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FACULTEIT INDUSTRIËLE INGENIEURSWETENSCHAPPEN
master in de industriële wetenschappen: biochemie

Masterproef

Evaluation of adsorption of various analytes in cerebrospinal fluid

Promotor :
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Delphine Verbeke

Scriptie ingediend tot het behalen van de graad van master in de industriële wetenschappen: biochemie

Gezamenlijke opleiding Universiteit Hasselt en KU Leuven

KU LEUVEN | universiteit hasselt

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Preface

My interests are with research and clinical biology. I discovered this during my studies.
When in the position to choose the place and subject for my thesis, I was made aware of the possibility to perform an internship with Janssen Pharmaceutica.

This fitted right in with my interests and I didn't hesitate to contact Janssen Pharmaceutica in order to secure an internship.

This internship was both very interesting and instructive and I would very much like to thank all involved for making this internship possible and for their continuous help and support during my time there. The possibility to participate in important research meant very much to me and to be able to do so in the very best conditions was a rare privilege.

I want to warmly thank Mr Hans Stieltjes and Mr Tom Verhaeghe for having given me the opportunity to do my internship at Bioanalysis with Janssen Pharmaceutica.
I also want to thanks Hans for his guidance and for helping me with reading my master's thesis.

I equally want to express my sincere appreciation to Doctor Kristel Sniegowski for her help with the writing of my thesis.

Furthermore I want to thank Tinne Huybrechts for her guidance in the practical part and her tips to process the results.

My thanks go to my parents for allowing me the opportunity to choose my studies and for their belief in me.

Finally I want to thank all those around me especially my sister Aurélie and my boyfriend Rutger, for their cheerfulness and unfailing patience during some challenging and stressful weeks.

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Glossary

BSA	Bovin Serum Albumin
CAD	Collisionally activated dissociation
CE	Collision energy
CSF	Cerebrospinal fluid
CUR	Curtain gas
CXP	Cell exit potential
DP	Declustering potential
EMA	European medicines Agency
EP	Entrance potential
ESI	Electro spray ionization
FDA	Food and drug administration
GS1	Ion source gas
HPLC	High pressure/performance liquid chromatography
HPLC-MS/MS	High pressure liquid chromatography tandem mass spectrometry
Ihe	Interface heater
IS	Internal standard
IS	IonSpray voltage
MS	Massa spectrometer
TEM	Temperature
QC	Quality control

Abstract

For drug development, especially for research on the central nervous system (CNS), it is necessary to measure the concentration of compounds in cerebrospinal fluid (CSF), next to the common body fluids such as plasma and urine. In urine and CSF the quantification of compounds is difficult because the risk of adsorption to sampling materials. For urine, additives can be used to avoid this. However, for CSF sampling this is not allowed because of patient safety reasons. In addition, the concentration in CSF is low, which makes detection and quantification extra difficult.

In this investigation, the impact of various materials on adsorption of the compounds during sampling and storage of CSF was evaluated and the adsorption is compared to the physiochemical properties (log P and pKa) of the compounds. This might help predicting adsorption based on the properties of the compounds. Also, the possibility to set up a generic procedure with useful materials for which the total adsorption is less than 15% was evaluated. For this purpose six compounds were selected based on their log P and pKa. These were tested for adsorption on the materials that are used for CSF sampling in various phase-I clinics.

From the results it was concluded that the choice of materials really can help to limit adsorption. However, the impact is not large enough to completely avoid adsorption for all compounds. It was also clear that log P is not the only physicochemical parameter to predict adsorption.

Abstract in het Nederlands

Voor de ontwikkeling van medicijnen, vooral voor het centraal zenuwstelsel is het belangrijk om de concentratie van componenten te kunnen meten in hersenvocht (CSF) naast ander lichaamsvochten zoals in plasma en urine. In urine en CSF wordt de kwantificatie van de componenten bemoeilijkt door adsorptie aan staalname materialen. In urine kunnen er aditieven toegevoegd worden om adsoprtie te vermijden. In CSF is dit moeilijk om wille van de veiligheid van de patient. Daarboven op is er maar een lage concentratie terug te vinden in CSF wat het meten van concentratie extra bemoeilijkt.

In dit onderzoek wordt de impact van adsoprtie op verschillende materialen bestudeerd. Dit gedurdende de staalname en de opslag van CSF. Hiervoor is de adsoprtie van de componenten vergeleken met hun physicochemische eigenschappen (log P en pKa), dit kan helpen bij het voorspellen van adsoprtie op basis van deze eigenschappen. Ook wordt de mogelijkheid geëvalueerd om een staalname kit op te stellen die minder dan 15 % adsorptie geeft. Voor dit doel werden er 6 componenten geselecteerd op basis de eigenschapen log P en pKa. Deze zijn getest in materialen die gebruikt worden in verschillende fase I klinieken.

Uit de resultaten is geconcludeerd dat de keuze van het materiaal van belang is om de adsotprie te limiteren. Maar de inpact is niet groot genoeg om de adsoprtie te vermijden voor ale componenten. Ook werd duidelijk dat log P niet de enige physicochemische eigenschap is die adsoprtie kan voorspellen.

1 Introduction

1.1 Situation

Bioanalysis means analysing small compounds in a biological material. Compounds smaller as 1000 Dalton[1] will be analysed with LC-MS/MS techniques, bigger molecules will be analysed with an immunoassay. The aim is to detect and quantify the amount of drug or metabolites in various matrices.
Although the majority of the analyses are done in plasma, analyses in various tissues, cerebrospinal fluid (CSF), blood and urine are also performed frequently. The primary technique for quantification of the analytes is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Before quantification in biological material can be done, it is important to remove potential interfering compounds such as proteins and peptides. Cleaning up the sample is needed to work selectively and to have enough sensitivity to analyse the samples [1].

For developing new drugs, especially in the neuroscience therapeutic area, the brain penetration of that specific drug is an important parameter. Therefore, the compound needs to be determined in brain samples (preclinical) and/or in cerebrospinal fluid (CSF) samples (preclinical and clinical).

1.2 Cerebrospinal fluid and sampling methods

Since it is very complicated to measure the drug directly in the human brain, CSF can be used as a surrogate to evaluate the working or the effect of a drug in the central nervous system. In preclinical and clinical studies it has been demonstrated that drug concentration in CSF is rather accurate in predicting unbound drug concentration in the brain. Based on this concentration, the amount of compound that can pass through the blood-brain barrier can be determined [2]. Cerebrospinal fluid is a secretion of the brain, more specifically of the choroid plexus. This is a special tissue in the brain that produces CSF in ventricles of the brain. CSF is a clear aqueous solution that contains ions such as Na^+, Cl^-, HCO^{3-}, K^+, Mg^{2+}, certain vitamins as well as a very small amount of peptides and proteins [3]. In CSF a small amount of CO_2 is also present. The concentration of CO_2 influences the pH of fluids. Because CSF and blood are both fluids of the body, the same pH can be expected. But literature research has showed that pH of CSF will be less alkaline than the pH in blood [4]. When the fluids are sampled the pH will change because the CO_2 disappears out of the fluids. This may influence the extent of ionization of compounds, in case their pKa is close to the physiological pH. A change in ionization may result in a change of adsorption behaviour.

For CSF sampling several procedures and various materials exist. CSF sampling can be divided in several stages based on the handling and use of material. In the first stage the doctor takes a CSF sample from humans by using needles and catheters. In the second stage the solution is collected

[1] Dalton = atomic mass unit, it is a way to express the molecule mass. 1 Dalton is equal to 1/12 times the mass of free carbon 12-atom [35].

into collecting tubes. The sampled CSF volume is mostly a few millilitres per time point that may be divided into smaller aliquots. In order to do this, the sample will come into contact with the pipet tips. And at the last stage the aliquots are stored into storage tubes. For each step, various products, from different materials and suppliers are available at the market. The collecting tubes and the storage tubes are not the same because the sample volume in stage two and four are different. In stage two it is possible to have more than 10 mL of CSF, in the fourth stage the volume of aliquots is about 100 to 500 µL. In all stages a risk of adsorption to sampling material is possible.

1.3 Adsorption

To measure concentrations in CSF, it is important to evaluate the potential risk of adsorption to the sampling materials. There is an important difference between absorption and adsorption (non–specific binding). Absorption means the uptake of a substance in tissue. Adsorption is the binding of compounds on the surface of a material. In bioanalysis, adsorption is a very important issue. In CSF the concentration of compounds is often low. If the adsorption is high, the concentration of the relevant compound cannot be measured accurately and precisely, or the compound will not even be detected. This potential underestimation can constitute an issue when accurate concentrations of the compound are necessary to investigate a disease or to evaluate the distribution of a compound in the central nervous system. This can result in wrong decisions regarding the disease or the treatment of a patient.

In biological matrices, many compounds tend to bind to a certain extent to peptides or to proteins present in samples. In urine and CSF only small amounts of proteins and peptides are present. This can create issues when drugs have to be quantified in these samples. The presence of high concentrations of proteins in matrices as plasma minimizes adsorption of the compounds to the sampling material. This is in contrast to matrices as urine and CSF that contains low concentrations of peptides and where adsorption to material will probably occur. Body fluids are polar fluids and the sampling materials are mostly non polar materials. Non polar compounds will be more attracted to the non-polar materials, this introduce the adsorption. The aim of this thesis will be to find materials minimize adsorption of compounds and to find a way to correct for the adsorption that has taken place during sample collection.

Accurately measuring concentrations of compounds in CSF is not easy. A lot of difficulties arise and are linked to each other. The following reasons describe the challenges to analyse concentrations in CSF.

Many compounds can adsorb to various materials that are used in the different sampling stages such as needles, recipients, pipets and tubing. This is especially more pronounced with low concentrations. The concentrations that can be found in CSF are similar to the concentration of the free fraction in plasma. The free fraction is the amount of compounds that does not bind to

proteins and peptides in plasma and can pass the blood brain barrier[2]. Because the concentrations of the target compounds in CSF are so low, adsorption will be more pronounced because active sites on container walls are relatively abundant, compared to high concentrations. With a high concentration of target compounds, the attraction to the active sites on the container walls will be barely notice and adsorption do not have an influence on the results.
Since a lot of parameters need to be measured and clinical investigators wants perform more clinical studies with CSF, the collected sample volume needs to be aliquoted[3]. Aliquoting samples increases the chance of adsorption. In addition, it limits the volume of sample that is available to determine each parameter because the total volume of CSF that can be collected is limited to a few mL per time point. This may result in relatively low volumes of CSF in tubes. The lower volume/surface ratio compared to a tube that is completely filled may result in more adsorption.
Not only is the concentration of the compound low but the concentration of proteins and peptides in CSF also. As explained before, this means that compounds rather bind to the various materials than to the proteins in the sampled plasma and thus more adsorption to the materials will occur.

