGURE 1.4: SCHEMATIC REPRESENTATION OF THE FORCES LEADING TO SURFACE TENSION**

### 1.3.2. Gibbs adsorption isotherm

As mentioned earlier (1.2.3), a surfactant has amphiphilic properties, a hydrophobic tale and a hydrophilic head, and it tends to accumulate in the air/water interface. The attraction between a water molecule and the hydrophilic head of the surfactant is weaker than the attraction between two water molecules. Hence a decrease of the surface free energy and consequently the inward force is noticed. As a result, the surface tension lowers. The concentration of these surfactant molecules is larger at the surface than in the bulk and is indicated as the surface excess concentration \( \Gamma \). (units: mol/area of the surface (Å² in this study)) The surface tension drops until the surface is saturated with surfactant molecules. A further increase of the concentration of these molecules will not influence the surface
tension. At this point the CMC is reached: the surfactant molecules in the bulk will start forming micelles (fig 1.5). The relationship between the surface excess concentration \( \Gamma \), the concentration \( c \) of the surfactant in the bulk and the surface tension \( \gamma \) is given by the Gibbs adsorption isotherm (eq 1.5).

\[
\Gamma = -\frac{1}{RT} \frac{d\gamma}{d \ln c}
\]  

(1.5)

Where:  
\( \Gamma \): surface excess concentration  
\( \gamma \): surface tension  
\( c \): concentration of the surfactant in the bulk  
\( R \): gas constant: 8.314 J mol\(^{-1}\) K\(^{-1}\)  
\( T \): temperature

This isotherm can generally be used for the determination of the solubility of amphiphilic compounds, such as most commonly used drugs. (Florence & Attwood, 2006)

**FIGURE 1.5: RELATIONSHIP BETWEEN SURFACE TENSION, CONCENTRATION AND CMC**

If surface pressure is applied, figure 1.6 shows a typical surface pressure isotherm as function of the drug concentration in the bulk. Its relation to the surface tension is defined in equation 1.6. The surface pressure is a pure theoretical term. It gives an indication of the surface tension difference between the solution with pure solvent and the drug solution.

\[
\pi = \gamma^0 - \gamma
\]  

(1.6)

Where:  
\( \pi \): surface pressure  
\( \gamma^0 \): surface tension without amphiphilic molecules in the surface  
\( \gamma \): surface tension with amphiphilic molecules in the surface
The difference between the surface concentration and the surface excess concentration is insignificant, if amphiphilic molecules are located in a monolayer at the surface. The more area taken in by one amphiphilic molecule in the surface, the less concentration is needed to form a monolayer. Thus, the molecular cross-sectional area $A_S$ of one molecule can be defined by equation 1.7. \cite{Suomalainen2003}

\begin{equation}
A_S = \frac{1}{N_A \Gamma}
\end{equation}

Where:
- $A_S$: molecular cross-sectional area (Å$^2$)
- $\Gamma$: surface excess concentration (mol/Å$^2$)
- $N_A$: Avogadro’s number: $6.022 \times 10^{23}$ mol$^{-1}$

Suomalainen et al. \citeyear{Suomalainen2003} investigated the relationship between the slope and the molecular cross-sectional area. At low surface pressure, there were no limitations for the concentration of the molecules in the surface. However, at a certain surface pressure (from where the slope reaches a constant value), the concentration in the surface was so high that an increase in molecular concentration in the surface depended on the molecular cross-sectional area. This slope was inversely proportional to the molecular cross-sectional area.

A drawback of the Gibbs adsorption isotherm is that the determination of the air/water partitioning coefficient ($K_{aw}^{-1}$) is difficult. As an alternative, an apparent partitioning
coefficient $K_{aw}^{-1}$ is defined. This is the concentration found after extrapolation of the constant part of the slope (fig 1.4). (Suomalainen et al., 2003)

The CMC limit or solubility limit can be calculated from the intersection between a line representing the constant slope and a line representing the horizontal part of the adsorption isotherm. The equilibrium between solid and solvated molecules develops starting from this point. Higher concentrations will not influence the surface pressure, given that molecules that exceed the solubility limit are in the solid phase. After all, a substance has to be solvated before it influences the surface tension. For most drugs, unlike surfactants, the solubility limit will be reached before the CMC limit. Thus, the solubility measurement based on the determination of the surface tension can be applied. If the CMC limit was reached first, molecules that exceed this limit would be in a micelle phase instead of a solid phase. So, CMC would be measured instead of solubility. Nevertheless, caution has to be taken, because it is impossible to distinguish both limits based on the adsorption isotherm. (Suomalainen et al., 2003; Heikkilä et al., 2008)

1.3.3. Measuring

The measurements of the surface tension are performed with a tensiometer. Wire probes execute the measurement and the technique is based on the Du Noüy ring method. This method is known as a maximum pull force method.

FIGURE 1.7: THE DU NOÜY TENSIOMETER (MARTIN ET AL., 1983)
The Du Noüy tensiometer (fig 1.7) has a vessel, containing a liquid, and a ring on a wire. At the start, the ring is immersed in the liquid. Then, the vessel is lowered slowly and as this happens, a force is exerted on the ring. The maximum pull force $F_{\text{max}}$ is recorded just before the ring detaches. This is due to the fact that the surface tension is a reflection of the strength of the bonds that must be broken in order to create a new surface. Therefore the value of the force measured just before detachment, gives the best value to calculate the surface tension (eq 1.8).

$$\gamma = \frac{F_{\text{max}}}{2(2\pi R)} f \tag{1.8}$$

Where: 
- $\gamma$: surface tension
- $F_{\text{max}}$: maximum force
- $R$: radius ring
- $f$: correction factor

The liquid is in contact with the inside and the outside of the ring (fig 1.8). This is the reason why the perimeter $2\pi R$ is multiplied with 2 in equation 1.8. A correction factor $f$ is needed to adjust for the shape of the liquid pulled up and for the radius $r$ of the wire (fig 1.8). In this method, the ring must be wetted with a contact angle close to zero. This is achieved by using a ring made of platinum or an alloy of platinum and iridium. (**Thiessen & Man, 1999; Martin et al., 1983; Heldman, 2003**)

FIGURE 1.8: DETAILS OF THE DU NOUY RING (MARTIN ET AL., 1983)

The tensiometer applied in this study uses probes instead of a ring to measure surface tension. The probes give the advantage that the surface of the liquid can be smaller.
These probes experience two forces: first, the buoyancy due to the volume of the liquid displaced by the probe and secondly, the mass of meniscus (the liquid) sticking on the probe. The buoyancy is the upward force caused by the pressure of a liquid. The force executed by the mass $m_m$ of the meniscus sticking on the probe is in equilibrium with the force executed by the surface tension $\gamma$ (eq 1.9).

$$2\pi r_p \gamma \cos \theta = m_m g$$ (1.9)

Where:
- $r_p$: radius probe
- $\gamma$: surface tension
- $\theta$: contact angle
- $m_m$: mass of the meniscus under the probe
- $g$: gravity constant: 9.81 m s$^{-2}$

The contact angle $\theta$ is zero. This is achieved, as mentioned earlier, by using probes made of a platinum/iridium alloy. These materials assure complete wetting and easy and reliable cleaning of the probes. Thus, the force measured by the tensiometer is given by equation 1.10.

$$F_p = m_m g + F_{Buoyancy} = 2\pi r_p \gamma + F_{Buoyancy}$$ (1.10)

Where:
- $F_p$: force acting on the probe
- $F_{Buoyancy}$: force due to buoyancy

The maximum force $F_{\text{max}}$ is seen just before the probe detaches (cf. Du Noüy). At this point, the buoyancy is minimized and the surface tension is given by equation 1.11.

$$\gamma = \frac{F_{\text{max}}}{2\pi r_p}$$ (1.11)

Figure 1.9a shows the principle of the measurement and the forces that have an effect on the probes. Figure 1.9b shows the situation just before the probes detach. (Johans et al., 2005)
Johans et al. (2005) showed that this tensiometer has some advantages compared to the traditional Du Nuôy ring method. First of all, the sample volume can be limited to 50 µl instead of volumes larger than 10,000 µl. Also this device is fully automatic and easy to use, whereas the traditional method has to be operated manually and is labor intensive. At last, the conventional technique takes about 15 min for one measurement, whereas this method can execute 96 measurements in less than 10 minutes.
2. AIM OF THIS STUDY

Heikkilä et al (2008) proved that microtensiometric methods are reliable for the determination of drug solubility. This technique is based on the determination of the surface tension of a drug solution. They used this technique for kinetic solubility identifications of ibuprofen and indomethacin. To broaden the application area of this method, more testing is needed, and this will be the main goal of this thesis.

First of all, the applicability and reliability of this technique for other single drug solutions will be investigated. The following drugs will be examined: ibuprofen, indomethacin, ketoprofen, naproxen, rifampicin, trimethoprim, piroxicam, carbamazepine, phenytoin, furosemide, spironolactone and hydrochlorothiazide. The measured solubility values will be compared to the results obtained with other 96-well plate HTS methods. These results were acquired from Heikkkilä et al. (2008).

Secondly, a possible application of this technique in the determination of binary mixtures of several drugs will be studied. Which mixtures will be examined, will depend on the results of the single drug solution tests. Research will be conducted to determine the minimal requirements that need to be fulfilled to detect the solution as a binary mixture. This will include measurements of different ratios of the two drugs in solution, and how this influences the Gibbs adsorption isotherm. Furthermore, the individual solubility of the drugs present in the mixture will be measured. The obtained values will be compared to the results of the tests for single drug solutions, in order to control the reliability of the measurements of binary mixtures.
3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Solvents

DMSO has been purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany).

![DMSO Molecular Structure](image1)

As mentioned earlier (1.2.3), DMSO acts as a cosolvent, hereby causing a better dissolution of poorly water-soluble drugs.

The ultrapure water used, has been acquired from Millipore (Molsheim, France).

3.1.2. Drugs

Ibuprofen has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: 206.2808 g/mol

pKₐ: 4.91

![Ibuprofen Molecular Structure](image2)
**Materials and methods**

Indomethacin has been purchased from Orion Pharma (Espoo, Finland).
Molecular weight: 357.7880 g/mol
\[pK_a: 4.5\]

![Indomethacin structure](image)

**FIGURE 3.3: MOLECULAR STRUCTURE OF INDOMETHACIN**

Ketoprofen has been purchased from Orion Pharma (Espoo, Finland).
Molecular weight: 254.2806 g/mol
\[pK_a: 4.45\]

![Ketoprofen structure](image)

**FIGURE 3.4: MOLECULAR STRUCTURE OF KETOPROFEN**

Naproxen has been purchased from Orion Pharma (Espoo, Finland).
Molecular weight: 230.2592 g/mol
\[pK_a: 4.15\]

![Naproxen structure](image)

**FIGURE 3.5: MOLECULAR STRUCTURE OF NAPROXEN**
**Materials and methods**

Rifampicin has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: $822.9402\ \text{g/mol}$

$pK_a$: 1.7 and 7.9

![Molecular structure of Rifampicin](image)

**FIGURE 3.6: MOLECULAR STRUCTURE OF RIFAMPICIN**

Trimethoprim has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: $290.3177\ \text{g/mol}$

$pK_a$: 7.3

![Molecular structure of Trimethoprim](image)

**FIGURE 3.7: MOLECULAR STRUCTURE OF TRIMETHOPRIM**

Piroxicam has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: $331.3460\ \text{g/mol}$

$pK_a$: 6.3

![Molecular structure of Piroxicam](image)

**FIGURE 3.8: MOLECULAR STRUCTURE OF PIROXICAM**
Carbamazepine has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: 236.2686 g/mol  
\( pK_a \): 13.9

![Molecular Structure of Carbamazepine]

Phenytoin has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: 252.2680 g/mol  
\( pK_a \): 8.33

![Molecular Structure of Phenytoin]

Spironolactone has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: 416.5730 g/mol  
\( pK_a \):

![Molecular Structure of Spironolactone]
Materials and methods

Hydrochlorothiazide has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: 297.7390 g/mol

$p_{K_a}$: 7.9

![Molecular structure of Hydrochlorothiazide]

FIGURE 3.12: MOLECULAR STRUCTURE OF HYDROCHLOROTHIAZIDE

Furosemide has been purchased from Orion Pharma (Espoo, Finland).