The most important limitation in designing optimal procedures is to respect the health of the volunteer, requiring sterile materials. Because of this, the use of additives to avoid adsorption in the second stage of sampling is not allowed by physicians because they have to guarantee the health of the volunteer or patient. Normally the collecting tube does not get into contact with the needle or tubes and will not represent a danger to the volunteer if an additive is used. In the worst case, however, the additive could contaminate the needle, which could be hazardous for the subject. Another possibility is to use some additives only in the fourth stage of sampling. But this may result in some logistical problems. An example of such a problem is when two aliquots needs additive 'A', another additive 'B' and some aliquots do not need an additive. This will make the sampling procedure complicated, with considerably more chance of mistakes, e.g. that the wrong aliquot goes into the wrong tube and/or to the wrong laboratory. In addition the added value could be questioned, because adsorption may already have been occurred significantly in the first three stages.

The maximum allowed adsorption in the total procedure of measuring CSF is limited to 15%. The sum of errors of the whole procedure (sampling, sample preparation and analysis) needs to remain under this 15%. The bias of 15% is determined by the bioanalytical guidelines (FDA,EMA) based on the complex matrix, the various steps needed in the procedure and the variation on the pharmacokinetic parameters [5], [6].

In literature a lot is known already about adsorption of proteins and biomarkers of CSF to several collection materials, e.g. for Tau proteins, amyloid β peptides, rhG [7], [8], [9], [10], [11], [12]. This confirms that adsorption in CSF is an actual problem. While these papers describe evaluation of adsorption to a single compound, or compound class, there is not much information regarding general principles of adsorption that can be used to predict adsorption, or to find generic

[2] This is a barrier that will separate the blood and CSF from each other in the brain.
[3] Aliquoting is dividing the sample in smaller volumes.

approaches to limit adsorption. Research in CSF matrix is of relatively recent date. CSF sampling was not common. This is in contrast to urine sampling and analysis. Urine is a matrix that is used for a very long time in research. It is a lot easier to sample, is more available and it is a good matrix to investigate how the molecules works in the body. Those are probably the reasons why research in urine is more developed. Therefore more information regarding adsorption of compounds in urine, how compounds react in recipient and how the effects of adsorption are mitigated is available. In addition, in urine various additives (tween, triton X100, BSA) can be used to reduce adsorption [13]. However, as described above, for CSF sampling this is not easy since the health of the volunteer has to be guarantee. The abundant information of adsorption of compounds in urine can be used in the investigation of adsorption of the compounds in CSF. When a compound adsorbs in a sampling recipient of urine, it will probably adsorb to CSF sampling material as well. The usefulness of this information may be limited because the amount of adsorption may differ from that in CSF due to the differences in composition, the sample volume and the use of other types of sample collection materials.

In one article [11] the use of polypropylene tubes is described. This is very relevant because these collecting tubes and storage tubes are also used in a standard protocol for CSF collection and storage. They investigated polypropylene tubes originating form 3 different suppliers: Fluidx, Eppendorf and Sarstedt tubes. The differences between suppliers were the type of coating inside the tubes or the chain length.

This study showed that adsorption in Sarstedt tube was more pronounced than in the other two types. These results are shown in figure 1.

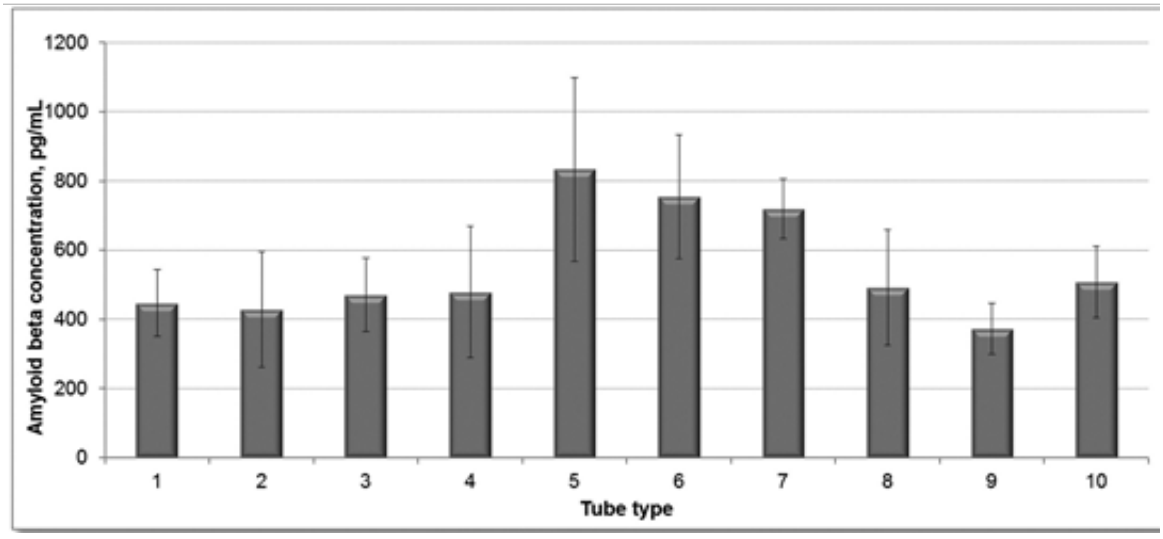


Figure 1: Influence of the adsorption in tubes. Concentration of $A\beta_{1-42}$, pg/ml (+/- STD) in polypropylene tubes of different suppliers with different sterilization cycles: (1-8) Fluidx tubes, (9) Sarstedt, (10) Eppendorf; (1-4) copolymer, (5-8) homopolymer, (1-5) unsterilized, (2,6) one sterilization cycle (irradiation with 25-40 kGy), (3,7) two sterilization cycles (irradiation with 50-80 kGy), (4,8) three sterilization cycles (irradiation with 75-120 kGy), (9,10) unknown numbers of sterilization cycles **[11].**

A second very interesting article [12] did some research about reducing adsorption in micro-dialysis. In this article some compounds were tested on adsorption in tubing and probe membranes. A suggestion in this article is that the drugs with a high lipophilicity will adsorb to the material and will give a risk of underestimating concentrations. During the adsorption evaluation of the compound, the compounds were divided into two groups, one group with low adsorption and one group with more adsorption to the tubing. The test was performed with artificial CSF and they concluded that an increase in BSA concentration in the matrix reduced the adsorption. The influence of adding BSA on the adsorption in tubing is shown in figure 2. These results confirm the hypothesis that there is relatively more adsorption when fewer proteins are present in the sample. This hypothesis will be studied as well in this thesis.

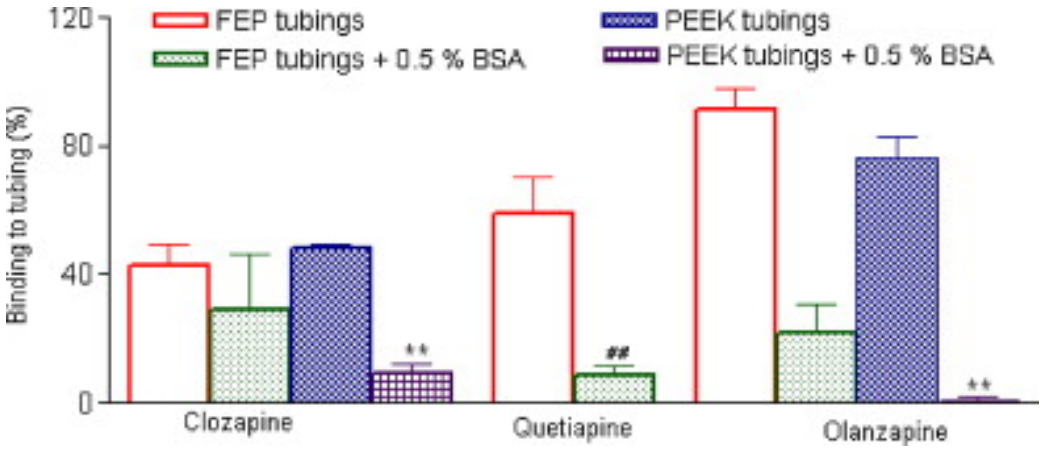


Figure 2: Influence of adding BSA on adsorption. Non-specific binding to fluorinated ethylene propylene (FEP) and polyetheretherketone (PEEK) tubing **[12]**.

1.4 Artificial CSF

Another limitation is the availability of only low volumes of CSF for all tests and for the use of reference matrix in quantitative experiments. There are some ethical aspects preventing the collection of large amounts of CSF from humans. It can be stressful for the patient and the safety of the volunteers has to be guarded during the whole sampling process.

The subjects are well paid to donate their CSF for human research. This can be seen as purchasing CSF. On the other hand this makes the investigation with CSF very expensive (remnant CSF: $25-$30/ mL per donor, prospective collected CSF is around $750/ mL). It would be helpful if a surrogate matrix could be used, not only for ethical reasons but also for economic reasons. An artificially prepared CSF could be used for this, provided that it would mimic real CSF sufficiently to enable representative use with respect to adsorption and/ or matrix effects. Therefore, the composition of this so called artificial CSF needs to be similar to real CSF. While one can buy artificial CSF, this does not contain any proteins and peptides. As these are important for influencing the extent of potential adsorption, it is preferably to add a protein, such as Bovin Serum Albumin (BSA).

1.5 Compounds

A lot of compounds are developed to have an impact in central nervous system. These compounds can be found in CSF and a lot of research is needed to this compounds. The reason why compounds are measured in CSF is to understand the reaction of the compound in CSF and to investigate if compounds do not have side effects. A few compounds will be explained below. Some compounds are already on the market and will have their own name, some compounds are not yet on the market and will be called compound A, B… The compounds of the list can be dived in three groups:

1. Compounds with a function in the central nervous system;
2. Oncology compounds;
3. Compounds with possible side effects in the brain.

1.5.1 Compounds with a function in the central nervous system

This group contains a lot of compounds. The group can be subdivided as well.