Molecular weight: 330.7440 g/mol

$p_{K_a}$: 3.9 and 9.9

![Molecular structure of Furosemide]

FIGURE 3.13: MOLECULAR STRUCTURE OF FUROSEMIDE
3.2. METHODS

3.2.1. Delta-8 multichannel microtensiometer

The tensiometer used in this study is the Delta-8 multichannel microtensiometer (Kibron Inc, Espoo, Finland) (fig 3.14).

FIGURE 3.14: DELTA-8 MULTICHANNEL MICROTENSIOMETER

The instrument consists of 8 parallel ultrasensitive microbalances containing 8 corresponding probes. The position of the balances matches the wells of a 96-well detection plate (fig 3.15a). Before every measurement, the probes are cleaned by heating them to approximately 1000 °C for about 10 seconds in an electric oven (fig 3.15b). Johans et al. (2005) proved that such heating is effective for removing residual organic compounds. This is necessary to avoid carry-over of organic substances between measurements. Also the errors that can be made by manual cleaning of the probes are avoided. Measuring of the surface tension can be started approximately 10 sec after this heating, since the small mass of the probes allows a fast cooling. The calibration of the device, using a liquid with known surface tension, has to be done before measurements. In this study, water is used, because all samples are aqueous solutions.

FIGURE 3.15: MEASUREMENT WITH (3.15a) AND CLEANING OF THE PROBES (3.15b)
As mentioned before (1.3.3), the technique to measure the surface tension is based on the Du Noüy ring method. This technique is known as a maximum pull force method. The Delta-8 multichannel microtensiometer reaches a maximum pull force by: immersing the probe into the well containing the sample (fig 3.16a), retrieval of the probe (fig 3.16b) and measuring of the maximum force needed to remove the probe from the sample (fig 3.16c).

FIGURE 3.16: MAXIMUM PULL FORCE METHOD PRINCIPLE IN THE DELTA-8 MULTICHANNEL MICROTENSIOMETER (CAMPBELL AND WEAVER, 2007)

### 3.2.2. Stock solution preparation

Stock solutions were made by dissolving an amount of the drug in 10.00 ml DMSO (100 %). Table 3.1 gives an overview of the exact concentrations of the stock solutions.

<table>
<thead>
<tr>
<th>drug</th>
<th>quantity weighed (g)</th>
<th>concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ibuprofen</td>
<td>0.9990</td>
<td>99.90</td>
</tr>
<tr>
<td>indomethacin</td>
<td>0.5007</td>
<td>50.07</td>
</tr>
<tr>
<td>ketoprofen</td>
<td>1.015</td>
<td>101.5</td>
</tr>
<tr>
<td>naproxen</td>
<td>0.5044</td>
<td>50.44</td>
</tr>
<tr>
<td>rifampicin</td>
<td>0.5064</td>
<td>50.64</td>
</tr>
<tr>
<td>trimethoprim</td>
<td>0.5064</td>
<td>50.64</td>
</tr>
<tr>
<td>piroxicam</td>
<td>0.5028</td>
<td>50.28</td>
</tr>
<tr>
<td>carbamazepine</td>
<td>0.5090</td>
<td>50.90</td>
</tr>
<tr>
<td>phenytoin</td>
<td>0.5048</td>
<td>50.48</td>
</tr>
<tr>
<td>furosemide</td>
<td>0.5045</td>
<td>50.45</td>
</tr>
<tr>
<td>spironolactone</td>
<td>1.011</td>
<td>101.1</td>
</tr>
<tr>
<td>hydrochlorothiazide</td>
<td>1.004</td>
<td>100.4</td>
</tr>
</tbody>
</table>
3.2.3. Kinetic solubility of single drug solutions

First, the kinetic solubility of single drug solutions was measured via microtensiometry.

A dilution series of the studied drug was made in a 300 µl 96-well plate (fig 3.17). 160 µl of the prepared stock solution of the drug was pipetted, using a single channel pipette (20-200 µl), into column 1, row A until H. Into the wells of columns 2-12, 80 µl of pure DMSO was pipetted, using a 8-channel pipette (30-300µl). Then, 80 µl of the solution in column 1 was brought into column 2. Solutions were mixed by aspirating and dispensing 7 times. Further, 80 µl of column 2 was transferred to column 3. This was continued until column 11. No drug containing solution was pipetted into column 12, because these wells were used as a blank. This plate was indicated as plate 1.

The dilution factor was 0.5 and was calculated using equation 3.1.

\[ DF = \frac{V_i}{V_f} \]  

(3.1)

Where:  
\( DF \): dilution factor  
\( V_i \): initial volume of the solution  
\( V_f \): final volume of the solution

This dilution factor of 0.5 means that the concentration of the solutions in the wells of one column was 0.5 times lower than the concentration of the solutions in the wells of the previous column.

Another 96-well plate of 300 µl, referred to as plate 2, was used to pipet 270 µl water, using the 8-channel pipette, into each row and column. Then 30 µl of the solution from plate 1 was pipetted into the corresponding wells in plate 2. New pipet tips were applied for every new column. Again, adequate mixing was required. Every well now contained drug concentrations that varied from 0.01 to 10 mg/ml or from 0.005 to 5 mg/ml in 10 % DMSO, respectively using stock concentrations of 10 and 5 mg/ml. A general view of the plate is given in figure 3.17.
Materials and methods

FIGURE 3.17: GENERAL VIEW OF A 96 WELL PLATE CONTAINING A DILUTION SERIES OF THE STUDIED DRUG DISSOLVED IN WATER AND 10 % DMSO

Finally 50 µl of the samples in plate 2 were transferred into the corresponding wells in a detection plate (with a well volume of 50 µl), once again using an 8-channel pipette, starting from column 12 and advancing to higher concentrations. An equilibration time of 10 minutes was respected to allow the non-solved drug to sediment and the surface to stabilize. The measurements were performed at room temperature (app. 24 °C) and repeated at least 4 times.