1.5.1.1 Antipsychotic

These drugs are used to treat symptoms of schizophrenia. The drugs change the effect of chemicals in the brain or it is able to block dopamine receptors in the brain [14], [15]. The follow compounds belong to this group of drugs:

- Risperidone
- Paliperidone

Other drugs in this group are primarily used to treat schizophrenia, but the medication can also be used with other medication in the treatment of depression, autistic disorder and bipolar disorder. These antipsychotics also can help to control aggressive behaviour and mood changes. This medication works by changing the activity of certain substance in the brain [16], [17], [18]. The following compounds belong to this group of drugs:

- Olanzapine
- Aripiprazole
- Quetiapine
- Clozapine

1.5.1.2 Compounds for other diseases

In this subdivision contains compounds to treat pain, Alzheimer, depression and so on. Follow compound belong to this group of drugs:

- Compound A
- Compound B
- Compound C
- Compound D
- Compound E

1.5.2 Oncology compounds

These are compounds that are used to treat al kind of cancers. There are a lot of cancers and medication to treat them but only a limited amount of compounds can pass the blood-brain barrier. One of the compounds that can pass is Decitabine. It is used to treat patients with myelodysplastic syndromes (MDS). Decitabine is a hypomethylating which is responsible for the re-expression of tumour suppressor genes. It is able to stop the growth of tumours and can be found in CSF [19].

1.5.3 Compounds with possible side-effects in the brain

For some compound there is seen in early studies that it is possible that a small amount of these compounds can pass the blood-brain barrier. These compounds could have some unwanted side-effects. To minimalize these side-effects, further investigations in CSF could be needed.

1.6 Physicochemical properties

Based on the physicochemical properties described below, six compounds were selected for this thesis. The possible compounds that were selected are described in 1.5.

1.6.1 Acid dissociation constant

The acid dissociation constant (Ka) is a parameter to quantify the strength of an analyte in a solution. The acid dissociate constant can be determine as follow:

$$Ka = \frac{[A^-][H^+]}{[HA]}$$

With [A-] the concentration of acid ions, [H+] the concentrations of the protons and [HA] the concentration of the acid. Mostly it will used as pKa, the relation of both is shown in the formula below

$$pKa = -\log Ka$$

The pKa could give an indication whether a compound will adsorb. The adsorption can be influenced by the pH of the matrix, the pH can change due to storage or aging. When the pH of the matrix is higher than the pKa value of the compound the concentrations of charged ions is higher

than non-charged ions in the solution, the compound will be more polar. This means it is easier to hold the compound in solution, resulting in less adsorption. When the compounds are neutral by the pH of the matrix, the compounds will be more non-polar and will probably adsorb. The relation of the pH, the pKa and charged molecules is given in the formula:

$$pH = pKa + \log\frac{[A^-]}{[AH]}$$

In this investigation the compounds are particularly basic and also expressed on pKa scale. In that case the theory is turned, when he pH is lower than the pKa the compounds will be more ionic.

1.6.2 Log P

Log P is the octanol/water partition coefficient, formula being shown below. The value is an indication of the degree of lipophilicity or hydrophobicity of a compound [20]. When the partition coefficient is high, the compound is more soluble in the octanol phase instead of the water phase and will be more lipophilic. As a result it will probably adsorb more because the compound has more affinity to a non-polar container wall, such as polypropylene, than to hydrophilic (aqueous) environments, such as CSF.

$$P_{ow} = \frac{C_{octanol}}{C_{water}}$$

With $C_{octanol}$ the molar concentration of compound in the octanol phase is described, and C_{water} is the molar concentration of compound in the water phase. P_{ow} is often expressed as log P.

Table 1: Log P values of some organic compounds [21].

molecule	Log P
Methanol	-0,77
Epoxy-ethane	-0,30
1,2- epoxypropane	0,03
2-butyne	1,46
1,4 pentadiene	2,48
n-pentane	3,39
1-heptene	3,99
1-octene	4,57
Eicosanoic acid	9,29

There is also a relation between log P and the pH. This relation is called log D.

$$D = \frac{C_{octanol}}{C_{water} + C_{ion\,,water}}$$

With $C_{octanol}$ the concentration of compound in its molecular form in octanol, c_{water} the concentration of compound in its molecular form in water and $C_{ion,\ water}$ the concentration of the compound in its ionized form in water.

Log D is in fact the partition of the ionized compounds in both phases (octanol and water). This partition depends on the pH value of the matrices. Log P = log D for unionized compounds at any pH while log P is fixed at a particular pH 7,4. When the pH has an influence, the graph of log D has to be evaluated to see if the pH of the matrix could have an influence. Quetiapine, in the figure below the log D graphic is shown as an example. In his graphic the relation between the pH and log D is given. CSF has a pH of 7,36. At this pH can be seen that the log D will change a lot if the pH change a bit. The lipophilicity of the compound could change what could impact the adsorption. For quetiapine could be see that when the matrix has a pH of 8 or more the log D is high and do not change anymore, on that point adsorption could be possible. When the matrices are less than 8 the compound will become more polar and could give less and less adsorption by decreasing the pH. An generic role that can be used on base compounds is when the pH is higher than the pKa the compounds will be less ionized, the log D will increases, the molecule will be less polar and this means there will be more adsorption to non-polar materials.

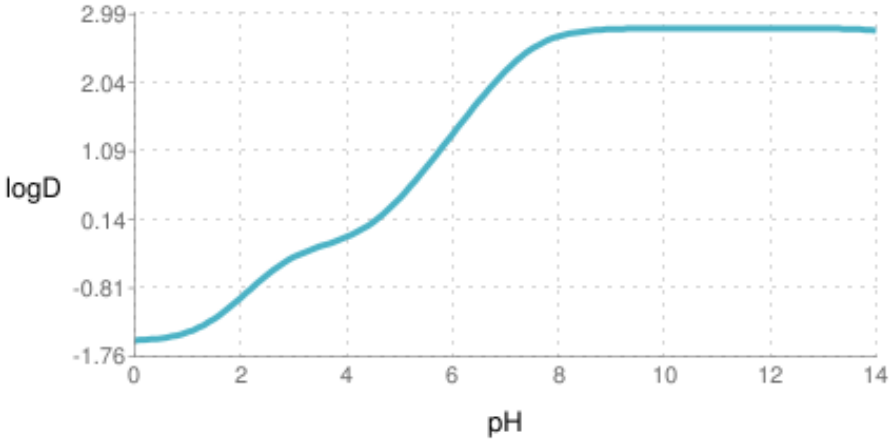


Figure 3: pH - log D relation of Quetiapine [22]

1.7 Materials

Also the compositions of the materials have a role in the amount of adsorption. Not every material has the same composition or has the same structure. It is important to investigate the relation between the different structures and the physicochemical properties of the compounds and matrix. It could be helpful if a relation can be found because the amount of adsorption of all kind of compounds could be predicted. This can an indication which kind of materials are desirable in use and which materials has to be avoid due to too much adsorption.

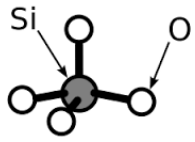
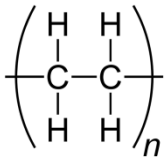
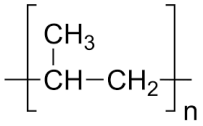
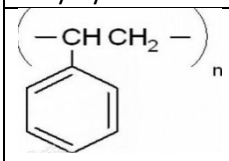
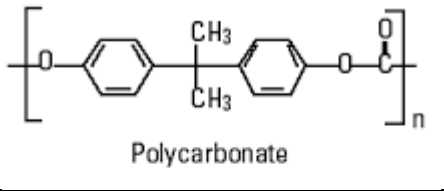
The most common materials used in laboratories and for sampling are poly-ethylene, polypropylene, polystyrene, glass, and polycarbonate. Materials could have the same chemical composition but the chain length, orientation of the chains and inside coating can be different and

can affect the extent of adsorption. In this thesis the focus will be on the chemical composition, because information about all other variables are very hard to obtain from manufacturers.

In the literature the composition of Fluidx, one of the tubes that is described in article [11] and that will be used is found. Fluidx had a special composition, the tube is made of a homopolymer (rasin), this materials provides lower binding and has a larger temperature tolerance than normal polypropylene [23]. This is already an explanation of the fact that Fluidx had the most recovery in article [11] what was discussed in '1.3 Adsorption'.

A hypothesis states that compounds with a high log P value will adsorb more to non-polar materials. This means the adsorption to polyethylene and polypropylene will likely be more than to glass. The opposite of this hypothesis is that compounds with a low log P value will adsorb more to glass. This different hypothesis will be investigated as well in the tests. The polarity of the materials is as follow, starting with the most polar material and ending with the most non polar material: glass, polycarbonate, polyethylene, polypropylene and polystyrene.

Table 2: Structure of the different kinds of materials.

Glass	Polyethylene	Polypropylene
		
Polystyrene	**Polycarbonate**	
		

1.8 Sample instrumentation

In this chapter the different steps of the analysing will be explained. The first step is the sampling preparation. When the sampling preparation is done the samples can be analysed in the HPLC-MS/MS. In that phase the molecules will be separated and detected by mass spectrometry.

1.8.1 Sample preparation

The sample has been cleaned to be sure that the method is specific and sensitive to find the desirable molecule [24]. Before analysis by LC-MS/MS, interfering elements need be removed otherwise the column will clogged very fast. The interfering elements can be removed by liquid-liquid extraction, solid phase extraction or by protein precipitation. The latter is the most common technique because it is the fasted technique and gives enough sensitivity to measure the desirable compounds. After sampling preparation the molecules will be separated and detected.

Protein precipitation is a technique that uses additives able to denature water-soluble proteins in matrix like plasma. The agents interact with the tertiary protein structure and produce an amorphous mass, this precipitates out of the solution. A solvent can be used to denature the proteins, mostly acetonitrile and methanol are used. This technique is the most common technique used by bioanalysis. It removes a minimal amount of interfering compounds and this is enough to work selectively and specifically [25].

During sampling preparation an internal standard (IS) is applied. An intern standard is a substance with similar physicochemical properties as the compound that will be analysed. A known and identical amount is added to all study samples to facilitate quantification of the analyte. Each compound has its own IS so a mix of IS will be made (comparable to the compound mix). Mostly a Stable isotopic labelled internal standard is used. The use of IS is needed to compensate the loss of compound during sample preparation or variation that occurs during measurement [26], [27].

1.8.2 Separation of molecules

In this study high pressure/ performance liquid chromatography is used. Liquid chromatography is a form of chromatography used to separate compounds using a mobile phase to push analytes through a column. In this case high pressure is used to push the mobile phase through the column. The sample is injected in a column, the column is called the stationary phase and are packed by particles. The molecules are eluted by a solvent (mobile phase) that is pumped into the column after injecting the sample. The stationary phase and the mobile phase depend of the analytes, these two can be changed depending on the characters of the analytes. Separating the molecules in the analytes is based on the affinity to the mobile phase meaning that the molecules are capable to interact with the mobile phase through non covalent binding. Non-covalent binding could be hydrogen bound, ion interaction or van der Waals forces. Molecules with greater affinity for the stationary phase elute later than molecules with a greater affinity to the mobile phase. The molecules need a while to go through the column. This is called the retention time. The retention time is specific for the molecules on a specific type of column and the composition of the mobile phase. Due to differences in retention time between the molecules they get separated into the column. The liquid flow from the column will be send to a detector. There are different mechanisms to separate the molecules in HPLC: reversed phase, normal phase, ion exchange, hydrophobic interaction and differentiation by size. The two first are the most used in bioanalyses so only those two will be explained [28].

1.8.2.1 Reversed phase chromatography

Reversed phase is the most used technique. For this technique the stationary phase is a hydrophobic stationary phase. The mobile phase is polar and non-polar molecules have the longest retention time because non-polar molecules are less attracted to this phase. The most used mobile phases to elute molecules from the column in bioanalyses are 0.1% ammonium carbonate, 0.1% formid acid or ammonium formate pH 4 and ammonium acetate. Often, the mobile phase exists of 2 parts (2 solvents) and will be used in a gradient. In the beginning solvent A (the aqueous solvent) will be more present in de gradient. In function of time solvent B (an organic

solvent; acetonitrile or methanol) will increase so the non-polar molecules can rid of the column as well. The most common stationary phases are C18 and C8. These are absorbent particles with a ligand of linear hydrocarbon chain. C8 and C18 are the length of the chains [24], [28].

1.8.2.2 Normal phase chromatography

Normal phase is the opposite of reversed phase chromatography, the stationary phase is hydrophilic and the mobile phase is hydrophobic. Polar molecules have the greatest affinity to the stationary phase so the retention time of polar molecules are the longest. The packaging includes silica, amino and alumina.

1.8.3 Detection of molecules

At bioanalysis mass spectrometry is often used as method to identify and quantify the molecules that were separated in the HPLC. The identification is based on the molecular mass of the parent ion and the product ion. The quantification of results will be done using a reference when the results are relative and using a standard curve when the results need to be exact. There are several steps to do:

- Ionisation
- Ion separation
- Ion detection

1.8.3.1 Ionisation

A technique that is most common to vaporise the molecules in bioanalyses is the electro spray ionisation (ESI). ESI is a soft ionisation method, this means that the ions stay intact and do not fragment in this stage. Figure 5 describes the working of ESI. The liquid flow from the HPLC has to be channelled into a capillary. At the end of the capillary a high voltage of about 3 to 5 kV is applied. There is also a nebulizer gas at the outside of the capillary to spray the sample. The nebulizer gas can be heated and ensures that a fine mist of charged droplets is created. The charged droplets move forward and solvent will evaporated. The charged droplets have the same polarity as the voltage that was applied on the capillary. The charge remains the same around the molecule, the solvent around the molecules will evaporate and the Coulomb force will increase. When at a certain point the mutual repulsive force of the charge exceeds the liquid surface tension, the droplets fall apart in smaller ions. This is also the coulomb explosion. Because of the heat the droplets of solvent that remain around the ions will vaporise so only the charged ions remains. These charged ions are present in a gaseous phase and will pass to the mass spectrometer [29].

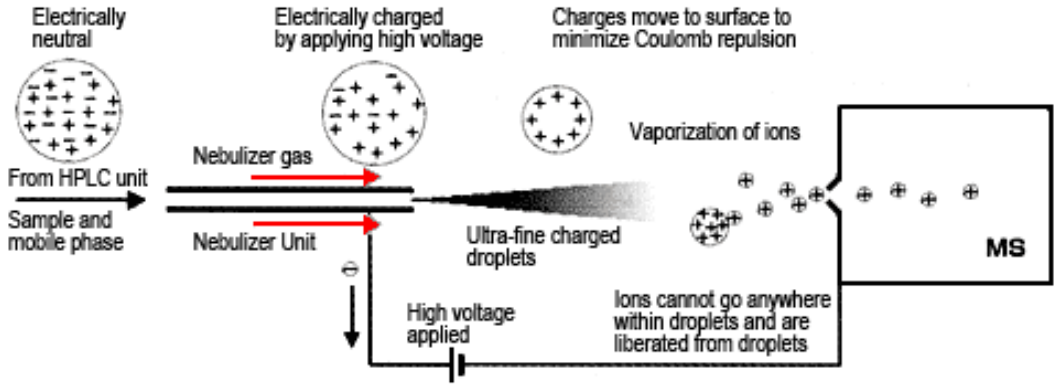


Figure 4: Schematic description of electro spray ionisation [29].

1.8.3.2 Ion separation

To separate ions an analyser is needed. There are a lot of different mass-analysers but for this study a triple quadrupole is used. The ions will be separated based on their mass/charge (m/z) ratio.

The spectrometer is divided into three parts (see figure 4). There are two selective quadrupoles for the selection of the molecules and one quadrupole which is used as collision cell. Each quadrupole consists of four parallel cylindrical rods. Each opposing pair of rods is connected electrically. An alternating current is applied to the rods. Ions travel down the quadrupole between the rods. Only ions with a defined m/z ratio will reach the detector for any given frequency. The other ions have unstable trajectories between the rods and will collide with the rods. This allows the selection of ions with a specific m/z ratio. Q1 and Q3 are quadrupoles. The different between Q1 and Q3 is that in Q1 the parent ion is selected and in Q3 the fragment (product) is selected that forms in the collision cell that is located between the two quadrupoles. In this cell there is a slightly higher pressure, due to the presence of nitrogen gas. The parent ions collide with the gas molecules with a lot of energy, therewith falling into fragments [26] [30].

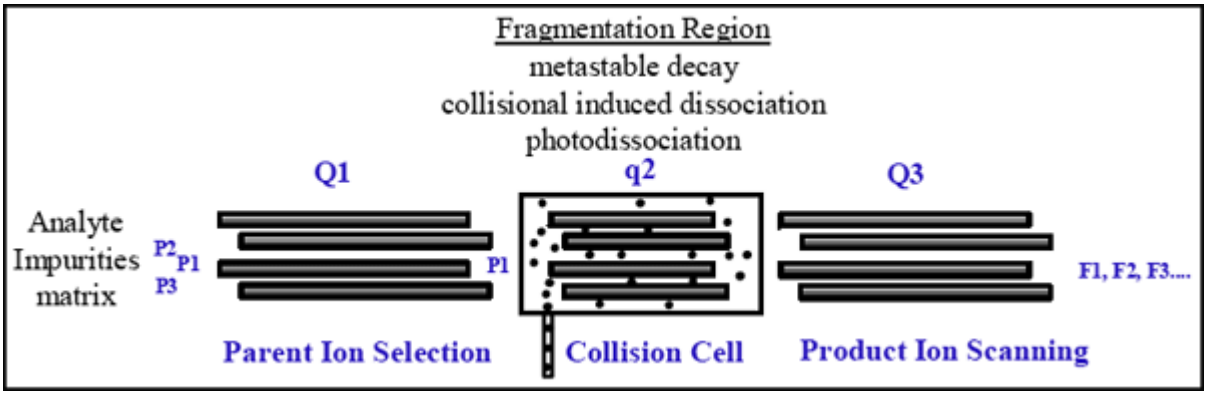


Figure 5: Principle of the tandem mass spectrometer [31].

1.8.3.3 Ion detection

The last part is the detection of the signal. In the instrument used in the lab, a channel electron multiplier is present. The charge will enter the multiplier, when it hits the surface of the electrode electrons are produced. These electrons generate the current. This will provide signals showing the peaks that can be seen at the screens of the computer. The size of the peak is determined by the amount of the molecules that can produce electrons [26].

2 Objectives

The aim of this study is to evaluate the possibility for a preferred generic procedure of materials to sample CSF for compounds known to adsorb to sampling material. The amount of this adsorption should be less than 15 %. To achieve this, some sub goals has to be determined.

- Making an inventory of materials used in clinics to sample CSF;
- Selecting compounds based on different physicochemical properties that will be evaluated on adsorption;
- Finding an optimal composition of artificial CSF to use as surrogate matrix for adsorption evaluation;
- A sample preparation and analysis method (LC-MS/MS) has to be developed to analyze those compounds, preferably in a combined assay.

After doing the adsorption test of the materials that are used in Phase I clinics, the laboratory should tell the clinics which materials are acceptable and which are not. The amount of adsorption in the materials that are used in the lab is known after the adsorption test. This will be used to calculate the exact concentration from the real samples. The information of adsorption to the materials will be used to tell phase I clinics which materials they can no longer use because of the amount of adsorption.

Also the influence of the physicochemical properties on the adsorption of the compounds to sampling materials will be evaluated to see if predictions of the amount of adsorption can be made based on the physiochemical properties of compounds.

3 Materials and methods

3.1 Tested sampling materials

An inventory of the different materials was made and tested. Materials of the stages mentioned in chapter 1.2 'Cerebrospinal fluid', used in the following clinics: Clinical Pathology Unit (CPU), Société Générale de Surveillance (SGS), Quality Performance Service (QPS) and Centrum for Human Drug Research (CHDR), were evaluated in the adsorption tests. Al these clinics investigate new drugs with volunteers for pharmaceutical companies. CPU and SGS are located in Antwerp, Belgium and the other two are located in the Netherlands: QPS in Groningen and CHDR in Leiden. These clinics were selected because they conduct Phase I clinical studies. This means that these clinics generally use their own materials. For clinics that are participating in Phase II or Phase III clinical studies, a central laboratory provides the materials that have to be used in a study. This means that all clinics use the same materials for a test so the variations of the different kinds of materials are more controlled. Because of the time and efficiency of these tests, not every material can be tested. However, for every stage of sampling some materials will be selected.

3.2 Compounds

The list of compounds is shown in table 1. These compounds are potentially interesting to test in combination with CSF and were therefore tested. More information about the application of the compounds is previously described in 1.5.

Table 3: Potential interesting compounds to be tested.

Compounds	
compound A	Deciatbine
compound B	Risperidone
compound C	Paliperidone
compound D	Olanzapine
compound E	Aripiprazole
Quetiapine	Clozapine

Having selected compounds based on their physicochemical properties (log P and pKa), a mix of these compounds was prepared. The concentration of each compound in the mix is based on the concentration that can be found in CSF. The concentration of the compounds already on the market can be calculated. For the calculation it is necessary to know the concentrations that can be found in plasma and in CSF and are depended on the dose taken by the patient. In this study the minimal detectable concentration of the compound in CSF has to be found, so the possible concentrations were calculated based on the C_{max} values of the compound that can be found in plasma when the lowest therapeutic dose is given to the patients. The plasma concentration of a compound at a certain dose level can be found in literature. Only the unbound drug concentration can pass through the blood-brain barrier therefore only a part of the plasma concentration can be

found in CSF. The fraction of unbound drug concentration can be found as well in the literature. For JNJ-compounds (A, B, C, D and E) all this information is still classified so only a concentration is given.