3.2.4. Kinetic solubility of drug solution mixtures

After finishing the kinetic solubility determination of the single drug solutions, mixtures of 2 drugs were prepared. Which mixtures to make, was determined by the results of the single drug solutions. Again stock solutions were produced in 10.00 ml DMSO. The combinations were made in different ratios: from 1:5 to 1:1 to 5:1. The applied dilution factor was 0.75 instead of 0.5 and dilutions series of 22 instead of 11 concentrations were prepared. In this way, more points were obtained. The solutions were mixed in the right proportion in the wells of column 1. The preparation was similar to the plate 1 production of the single drug solutions (3.2.3). The difference, however, was that only wells A, C, E and G contained the most concentrated mixture. In this way, it was possible to get 22 concentrations for one dilution series. Also the volumes of these wells were not always 160 µl. Afterwards the other plates were made like before (3.2.3).
3.2.5. Calculations

The software applied was the Delta-8 manager 2.73. This programme analyzed the Gibbs adsorption isotherm. The values of the CMC or solubility limit, the maximum surface tension drop $\Delta \gamma$, the apparent air/water partitioning coefficient $K_{aw}$ and the molecular cross-sectional area $A_s$ were calculated automatically.

The solubility limit of a drug was calculated by making the average $\bar{x}$ (eq 3.2) of the solubility limits obtained from the different detection plates. The 95 % confidence interval (eq 3.4) of every solubility limit was determined. Therefore the standard deviation $s$ had to be found (eq 3.3).

\[
\bar{x} = \frac{x_1 + x_2 + \ldots + x_n}{n} = \frac{1}{n} \sum_{i=1}^{n} x_i \tag{3.2}
\]

\[
s = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}} \tag{3.3}
\]

\[
95 \% CI = [\bar{x} - 1.96s, \bar{x} + 1.96s] \tag{3.4}
\]
4. RESULTS AND DISCUSSION

Heikkilä et al. (2008) started the first solubility tests with this microtensiometer. They tested 3 cosolvents: methanol, ethanol and DMSO. The type of cosolvent affected the values acquired for the solubility of a drug and the reliability of the measurement. Methanol and ethanol proved to be unuseful as cosolvents in this type of measurements. This could be explained by their high vapour pressure, which caused them to evaporate easily. Since the sample volume was rather small (50 µl in these experiments), limited changes in the sample volume caused large variations in the results. On the other hand, good reproducible results were obtained with DMSO, so this cosolvent was chosen for further tests.

Some disadvantages of this technique were also examined. As mentioned earlier (1.3.2), it was impossible to distinguish between the CMC and the solubility limit based on the Gibbs adsorption isotherm. Most drugs have more polar groups and a more complex shape than surfactants. Therefore, most drug substances will rather precipitate than form micelles. Both highly soluble and very poorly soluble drugs led to unreliable results, as they were insufficiently partitioned in the water/air interface. Therefore the surface tension decrease caused by these drug solutions is too low to obtain adequate results. Other problems that could affect this method were supersaturation and cosolvent effects, but this was also the case in other 96-well plate HTS methods.

When determining the drug solubility by using kinetic solubility measurements with 96-well plate methods, some problems can arise (this was discussed previously in 1.2.5). These can be avoided with this new technique. Moreover, filtration becomes unnecessary as solid particles do not have an influence on the measurements. So, if problems occur in, for example, an UV-measurement, replacement by, for instance, a HPLC method is necessary. This technique however is more time consuming and executing concentration determinations in 96-well plates are not possible. These disadvantages do not occur when using the microtensiometer.
4.1. SINGLE DRUG SOLUTIONS

The drugs that decreased the surface tension by less than 3 mN/m were not analyzed. Suomalainen et al. (2004) proved this restriction, because the results were not reliable to describe the surface activity parameters (1.3.2) clearly. They demonstrated this by calculating the cross-sectional area of the drug. The areas they found were larger than the actual sizes of these drugs. The drugs that lowered the surface tension with less than 3 mN/m in this study were: naproxen, trimethoprim, piroxicam, carbamazepine, phenytoin, furosemide and hydrochlorothiazide. Good data were obtained with the other drugs: ibuprofen, indomethacin, ketoprofen, rifampicin and spironolactone.

4.1.1. Ibuprofen

4 different detection plates were made. Table 4.1 shows the solubility limits that were acquired and the maximum drop in surface tension.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Solubility limit (mg/ml)</th>
<th>Δγ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2205</td>
<td>30.70</td>
</tr>
<tr>
<td>2</td>
<td>0.2291</td>
<td>30.00</td>
</tr>
<tr>
<td>3</td>
<td>0.2550</td>
<td>30.50</td>
</tr>
<tr>
<td>4</td>
<td>0.2542</td>
<td>31.40</td>
</tr>
<tr>
<td>Average</td>
<td>0.2397 ± 0.01756</td>
<td>30.65 ± 0.5802</td>
</tr>
</tbody>
</table>

The 95 % confidence interval is calculated: [0.2053 ; 0.2741]. The kinetic solubility obtained previously with other 96-well plate methods was 0.245 mg/ml. The calculated confidence interval contains this value, therefore, the conclusion can be made that no significant difference is detected when comparing the results of this technique with those of other 96-well plate HTS methods. Figure 4.1 shows the Gibbs adsorption isotherm for ibuprofen. The intersection between the 2 lines gives the solubility limit.
FIGURE 4.1: GIBBS ADSORPTION ISOTHERM FOR IBUPROFEN IN WATER. DMSO WAS USED AS COSOLVENT (n=4)

4.1.2. Remaining drugs

Table 4.2 gives the average solubility limits and the kinetic solubility values obtained with other 96-well plates methods of the remaining drugs that affected the surface tension. Table 4.3 shows the 95% confidence intervals, CI’s, and the maximum drop in surface tension $\Delta \gamma$ of these drugs.

### TABLE 4.2: AVERAGE SOLUBILITY LIMITS AND KINETIC SOLUBILITY VALUES FROM OTHER 96-WELL PLATE METHODS

<table>
<thead>
<tr>
<th>Drug</th>
<th>Average solubility limit (mg/ml)</th>
<th>Solubility, other 96-well plate methods (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indomethacin</td>
<td>$0.08025 \pm 0.006352$</td>
<td>0.068</td>
</tr>
<tr>
<td>ketoprofen</td>
<td>$0.2756 \pm 0.01291$</td>
<td>0.257</td>
</tr>
<tr>
<td>rifampicin</td>
<td>$1.841 \pm 0.07544$</td>
<td>1.910</td>
</tr>
<tr>
<td>spironolactone</td>
<td>$0.3767 \pm 0.1302$</td>
<td>0.206</td>
</tr>
</tbody>
</table>
Results and discussion