An example of the calculation of the concentration of a drug that can be found in CSF, is explained with the test compound Quetiapine. The unbound plasma fraction (FU) of quetiapine is 17%, the maximum concentration that can be found in plasma is 80 ng/ml when a tablet of 25mg is taken, and this is the smallest dose that exists of that compound. To calculate the possible concentration in CSF, the concentration in plasma has to be multiplied by the unbound fraction in plasma [32].

3.3 Test setup

First an analysis method (LC-MS/MS) was developed, to detect the six selected compounds in CSF samples at the same time. The settings of the developed method are described in 3.4.

Before CSF samples can be measured at the LC-MS/MS, a sample preparation is needed to remove the proteins. From some preliminary adsorption tests it was learned that it was necessary to optimize the original preparation method in order to have less variation to be able to observe the small differences in adsorption. Optimization was performed by increasing the volume (method 2) or replication (method 3). This resulted in the fact that different sample preparation methods were used in different tests.

For preparation method 1, five volumes of acetonitrile were added to the sample to precipitate the proteins. Afterwards the plate needs to be vortex mixed and centrifuged. Three replicates where taken from three different tubes and of every replicate one preparation was made. The sample preparation was evaluated in a reproducibility test and used in a 96 well plate.
A reproducibility test was performed for this method to see if analysing six compounds at once is reproducible and accurate and to determine the minimal concentration of the compounds that can be detected with LC-MS/MS. Plasma was used in this test because compounds are stable in plasma and there will be no adsorption. During sample preparation, the IS-mix was added.
To test the reproducibility of preparation method 1 a standard curve and some quality controls samples were analysed. By using the standard curve the measuring range was investigated. The QC's were used to investigate the accuracy of the test and the reproducibility. The concentrations of the compound for the standard curve were within the range of 1% - 1000% of the actual concentration of the compound that is used in this investigation. The 1% value is chosen to be able to determine the minimal concentration of the compounds that still can be detected with LC-MS/MS when adsorption occurs. The 10% above the real concentration is to have a marge. All compounds are dissolved together in a stock solution, the solution is dissolved in DSMO. The mix of all IS are dissolved in DMSO as well. To mimic the samples that are coming from clinics (study samples) only 2 % solvent is allowed in the QC's samples. The QC is made in plasma. The sample preparation of the QC sample is shown in table 4.

Table 4: Sample preparation method 1 that was used in the first experiment to evaluate the sample preparation. The standard curve and the QC's are used only in the reproducibility test, the method of the studied samples are used in the other test.

Studied samples	Standard curve	QC
25 µL plasma	25 µL plasma	25 µL plasma + compounds
25 µL sample with compound	25µl DMSO + compound	25 µL DMSO
25 µL IS in acetonitrile	25 µL IS in acetonitrile	25 µL IS in acetonitrile
Vortex		
125 µL acetonitrile		
Vortex + centrifugation		

Based on preliminary adsorption experiments it was necessary to minimize the variation caused by preparation method 1. Therefore preparation method 1 was adapted to method 2 in order reduce variation by having a better mixing of the solution. It was assumed that in a 96 well plate the solution could not be vortex mixed good enough and that 125 µL of acetonitrile was not enough to reach the top of the well to get a homogenous solutions. Therefore, the 96 well was changed in Micronics micro tubes and the volume of acetonitrile is increased to 250 µL. The adapted preparation method is described in the table 6. As in preparation method 1 three replicates where taken from three different tubes and of every replicate one preparation was made. This preparation method was used for the first test of the needles, the pipet-tips, the pH test and the surrogate matrix test.

Table 5: The second preparation method.

250 µL Acetonitrile
20 µL IS in DMSO
20 µL sample with compound
Vortex + centrifugation

In method 3 the preparation stays the same as method 2 but the number of aliquots is increased. Results showed that when 10 replicates were taken with for every replicate 3 preparations were made, the variation on the results were better. This was needed the test the tubes, the pipet-tips that are used in the clinic and the needle test otherwise small amount of adsorption could be missed.

3.3.1 Selection of most optimal surrogate matrix

The evaluation of the adsorption of the compounds by the different materials was done in different steps. First an appropriate composition of artificial CSF was selected that can be used as a surrogate of real CSF. Therefore different compositions of artificial CSF were prepared, varying in their amounts of peptides and pH, and the adsorption was evaluated in different materials: polypropylene, polyethylene, polystyrene and glass. The concentration of the compounds in the different materials was compared to the concentration of the compounds in real CSF. The test is

done by transferring the fluids 5 times. The amount of adsorption was calculated relatively by comparing tube 6 by tube 1. The use of the different materials could give an indication if there is a relation between the physicochemical properties of the compound and the material that will be used.

Artificial CSF is made in the laboratory and contains: NaCl 8,59 g/L; KCl 0,20 g/L; $CaCl_2.6H_2O$ 0,26 g/L $MgCl_2.6H_2O$ 0,17g/L and $Na_2HPO_4.2H_20$ 0,18 g/L [33]. To this composition different concentrations of BSA (0%, 0,25%, 0,5%, 1% and 2%) were added to evaluate which is the most appropriate to simulate real CSF.

Before an optimal surrogate can be found, the pH of the composition needs to be adjusted to the pH of real, fresh sampled CSF, which is +/-7,36 [4]. The pH of real CSF can change during storage because CO_2 will dissipate slowly out of the sample. The pH of the samples becomes more alkaline. The pH of the fresh sample has to be compared with the stored sample because the pH of the matrices can influence the amount of adsorption of the compounds. The possible influence of the pH on adsorption is tested. The adsorption of the six compounds in a matrix with a pH of fresh CSF is compared to the adsorption of compounds in a matrix with a pH of stored CSF. The test is important to determine if there is more adsorption in stored CSF so this can be considerate.

3.3.2 Adsorption evaluation

Adsorption of the compounds was determined by measuring the compounds in the artificial CSF before and after transferring the solution to the various materials. For every stage of the sampling procedure, several materials were used that need to be tested. At the sampling stage, needles and catheters were tested, at the collecting stage CSF tubes of 10 ml are used. In the third phase all kind of pipets are evaluated and in the aliquots phase small collecting tubes were used. The sequence of the adsorption tests is different than the sequence of sampling. To measure the adsorption in all these materials several methods are used. The applied methods are described below. The adsorption of all tests is calculated relatively, the concentration in the last aliquot is always compared to the concentration of the first aliquot which is taken at the beginning of the tests. Afterwards, the adsorption results of the different materials are compared to each other in order to develop a sampling CSF procedure with a minimal of adsorption.

The sequences of the tests are done in function of the relevance in the laboratory. First the pipet-tips that are commonly used in the lab are tested. Afterward the sequence of the sampling procedure is followed.

3.3.2.1 Adsorption test for pipet-tips

First the adsorption in the tip is evaluated. The pipet tip is used in stage 3 of CSF sampling and for sample preparation. Figure 6 describes the first test. The aim of this test is to test the adsorption in the pipet and the saturation of the tip after a certain amount of sampling. The container is filled with a known concentration of compound and with artificial CSF. From this container five aliquots from 20 µL were taken. A very important issue in this test is that all aliquots were taken with the

same pipet tip and the first aliquot could not be prewetted[4]. Otherwise saturation cannot be determined. Also all aliquots need to be measured. When the concentration of the compound does not change anymore, saturation has occurred. If all the concentrations are lower, there is no saturation in the pipet tip. It is important that the tip is not inserted too deep into the solutions because there could be adsorption at the outside of the tip as well. In this test the adsorption is calculated compared to a standard point. The standard point is a DMSO solution spiked with the six compounds. The concentrations of the compounds are the same as (a)CSF used for the adsorption test. Every aliquot of matrix is regard to the standard point in DMSO. The reason why a standard point in DMSO is chosen is because normally there is no adsorption in DMSO.

The set-up for the tips used in clinics is a bit different. The amount of aliquots is increased to 7 aliquots and the volume is increased as well, from 20µL to 500µL. These volume and amount of aliquots are more used in the clinic. In clinics they take aliquots of 500µL and mostly the amount of volume that is sampled is between 10-15 mL.

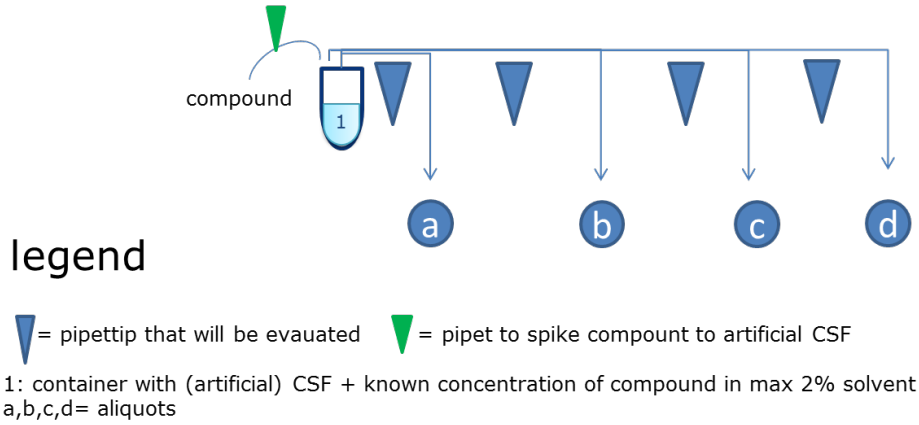


Figure 6: Evaluation of the adsorption and saturation to a pipet tip

3.3.2.2 Adsorption test for needles and tubing

Needles and tubing are tested on adsorption by rinsing the material with a specified volume of CSF. The aim is to test after how many volumes of the fluid the material is saturated. The solution that passes through the material is collected in a tube. When the concentration in the solution remains the same, the material is already saturated or no adsorption was measured. When it is not (yet) saturated, the percentage of adsorption can be measured and must be taken into account in the further procedure. The figure 7 explains the test set up. In this test a reference sample is used to compare the results of the samples that went through the needle. The reference sample is an aliquot taken from the first container. After taking the aliquot, the sample is put into the needle by using a pipet, amounts of 500 µL per time will pass through the needle and this is done 10 times. In total one mL has to pass through the needle. After passing the needle the sample is collected in a tube. From these tubes 20 µL aliquots were analysed.