<table>
<thead>
<tr>
<th>Drug</th>
<th>95% CI</th>
<th>Δγ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indomethacin</td>
<td>[0.06780 ; 0.09270]</td>
<td>10.75 ± 0.6024</td>
</tr>
<tr>
<td>ketoprofen</td>
<td>[0.2503 ; 0.3009]</td>
<td>12.20 ± 0.4113</td>
</tr>
<tr>
<td>rifampicin</td>
<td>[1.693 ; 1.989]</td>
<td>13.00 ± 0.5124</td>
</tr>
<tr>
<td>spironolactone</td>
<td>[0.1215 ; 0.6319]</td>
<td>4.0 ± 0.1581</td>
</tr>
</tbody>
</table>

The kinetic solubility values obtained for the remaining drugs are also part of the 95% confidence interval. So, there is no significant difference between this method and other HTS techniques that apply 96-well plates. Attention has to be paid for the results found with spironolactone. The confidence interval is quite large compared to the other drug intervals. Probably the reason is that the surface tension is decreased by 4 mN/m, which is very close to the 3 mN/m limit. The Gibbs adsorption isotherms of indomethacin, ketoprofen, rifampicin and spironolactone are shown in respectively figures 4.2, 4.3, 4.4 and 4.5. The intersection between the 2 lines gives the solubility limit.

FIGURE 4.2: ADSORPTION ISOHERM FOR INDOMETHACIN IN WATER. DMSO WAS USED AS COSOLVENT (n=8)
Results and discussion

**FIGURE 4.3:** GIBBS ADSORPTION ISOTHERM FOR KETOPROFEN IN WATER. DMSO WAS USED AS COSOLVENT (n=4)

**FIGURE 4.4:** GIBBS ADSORPTION ISOTHERM FOR RIFAMPICIN IN WATER. DMSO WAS USED AS COSOLVENT (n=4)
Results and discussion

FIGURE 4.5: GIBBS ADSORPTION ISOTHERM FOR SPIRONOLACTONE IN WATER. DMSO WAS USED AS COSOLVENT (n=5)

The results found with the shake-flask method for ibuprofen (0.090 mg/ml), indomethacin (0.037 mg/ml), ketoprofen (0.128 mg/ml), rifampicin (0.623 mg/ml) and spironolactone (0.042 mg/ml) were lower than the values obtained with the microtensiometer. As mentioned earlier (1.2.5), this was mainly due to the effect of the supersaturation and less due to the cosolvent (DMSO) effect. To find out if this supersaturation had further effects, Heikkilä et al. (2008) examined the influence of time during 5 h. The solubility results did not change too much during this period. So, the conclusion was that results obtained with this technique were from the same magnitude even when the measurements were not performed directly after the detection plate preparation. On the other hand, the best reliability is reached when every detection plate is analyzed the same time after preparation. In this case, the measurements were performed 10 minutes after the preparation.

As an overall conclusion, it can be stated that this technique is reliable in measuring the solubility of single drugs in solution. Results obtained with this new method prove to be not significantly different (calculated for a 5 % significance level) from results obtained with other 96-well plate HTS methods. The measurements were fast (less than 5 minutes for 1 detection plate) and were easy to repeat. Also, the solubility determinations are executed automatically by the software and only small sample amounts are needed. The most important disadvantage of the method is the fact that a studied drug has to be surface
active: it has to cause at least a surface tension decrease of 3 mN/m. Other problems can be affected by an insufficient participation in the water/air interface. This is the case for both highly soluble and very poorly soluble drugs, because the surface tension caused by these drugs is too low to get reliable results. Finally, as mentioned earlier (1.3.2), it is not possible to distinguish the CMC from the solubility limit. This is not common, but attention has to be paid if lipophilic drugs are investigated.

4.2. MULTIPLE DRUG SOLUTIONS

4.2.1. Difference between solubility limits

These experiments were executed to discover the minimal difference in kinetic solubility limits between the 2 drugs in a binary mixture. This is needed to give a graph in which a binary mixture can be distinguished and in which the solubility limits of both drugs can be measured.

The first mixture prepared, consisted of ibuprofen and indomethacin. This combination was made in 9 different ratios. At least 4 detection plates per ratio were made. The stock solutions of ibuprofen and indomethacin had a concentration of respectively 50.40 mg/ml and 50.03 mg/ml. Table 4.4 gives the maximum drug concentrations in the detection plates.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>C ibuprofen (mg/ml)</th>
<th>C indomethacin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>0.8400</td>
<td>4.169</td>
</tr>
<tr>
<td>1:4</td>
<td>1.008</td>
<td>4.002</td>
</tr>
<tr>
<td>1:3</td>
<td>1.260</td>
<td>3.752</td>
</tr>
<tr>
<td>1:2</td>
<td>1.680</td>
<td>3.335</td>
</tr>
<tr>
<td>1:1</td>
<td>2.520</td>
<td>2.502</td>
</tr>
<tr>
<td>2:1</td>
<td>3.360</td>
<td>1.668</td>
</tr>
<tr>
<td>3:1</td>
<td>3.780</td>
<td>1.251</td>
</tr>
<tr>
<td>4:1</td>
<td>4.032</td>
<td>1.001</td>
</tr>
<tr>
<td>5:1</td>
<td>4.200</td>
<td>0.8338</td>
</tr>
</tbody>
</table>
Figure 4.6 shows the Gibbs adsorption isotherm of the 1:5 mixture. It is clear that 2 slopes can be seen, separated by a horizontal section, as indicated in the graph. The first slope (seen at the lowest concentration values) is the result of the presence of indomethacin in this sample, because there is a solubility limit found with a value of 0.09147 mg/ml, when this part of the adsorption isotherm is analyzed (table 4.5). This result is part of the 95 % CI of the indomethacin solution (4.1.2). So, this solubility value does not significantly differ from the single drug solution value.