[4]Prewetting means rinsing the pipet in advance before taking an aliquot.

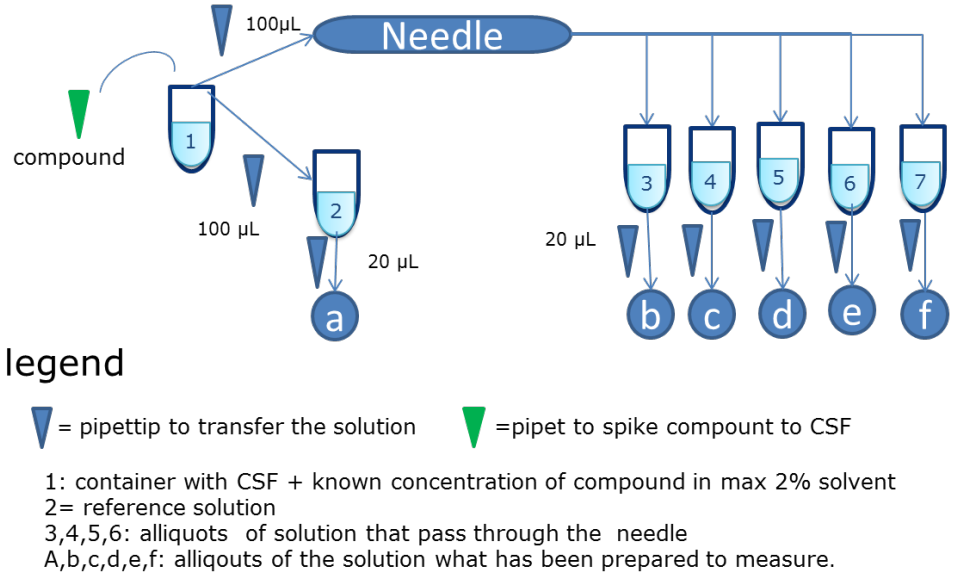


Figure 7: Principle of the adsorption test for needles

3.3.2.3 Adsorption tests for tubes:

This method describes the adsorption in tubes. In figure 9 the adsorption test with tubes is described. From the first container an aliquot (25 µL) is taken before the test can proceed, this aliquot was used as reference. The amount of sample in the first container is different if the container is a storage container or a collecting container. Collecting containers were filled to 50% of the max volume and in the storage containers the tests are done with an amount of 20% of the max volume. After this first step, the solution of container one is transferred several times (mostly 5 times) from container to container until all the volume is transferred. The transfer is done by decanting and not by a pipet to avoid adsorption to the pipet tip. From the last container a new aliquot (25 µL) is taken. The results of these two aliquots can be compared and it will produce a relative value of adsorption in the container. With this setup, the problem is enlarged this is done to be sure to pick up the adsorption if it is present and that the variation of the method is not seen as possible adsorption. Another reason to apply this is to cover variations of the clinic: the difference in temperature, the time that samples remain at room temperature, the different ways of sampling…

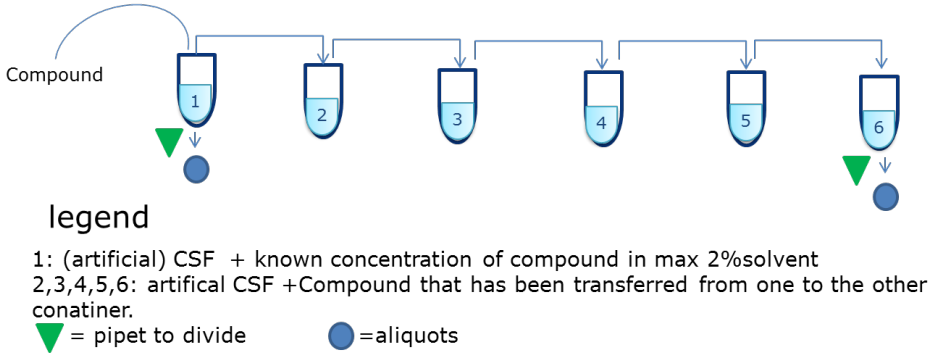


Figure 8: Test to evaluate adsorption to the containers

3.4 Analysing parameters

An LC-MS/MS method was developed to analyse the selected compounds. The specific conditions of the HPLC and the mass spectrometer are described below.

3.4.1 HPLC

For this study a Shimadzu is used. To eluate the compounds from the column a gradient is used, so the different compounds are separated from each other. This will be easier for the MS analyser to detect the different compounds. Following settings are used in this method:

LC parameters

Pump n°	LC-20AD Shimadzu
column	Waters Xbridge C18 3.5µm 2.1*50mm
flow rate (µl/min)	500
solvent A	0.01M Ammonium carbonate in milli-Q water
solvent B	acetonitrile

gradient profile

time (min)	% A	% B
0.00	70	30
2.50	15	85
2.51	2	98
3.00	2	98
3.01	70	30
4.00	70	30

Auto sampler

equipment SIL-HTC Shimadzu

injection volume (µl) 2 µl

needle stroke 48 mm

sampling speed 5µL/sec

cooler temperature 4°C

Column oven

equipment CTO- 20 AC

temperature 40 °C

3.4.2 Mass Spectrometer

The type of analyser used to detect the molecules is an API 4000 from AB Sciex instruments, this is a triple quadrupole mass spectrometer. The ion source that is used is a Turbo Ion Spray, is the same as electro spray ionisation (ESI). Because six compounds with their internal standard were evaluated in this study, the mass spectrometer measures in two time periods. The first period analysed from 0-2 minutes and the second period analyse from 2-4 minutes. This is needed to have enough data points across the peaks. The amount of data points is important for good quantification of the peak (at least 15-20 data points). When there are not enough data points it is possible that information will be lost, the top of the peak could be missed and there will be an underestimationt of the results [34]. Follow setting were used for the mass spectrometer:

MS parameters

period 1 0-2 min

compound	Q1 mass	Q3 mass	dwell time[5]	DP	CE	CXP
	m/z	m/z	msec			
compound A	368.1	292	40	71	39	18
IS compound A	374.1	295	40	71	39	18
Quetiapine	384.2	253	40	16	33	18
IS quetiapine	392.2	258	40	16	33	18
compound B	441.1	190	40	90	35	12
IS compound B	425.1	191	40	90	31	12
Paliperidone	427.2	207	40	86	39	6
IS Paliperidone	431.2	209	40	86	39	6

period 2 2-4 min

compound	Q1 mass	Q3 mass	dwell time	DP	CE	CXP
	m/z	m/z	msec			
Aripiprazole	448.2	285	50	41	37	8
IS Aripiprazole	456.2	293	50	41	37	8
compound D	345	289	50	92	39	15
IS compound D	354.2	290	50	92	39	15

Parameters

CUR: 30
GS1: 40
GS2: 50
IS: 4000
TEM: 650
ihe: ON
CAD: 6
EP: 10

[5] This is the time that is spent in a cycle to scan the mass of the compounds.

4 Results

4.1 Materials

After contacting the clinics, an inventory list of materials (table 6) has been composed. All these materials have been tested. In table 7 extra materials have been shown. These materials are materials that are used in the laboratory of Janssen Pharmaceutica. These materials are common in use so they needed to be evaluated as well.

Table 6: Inventory of materials used in clinics

Material	References	Company	Composition
Needle and catheters	Pencan: 4502035-13	B Braun Melsungen	Not available
	Spinocath catheter kit 4517725	B Braun Melsungen	Not available
Collecting tubes	10 ml: 62.610 201	Sarstedt AG&Co	Polypropylene
	15 ml Falcon tube 352097	Corning	high-clarity polypropylene
Pipets and pipet tips	3.5 ml Pipets 861172	Sarstedt AG&Co	Low Density Polyethylene (LD-PE)
	polypropylene pipette tip		
Storage tubes	2.0 ml micro tubes with screw cap	Sarstedt AG& Co	Polypropylene
	0.5 ml tubes Article nr 72.730	Sarstedt AG & Co	Polypropylene
	1.4 ml non-coded tubes U-bottom, Cat No. MP22502	Micronic	polypropylene

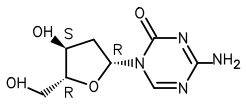
Table 7: Materials used in laboratories

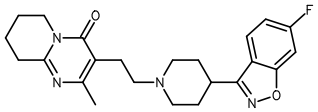
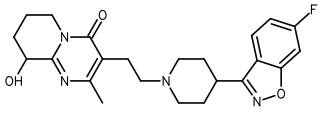
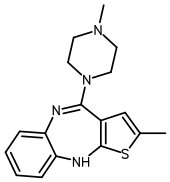
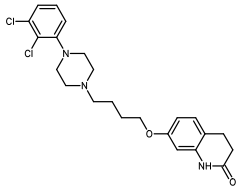
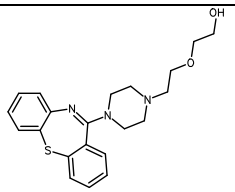
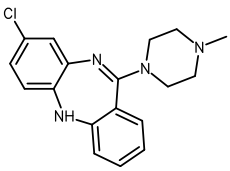
Material	References	Company	Composition
Pipet tip	Mastertips, Pos.Displacement tip	Eppendorf	Virgin polypropylene and polyethylene
	Displacement pipet tip	Gilson	
	Combi-tips advanced	Eppendorf	Virgin polypropylene
	Epp tips reload	Eppendorf	Virgin polypropylene and polyethylene
	Low binding pipet tips 4148	Corning	Polypropylene
	Air column tip low retention	Sartorius	
Storage tubes	1.4 ml tube	Fluidx	Polypropylene: homopolymer (rasin)
	Lo bind DNA	Eppendorf	Polypropylene
	Lo bind protein	Eppendorf	Polypropylene

4.2 Compounds

Of the list of compounds (table 3) the following compounds were selected: compound A, compound B, compound C, Quetiapine, Paliperidone and Aripiprazole. The compounds where selected because of the difference of their properties especially on their log P. The range of log P is chosen from the lowest to the highest log P of the given compounds, to get some more variation in the properties of the compounds different pKa's were chosen as well. In table 8 all compounds with their physicochemical properties are given. The compounds are basic and the pKb's were converted to pKa's.

Table 8: structure and physicochemical properties of the compounds.