In the Gibbs adsorption isotherm of the 1:4 mixture (fig 4.7), there is also a visible horizontal section. This section however gets smaller. In the 1:5 mixture this part goes from concentration 0.23 mg/ml until 0.10 mg/ml (difference 0.13 mg/ml). On the other hand in the 1:4 mixture, this section goes from concentration 0.23 mg/ml until 0.13 mg/ml (difference 0.10 mg/ml). The reason for this change is that in the 1:4 mixture there is more ibuprofen than in the 1:5 mixture. This is confirmed by the other combinations 1:3 and 1:2, where the value of the section decreases to respectively 0.07 mg/ml and 0.04 mg/ml. The values of the 2 slopes stay approximately the same in these combinations, so only the horizontal part changes. The different adsorption isotherms tend towards the isotherm of pure ibuprofen.
Results and discussion

A little change in the slope can be seen if ibuprofen and indomethacin have the same concentration in a mixture (fig 4.9). Also it must be noticed that the overall surface tension decrease is larger than in the 1:5 ratio (21.5 mN/m in 1:5, 27.5 mN/m in 1:1). Again, this confirms that the different Gibbs adsorption isotherms tend towards the isotherm of pure ibuprofen.
Results and discussion

Figure 4.10 shows the graph when ibuprofen and indomethacin are mixed in a 2:1 ratio. It is clear that no horizontal part can be distinguished. The surface tension decrease is 30.0 mN/m and almost the same value as the decrease caused by an ibuprofen solution. This is confirmed by the other mixtures (3:1, 4:1 and 5:1) that have approximately the same isotherms.

Table 4.5 gives the solubility limits found in the different mixtures. All of these limits are part of the 95 % CI found for the single drug solutions (4.1.1 and 4.1.2). So, these solubility values do not significantly differ from the single drug solution values.

**TABLE 4.5: SOLUBILITY LIMITS IN DIFFERENT IBUPROFEN/INDOMETHACIN MIXTURES**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Solubility limit ibuprofen (mg/ml)</th>
<th>Solubility limit indomethacin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>0.2726</td>
<td>0.09156</td>
</tr>
<tr>
<td>1:4</td>
<td>0.2731</td>
<td>0.09257</td>
</tr>
<tr>
<td>1:3</td>
<td>0.2697</td>
<td>0.09167</td>
</tr>
<tr>
<td>1:2</td>
<td>0.2730</td>
<td>0.09247</td>
</tr>
<tr>
<td>1:1</td>
<td>0.2740</td>
<td>0.07608</td>
</tr>
</tbody>
</table>

As proven in earlier experiments, the kinetic solubility values of ibuprofen and indomethacin are respectively 0.2397 mg/ml and 0.07143 mg/ml. The difference in the
kinetic solubility between those 2 results is 0.1683 mg/ml. It can be concluded now that a minimal difference of approximately this value is necessary to be able to measure the solubility limits of both drugs in a 1:1 mixture.

![Graph showing surface tension decrease vs. concentration of ibuprofen.](image)

**FIGURE 4.10: IBUPROFEN-INDOMETHACIN (RATIO 2:1) GIBBS ADSORPTION ISOTHERM (n=4)**

The necessity of a minimal difference in kinetic solubilities of the drugs in a mixture can be demonstrated when examining a combination of, in a 1:1 relation, ibuprofen and ketoprofen. Both drugs have approximately the same kinetic solubility: respectively 0.2397 mg/ml and 0.2756 mg/ml. Also the difference in surface tension decrease is not a limiting factor (4.2.2). New stock solutions were made, with concentrations of respectively 50.00 mg/ml and 50.26 mg/ml ibuprofen and ketoprofen. Again 4 detection plates were prepared. The maximum concentrations of ibuprofen and ketoprofen in these plates were respectively 2.500 mg/ml and 2.513 mg/ml. Notice that there is no plateau in the graph (fig 4.11), so it is impossible to measure the solubility limits of both drugs in a 1:1 mixture. As a result, there has to be a significant difference in kinetic solubility between the 2 drugs. To distinguish clearly the solubility limits of the 2 drugs in a 1:1 ratio, this difference has to be approximately 0.1683 mg/ml. This value however has to be approached critically. It makes only sense to have a difference of this magnitude if the drug with the lowest kinetic solubility has the same or a higher concentration in the test sample than the drug with the highest kinetic solubility. This is shown with the combination of ibuprofen and indomethacin in a 2:1 ratio (fig 4.10). The difference between the kinetic solubility values is high enough, but,
Results and discussion

because the concentration of ibuprofen is higher than the indomethacin concentration, it is impossible to detect the 2 solubility limits.

Further analysis of fig 4.11 shows that the slope of the isotherm has a value of app. -24, which is situated in between the slopes of ibuprofen (app. -29) and ketoprofen (-19). This can be explained if the cross-sectional area $A_s$ of both drugs is analyzed. The values were respectively $52 \text{ Å}^2$ and $66 \text{ Å}^2$. As mentioned earlier (1.3.2), the slope is inversely proportional to the molecular cross-sectional area $A_s$. This slope increases from -19, with pure ketoprofen, to -24 in the mixture and the cross sectional area $A_s$ will decrease to a value between $66 \text{ Å}^2$ and $52 \text{ Å}^2$. This is the average of both individual values, because the amount of ibuprofen and ketoprofen molecules in the water/air interface will be the same. This proves that this is an adsorption isotherm of binary mixture in a 1:1 ratio of ibuprofen and indomethacin. The main disadvantage is that it is impossible to know that this is a binary mixture, if an unknown sample is measured. After all the value of the slope can also be the same as the result found for a single drug solution.

FIGURE 4.11: IBUPROFEN-KETOPROFEN (RATIO 1:1) (☻), IBUPROFEN (◼) AND KETOPROFEN (▲) GIBBS ADSORPTION ISOTHERM (n=4)
4.2.2. Difference between surface tension decreases

Both drugs in a binary mixture cause a surface tension decrease. Following experiments were performed to determine the difference between these drops, necessary to get a Gibbs adsorption isotherm in which a binary mixture can be distinguished and in which the solubility limits of both drugs can be detected.

The solution was a mixture between rifampicin and ketoprofen. This combination was made in 2 different combinations, 1:1 and 1:5 ratios. At least 4 detection plates per ratio were made. The stock solutions of rifampicin and ketoprofen had a concentration of respectively 80.23 mg/ml and 80.42 mg/ml. Table 4.7 gives the maximum drug concentrations in the detection plates.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>C rifampicin (mg/ml)</th>
<th>C ketoprofen (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>1.337</td>
<td>6.702</td>
</tr>
<tr>
<td>1:1</td>
<td>4.012</td>
<td>4.021</td>
</tr>
</tbody>
</table>

The kinetic solubility values of rifampicin and ketoprofen are respectively 1.841 mg/ml and 0.2756 mg/ml. The difference (1.565 mg/ml) between these two values is high enough to distinguish the solubility limits of both drugs in the binary mixture (4.2.1). Figure 4.12, however, shows us that this difference is not the only limiting factor. No horizontal section can be seen, despite the fact that the difference in kinetic solubility is high enough. On the other hand, rifampicin and ketoprofen cause approximately the same decrease in surface tension (respectively 12.00 mN/m and 12.20 mN/m). This proves that there also must be a difference between the decrease in surface tensions to get a Gibbs adsorption isotherm on which the 2 solubility limits can be seen.

Further analysis of fig 4.12 shows that the slope of the isotherm has a value of app. -13, which is situated in between the slopes of rifampicin (app. -8) and ketoprofen (-19). This can be explained if the cross-sectional area \( A_s \) of both drugs is analysed. The values were respectively 89 Å\(^2\) and 66 Å\(^2\). As mentioned earlier (1.3.2), the slope is inversely proportional to the molecular cross-sectional area \( A_s \). This slope decreases from -19, with pure
ketoprofen, to -13 in the mixture and the cross sectional area $A_s$ will increase to a value between 66 Å² and 89 Å². This is the average of both individual values, because the amount of rifampicin and ketoprofen molecules in the water/air interface will be the same. This proves that this is an isotherm of a binary mixture in a 1:1 ratio of rifampicin and indomethacin. Again, the main disadvantage is that it is impossible to know that this is a binary mixture, if an unknown sample is measured.

The Gibbs adsorption isotherm of the 1:5 mixture (fig 4.13) confirmed that also the difference in surface tension decrease is a limiting factor. The concentration of ketoprofen is 5 times higher than the rifampicin concentration. According to the results obtained with the mixture of ibuprofen and indomethacin in a 1:5 ratio, a horizontal section had to be seen if just the kinetic solubility differences were taken into account. Again no plateau can be distinguished. This shows once more that the difference in surface tension decrease is a factor that must be taken into account. The only difference between the 2 isotherms is the slope. The slope in fig 4.13 is steeper (app. -16), because the concentration of ketoprofen is 5 times higher than the rifampicin concentration. As seen in the single drug solution experiments, the ketoprofen isotherm (slope app. -19) has a steeper slope than the rifampicin adsorption isotherm (slope app. -8).
Results and discussion

The next experiments were done to determine the minimal required difference in surface tension decrease. Ketoprofen and indomethacin were mixed in 2 different ratios. Again 4 detection plates of each mixture were prepared. The kinetic solubility of ketoprofen and indomethacin were respectively 0.2756 mg/ml and 0.07143 mg/ml. The difference is high enough and is consequently not a limiting factor. The stock solutions had concentrations of correspondingly 50.00 mg/ml and 50.26 mg/ml. Table 4.8 shows the maximum drug concentrations in the detection plates.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>C ketoprofen (mg/ml)</th>
<th>C indomethacin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>0.8333</td>
<td>4.188</td>
</tr>
<tr>
<td>1:1</td>
<td>2.500</td>
<td>2.513</td>
</tr>
</tbody>
</table>

The Gibbs adsorption isotherm of the 1:1 combination (fig 4.14) shows clearly no horizontal section. The surface tension decreases of ketoprofen (12.20 mN/m) and indomethacin (10.75 mN/m) were found in previous single drug solution tests. This difference of 1.45 mN/m is obviously not enough to determine the 2 solubility limits.

FIGURE 4.13: RIFAMPICIN-KETOPROFEN (RATIO 1:5) GIBBS ADSORPTION ISOTHERM (n=4)
Results and discussion

Again the slope is analyzed (cf. ibuprofen-ketoprofen and rifampicin-ketoprofen). A value of app. -17 was found. The slope values of ketoprofen and indomethacin are respectively app. -19 and app. -17. These results are app. the same and this is also confirmed by the cross sectional areas (respectively 66 Å² and 69 Å²). It can be concluded that it is impossible to detect a binary mixture if the slopes, and consequently the cross sectional areas, of both drugs are more or less the same.

The confirmation that the difference is too small is again given by the 1:5 mixture. According to differences in the kinetic solubility a horizontal section had to be seen, but this was not the case (fig 4.15). The adsorption isotherm is also very similar to the one found for the 1:1 combination, because the slope does not change (see above). The only difference is the smaller drop in the surface tension (app. 10.5 mN/m instead of 12 mN/m in the 1:1 ratio), because the concentration of indomethacin is five times higher than the ketoprofen concentration.
Results and discussion

FIGURE 4.15: KETOPROFEN-INDOMETHACIN (RATIO 1:5)
GIBBS ADSORPTION ISOTHERM (n=4)

The single drug solution experiments give surface tension decreases of 10.75 mN/m and 4.00 mN/m for respectively indomethacin and spironolactone. The stock solution concentration of indomethacin was 50.00 mg/ml. Spironolactone had a concentration of 50.08 mg/ml. Two mixtures, ratio 1:1 and ratio 5:1, were produced. Four detection plates of each combination were made. Table 4.9 shows the maximum drug concentrations in the detection plates.

TABLE 4.9: MAXIMUM CONCENTRATIONS OF INDOMETHACIN AND SPIRONOLACTONE IN THE DETECTION PLATES

<table>
<thead>
<tr>
<th>Ratio</th>
<th>C indomethacin (mg/ml)</th>
<th>C spironolactone (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>4.167</td>
<td>0.8347</td>
</tr>
<tr>
<td>1:1</td>
<td>2.500</td>
<td>2.504</td>
</tr>
</tbody>
</table>

No horizontal part can be seen in the 1:1 Gibbs adsorption isotherm (fig 4.16). So, the difference of 6.75 mN/m in surface tension decrease is not enough to determine the 2 solubility limits in a 1:1 combination. Spironolactone has an kinetic solubility of 0.3767 mg/ml. The value of indomethacin is 0.07143 mg/ml. The difference in kinetic solubility (0.3053 mg/ml) is thus no limiting factor.
Results and discussion

FIGURE 4.16: INDOMETHACIN-SPIRONOLACTONE (RATIO 1:1)
GIBBS ADSORPTION ISOTHERM (n=4)

Also here, the slope is analyzed (cf. ibuprofen-ketoprofen and rifampicin-ketoprofen). A value of app. -11 is found. The slope values of indomethacin and spironolactone are respectively app. -17 and app. -5. The cross sectional areas are respectively 66 Å² and 187 Å². The same argumentation found for the ibuprofen-ketoprofen 1:1 mixture and the rifampicin-ketoprofen 1:1 mixture can be made. Thus, this is an isotherm of a binary mixture in a 1:1 ratio of indomethacin and spironolactone. However, the main disadvantage is that it is impossible to know that this is a binary mixture, if an unknown sample is measured. After all the value of the slope can also be the same as the result found for a single drug solution.