Compound	structure	Molecular mass (g/mol)	Log P	pKa
Compound A		367,41	1,27	19,4
Compound B		440,78	2,98	11,02
compound C		344,89	4,86	12,85
Compound D		386,38	3,26	10.14
Compound E		477.47	2,14	15,88
Decitabine		228,21	-2.16	13,98

Risperidone		410,48	2,63	4,41
Paliperidone		426,48	1,07	4,41
Olanzapine		312,14	2,86	7,52
Aripiprazole		448,39	5,31	4,29
Quetiapine		383,51	2,99	5,87
Clozapine		326,82	3,56	7,67

4.3 Variation of the HPLC-MS/MS method

In order to interpret the results of the adsorption tests it is necessary to find out the variation of the HPLC-MS/MS analysis method. The variation was determined on three levels, i.e., the injection by the HPLC-MS/MS auto sampler from one aliquot a, the number of aliquots from one tube and the number of independent preparations in different tubes. For the first level, 3 different injections were taken from one aliquot (3 injections from every aliquot a). For the second level for each of three tubes, the variation of three different aliquots (aliquot a, b and c from every tube) was calculated. Finally, the variation of the use of 3 different aliquots taken from 3 different tubes was determined (for calculating this aliquot b or c can be used of every tube). A scheme of test set-up is given in figure 9. The results of those tests are shown below.

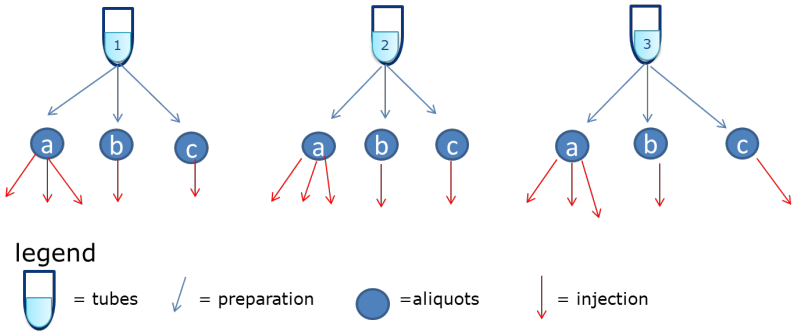


Figure 9: The setup of the test to find out the cause of variation. For the first level, 3 different injections were taken from one aliquot (3 injections from every aliquot a). For the second level for each of three tubes, the variation of three different aliquots (aliquot a, b and c from every tube) was calculated. Finally, the variation of the use of 3 different aliquots taken from 3 different tubes was determined (for calculating this aliquot b or c can be used of every tube).

In the first four tables the variation on the results are given of the test where the variation of the method are tested. In this test 3 injections were taken from the same aliquot. This test is done in triple: three aliquots were made, from every aliquot three injections were made, and with two different matrices: real CSF and aCSF with 0% BSA. The difference between tables 9-10 and tables 11-12 are the volume of the tubes. In one series the tubes are filled with 500 µL and the other series is filled with 200µL. This was done to find out if there is a difference in variation with use of different volumes. The principle of the test was transferring the liquids 3 times, the variation on the results from tube 1 and tube 4 are calculated and shown.

Table 9: variation of the injections (n=3)/ aliquot (transfer0) (CV %) 500µL

Aliquot	Compound A		Quetiapine		Compound B		Aripiprazole		Compound B		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
1	7.4	0.7	0.4	1.4	2.2	2.3	2.3	1.5	1.3	3.0	1.7	3.5
2	0.8	7.4	2.0	4.9	5.5	2.9	3.4	3.7	2.0	0.6	2.9	3.0
3	6.7	8.8	2.0	3.8	0.7	2.4	3.8	1.7	3.7	4.2	0.5	5.1

Table 10: variation of the injections (n=3)/ aliquot (transfer 3) (CV %) 500µL

Aliquot	Compound A		Quetiapine		Compound B		Aripiprazole		Compound B		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
1	2.1	3.1	5.5	2.7	2.1	4.4	3.2	4.0	3.2	1.5	1.3	3.2
2	5.3	3.1	2.7	1.9	4.8	4.0	1.9	0.0	1.3	3.2	1.9	1.9
3	4.4	7.6	2.0	4.4	2.1	1.3	2.7	11.0	1.5	1.4	2.5	3.0

Table 11: variation of the injections (n=3)/ aliquot (transfer 0) (CV %) 250µL

Aliquot	Compound A		Quetiapine		Compound B		Aripiprazole		Compound B		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
1	5.7	0.9	2.1	5.3	4.7	4.9	0.7	5.0	1.9	1.2	3.2	3.3
2	9.8	5.5	2.0	2.1	5.3	5.1	3.7	4.2	5.2	2.5	1.9	3.7
3	2.7	3.4	4.8	0.5	3.8	0.5	1.0	6.6	2.5	4.0	1.8	3.7

Table 12: variation of the injections (n=3)/ aliquot (transfer 3) (CV %) 250µL

Aliquot	Compound A		Quetiapine		Compound B		Aripiprazole		Compound B		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
1	1.7	5.9	2.7	4.5	3.9	4.9	0.1	13.0	0.4	3.8	3.2	5.2
2	8.3	9.2	5.5	1.1	2.4	0.7	3.9	19.1	3.3	1.3	4.5	4.5
3	4.1	3.8	2.5	3.1	0.6	2.9	4.3	7.7	4.1	4.5	4.5	5.5

These results show the variation of the HPLC-MS/MS method. The mean variation on the results from the smaller volumes is bigger than the mean variation of the bigger volumes. This can be seen because the results in the first 2 tables are smaller than the last 2. The variations on the results between both matrices are comparable.

In the next tables 13 and 14: the results are represented of the variation of using 3 tubes but only one aliquot per tube and one injection per aliquot. This was tested because the previous tests were done in that principle. It means working in triplicate but a single sample preparation.

Table 13: Variation on the use of 3 tubes, 1 aliquot and 1injection. (transfer0) (CV %)

Volume	Compound A		Quetiapine		Compound B		Aripiprazole		Compound C		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
500µL	2.5	7.4	1.6	5.4	2.6	9.0	4.9	13.7	1.9	10.2	2.4	6.3
250µL	3.6	10.2	4.2	13.6	4.2	8.0	2.0	18.3	0.9	14.9	1.0	16.7

Table 14: Variation on the use of 3 tubes, 1 aliquot and 1injection (transfer3) (CV %)

Volume	Compound A		Quetiapine		Compound B		Aripiprazole		Compound C		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
500µL	1.8	8.1	6.9	9.3	3.0	3.3	12.6	14.2	7.0	8.1	6.3	1.3
250µL	2.4	7.3	2.5	8.7	3.6	6.5	8.1	22.5	3.6	2.4	4.0	12.8

When these results were studied, there is no much difference between the variation on the results of 250 µL and 500 µL. The results do represent a difference between the two different matrices. In real CSF there is less variation than in aCSF.

In follow tables 15 and 16 the results are represent of a test where more aliquots where taken from one tube. This test only was done with a tube what was filled with 500 µL. For the test 3 aliquots were taken from one tube. This test was also done in triplicate: 3 tubes, 3 aliquots per tube and one injection per aliquot.

Table 15: Variation on different aliquots from one tube (transfer 0) (CV %) (n=3)

Tube	Compound A		Quetiapine		Compound B		Aripiprazole		Compound C		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
1	4.8	6.6	3.3	4.8	2.8	6.7	8.8	11.8	6.2	6.3	3.6	2.8
2	6.3	13.4	4.3	10.8	5.7	7.8	7.1	1.9	5.9	6.1	3.8	8.4
3	6.1	9.6	5.3	1.7	5.1	4.4	9.2	8.8	6.3	9.6	3.5	7.3

Table 16: Variation on different aliquots from one tube (transfer3) (CV %) (n=3)

Tube	Compound A		Quetiapine		Compound B		Aripiprazole		Compound C		Paliperidone	
	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF	CSF	aCSF
1	2.7	3.6	4.4	5.1	2.2	6.6	9.0	15.5	5.1	8.3	7.7	5.2
2	3.2	2.9	7.2	6.0	5.2	2.7	9.4	9.2	9.0	3.7	6.6	2.4
3	1.6	11.1	3.1	4.7	0.8	5.6	1.6	13.2	5.1	9.8	4.5	2.1

In these tables there could be seen that there is a lot of variation between different aliquots coming from one tube because the most results are above 5%. What can also be seen is that Aripiprazole has always the most variation. This could be explained by the fact that for Aripiprazole the IS is not a STIL but an analog. This can differ in compensation of the peak areas. There is no difference between the two matrices.

4.4 Reproducibility test

This test was done in plasma. A curve was made with different concentrations of compounds in plasma to found out which minimal concentration the analysis method can measure. The QC's were analysed to test the reproducibility and accuracy of the method. Preparation method 1 was used, for every standard point three replicates were made. The results of the test are given in table 17-19.

Table 17: Reproducibility test of Compound A and Aripiprazole. Some concentrations could not be measured, because the response was too low. (n=3)

Compound A			Aripiprazole		
Conc (ng/ml)	Mean accuracy (%)	Variation (%)	Conc (ng/mL)	Mean accuracy (%)	Variation (%)
0.05	NR	NR	0.01	NR	NR
0.1	112.5	12.0	0.02	NR	NR
0.2	101.3	7.1	0.04	NR	NR
0.5	94.5	8.4	0.1	111.3	6.7
1.0	95.1	6.8	0.2	94.7	10.1
2.0	93.1	3.5	0.4	94.4	13.0
5.0	100.4	3.3	1.0	98.4	3.1
10	95.6	4.4	2.0	98.8	10.0
20	101.4	5.8	4.0	101.3	9.9
50	109.3	3.2	10	104.1	6.0
QC (5.0)	97.0	5.6	QC (1.0)	100.7	11.0

Table 18: Reproducibility test of Compound B and Quetiapine. (n=3)

Compound B			Quetiapine		
Conc (ng/ml)	Mean accuracy (%)	Variation (%)	Conc (ng/mL)	Mean accuracy (%)	Variation (%)
0.1	105	2.1	0.3	107	0.88
0.2	97	10.4	0.6	95.6	2.19
0.4	99	5.4	1.2	94.9	2.48
1.0	103	2.8	3.0	101	3.83
2.0	98	3.0	6.0	101	2.42
4.0	95	2.1	12	95.8	0.84
10	102	1.4	30	105	1.78
20	100	3.7	60	102	1.54
40	98	0.21	120	98.6	0.58
100	103	1.0	300	99.6	0.90
QC (10.0)	97.4	0.95	QC (30.0)	101	1.5

Table 19: Reproducibility test of Compound C and Paliperidone. Some concentrations could not be measured, because the response was too low. (n=3)

Compound C			Paliperidone		
Conc (ng/ml)	Mean accuracy (%)	Variation (%)	Conc (ng/mL)	Mean accuracy (%)	Variation (%)
0.05	NR	NR	2.5	102	16
0.1	NR	NR	5.0	101	5.3
0.2	104	13.7	10	94	4.2
0.5	103	6.1	25	103	2.3
1.0	96	3.2	50	103	1.6
2.0	95	2.2	100	99	1.9
5.0	101	6.1	250	103	4.8
10	100	2.8	500	98	0.08
20	100	2.6	1000	96	0.89
50	102	2.1	25000	102	1.6
QC (5.0)	101	2.8	QC (250.0)	100	1.8

In those tables the accuracy and the variation are shown. The variation is the variation coefficient (CV %). This value needs to be less than 15%. For most compounds the CV% stays under this limit except for the lowest concentration of Paliperidone. The accuracy was calculated with a regression line. The concentration obtained with the regression were compared with the effective concentration that was made. The mean accuracy is the average of the three replicates. The accuracy also needs to be between the 15% limit. The results showed for all compounds an accuracy between 85-115%. This means the accurancy of the method is good. The results of the QC's could been used to analyse the reproducibility. The variations (%CV) of QC's of al compound were less than 15% and the accuracy differs only 3% of the nominal concentration.