The confirmation that the difference between surface tension decreases has to be higher than 6.75 mN/m is given by figure 4.17. Again no plateau can be noticed, despite the fact that the difference in the kinetic solubility is high enough. The difference between the 2 isotherms can be clearly seen if is looked to the surface tension decrease. The 5:1 isotherm has a larger decrease in surface tension. This is due to the influence of indomethacin that is present in a 5-fold higher concentration than spironolactone.
The single drug solution tests give surface tension decreases of 12.00 mN/m and 4.00 mN/m for respectively rifampicin and spironolactone. New stock solutions were produced with a concentration of 80.23 mg/ml for rifampicin and 80.72 mg/ml for spironolactone. Two mixtures, ratio 1:1 and ratio 1:5, were prepared. Eight detection plates of each combination were made. Table 4.10 shows the maximum drug concentrations in the detection plates.

**TABLE 4.10: MAXIMUM CONCENTRATIONS OF RIFAMPICIN AND SPIRONOLACTONE IN THE DETECTION PLATES**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>C rifampicin (mg/ml)</th>
<th>C spironolactone (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>1.337</td>
<td>6.727</td>
</tr>
<tr>
<td>1:1</td>
<td>4.012</td>
<td>4.036</td>
</tr>
</tbody>
</table>

Clearly, a horizontal section is noticeable in the 1:1 Gibbs adsorption isotherm (fig 4.18). The first slope (seen at the lowest concentration values) is the result of the presence of spironolactone in this sample, because there is a solubility limit found with a value of 0.1741 mg/ml, when this part of the adsorption isotherm is analysed. The graph shows that a difference of 8 mN/m is enough to distinguish a mixture in a 1:1 ratio and to determine the individual solubility values. Also the horizontal part is larger than the section seen in a 1:1 mixture of ibuprofen and indomethacin (fig 4.9). This is due to the higher difference in
kinetic solubility between rifampicin and spironolactone (1.464 mg/ml instead of 0.1683 mg/ml for ibuprofen-indomethacin mixture).

The solubility limits detected in this isotherm are respectively 0.1741 mg/ml for spironolactone and 1.718 mg/ml for rifampicin. Both values are part of the 95 % CI (4.1.2). So, these solubility values do not significantly differ from the single drug solution values.

It can be concluded that the difference between surface tension decreases has to be approximately 8 mN/m. With this difference, the technique can be used to distinguish the 2 solubility limits in binary mixture. This result however has to be approached critically. Tests were done with spironolactone to get this value. On the other hand, spironolactone causes a surface tension decrease of 4 mN/m, which is very close to the limit of 3 mN/m. As mentioned previously, this restriction is made, because surface tension parameters cannot be unambiguous described when surface tension is only lowered with this magnitude. So, because spironolactone is very close to this restriction, it might be possible that a difference in surface tension decrease of 6.75 mN/m (as seen in indomethacin-spironolactone mixture) makes it nevertheless possible to distinguish the 2 solubility limits in a binary mixture. This will require further investigation.
5. CONCLUSION

Surface tension measurements in 96-well plates proved to be reliable for drug solubility determinations by using kinetic solubility measurements. Heikkilä et al. (2008) had already demonstrated this for ibuprofen and indomethacin. In this study, the applicability and the reliability of this technique for other drugs were tested. Also, the determination of the individual solubility of both drugs in a binary mixture was investigated.

First of all, solubility determinations were executed on other single drug solutions. These results proved to be not significantly different (calculated for a 5% significance level) from results obtained with other 96-well plate HTS methods. This demonstrates that microtensiometric measurements are applicable and reliable for other drugs than ibuprofen or indomethacin. However, there is a limitation to which drugs can be used. The analyzed substance has to be surface active: it has to lower the surface tension with at least 3 mN/m. If this is not the case, the measurements are not reliable, which implies that the surface activity parameters (1.3.2) cannot be described clearly.

Several binary mixtures were analyzed. It is clear that binary mixtures can be detected and that the individual solubility values of both drugs can be distinguished, if the differences in solubility and in surface tension decrease are sufficiently high. When this is the case, a Gibbs adsorption isotherm with 2 slopes and a horizontal section between them is obtained. The difference between the solubility limits has to be approximately 0.1683 mg/ml, to distinguish a 1:1 mixture. Nevertheless, this value only makes sense if the drug with the lowest kinetic solubility has the same or a higher concentration in the test sample than the drug with the highest kinetic solubility. The surface tension decreases have to differ at least 8 mN/m. This last result, however, has to be interpreted critically, because it was found for a rifampicin-spironolactone mixture. The last drug causes a surface tension drop of 4 mN/m, which is very close the restriction of 3 mN/m. This implies that lower drops could be possible if other drugs are used. This will require further investigation.

The individual solubility values of both drugs in a mixture do not differ significantly (calculated for a 5% significance level) from the results found for the single drug solutions. This proves that the microtensiometer is applicable and reliable for the determination of the individual solubility of both drugs in a binary mixture.
Conclusion

At last, it is still possible to distinguish a binary mixture if these two limits are not reached. This can be done by analyzing the slope. When examining the slopes, the slope of a binary mixture proved to be situated in between the slopes of the single solutions of the drugs in the mixture. This implicates that it is impossible to detect a mixture, if the individual slopes of the drugs are equal. This can also be concluded if an unknown sample is measured. It is necessary to know the composition of the mixture. After all, the value of the slope can also be the same as the result found for a single drug solution.

This method has some problems, but is very fast (less than 5 minutes for 1 detection plate) and easy repeatable. Also, solubility determinations are executed automatically by the software and only small sample amounts are needed. Microtensiometric measurements are reliable for solubility determinations of single drug solutions and individual solubility values in binary mixtures.

The minimal requirements to detect a binary mixture (as described above), were determined for neutral solutions. More research and testing will be necessary to investigate the use of this technique and the minimal requirements of solutions with different pH values.
6. LITERATURE


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