For Aripiprazole, compound A and compound C the HPLC-MS/MS method was not very sensitive. The low concentration could not be measured. The response was too low. But the method was found suitable for this investigation because if the concentration of the compound after the adsorption test falls under the lowest sensitive concentration the adsorption will be significant.

4.5 Selection of most optimal surrogate matrix composition

In this stage of the investigation an optimal surrogate matrix was searched to work with. This is necessary because of the ethical and economical reasons described in '1.3 Adsorption'. First the influence of the pH was tested. Afterwards on optimal surrogate matrix was searched.

4.5.1 Influence of the pH

According to literature the pH of fresh CSF is 7.36, which is about the same as the pH of blood. [4]. The available (purchased) CSF in the lab had a pH of 8.41. It is likely that the higher pH is caused by the same effect as in blood: the release of CO_2 upon storage, resulting in an increase in pH.

Knowing that the pH of the fluid changes over time, the adsorption of the compounds to various materials was tested at both pH values. This was done by adjusting the pH of artificial CSF to a pH of 7,36 and a pH of 8,42. Preparation method 1 was used in this test.

The results of the influence of the pH on the recovery of the compounds are shown in follow figures 10-15.

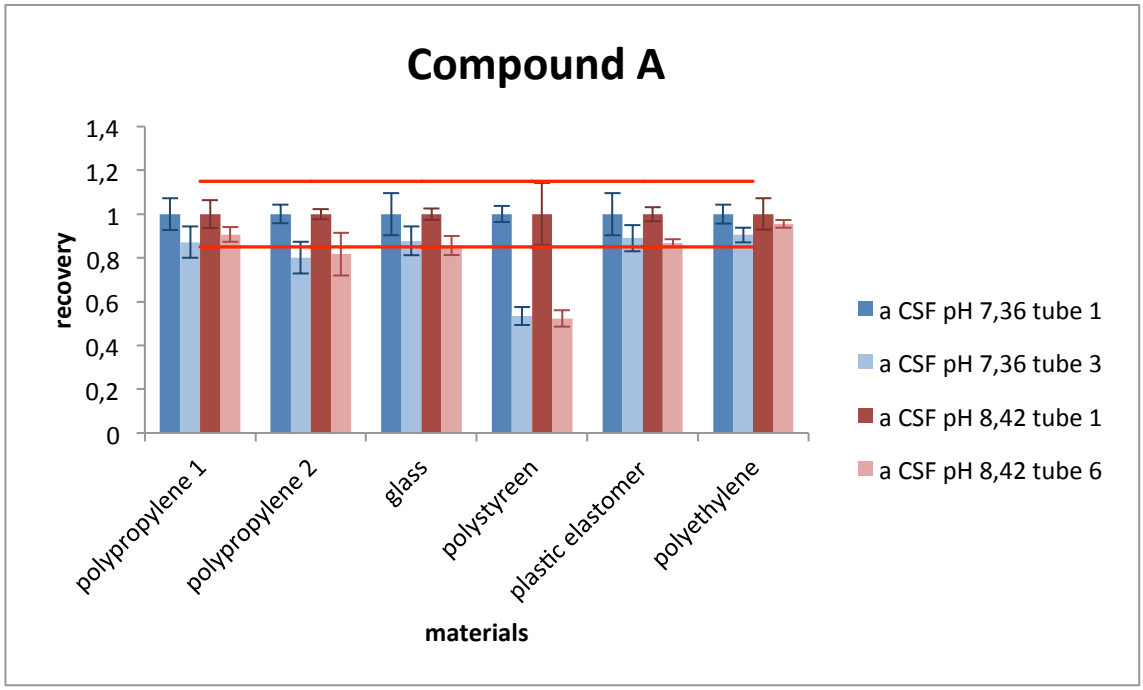


Figure 10: Influence of the pH of the matrix and the amount of recovery of compound A. The red lines are the 15% limits. Polypropylene 1= Fluidx tube, Polypropylene 2= Micronic tube.

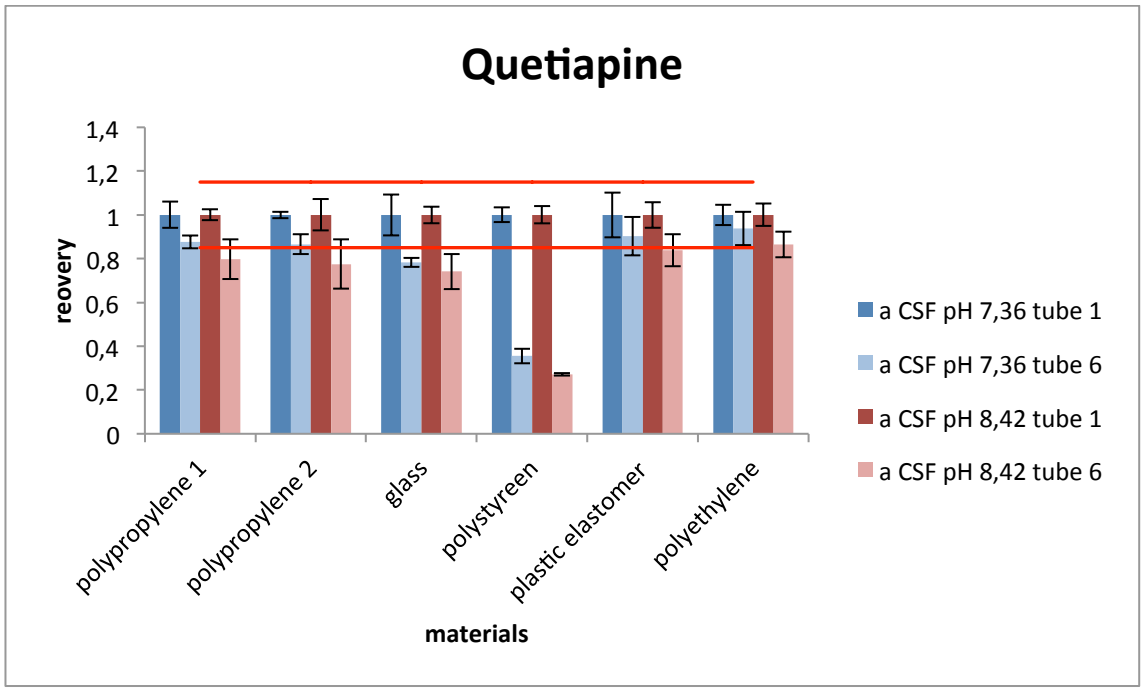


Figure 11: Influence of the pH of the matrix and the amount of recovery of compound Quetiapine. The red lines are the 15% limits. Polypropylene 1= Fluidx tube, Polypropylene 2= Micronic tube.

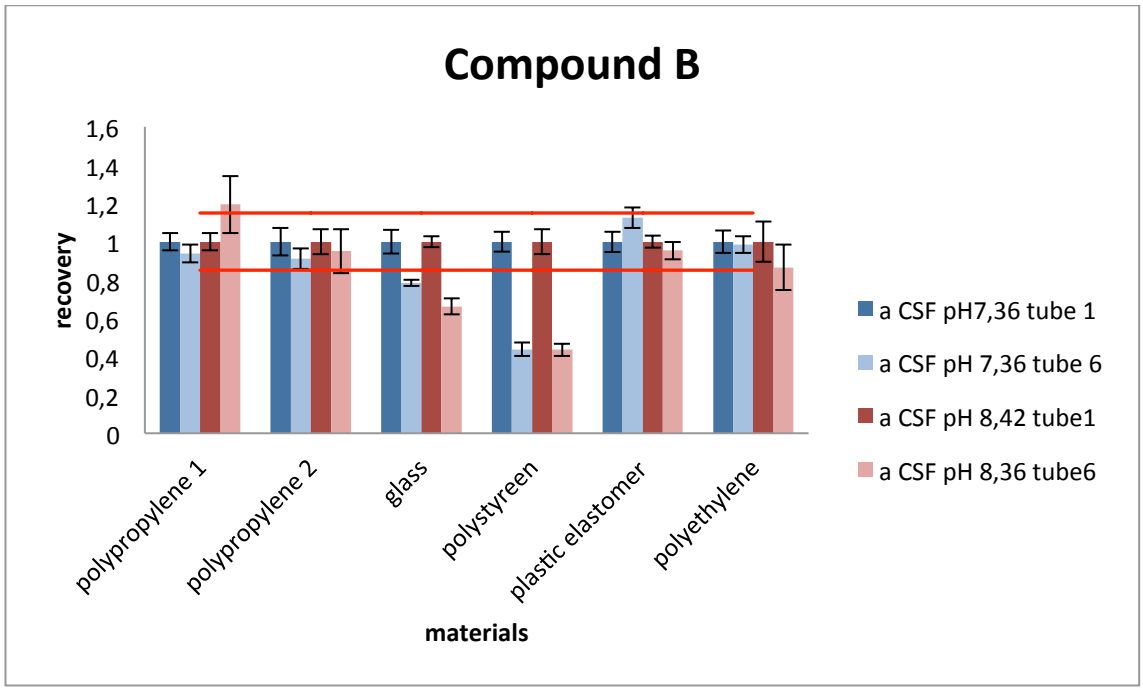


Figure 12: Influence of the pH of the matrix and the amount of recovery of compound B. The red lines are the 15% limits. Polypropylene 1= Fluidx tube, Polypropylene 2= Micronic tube.

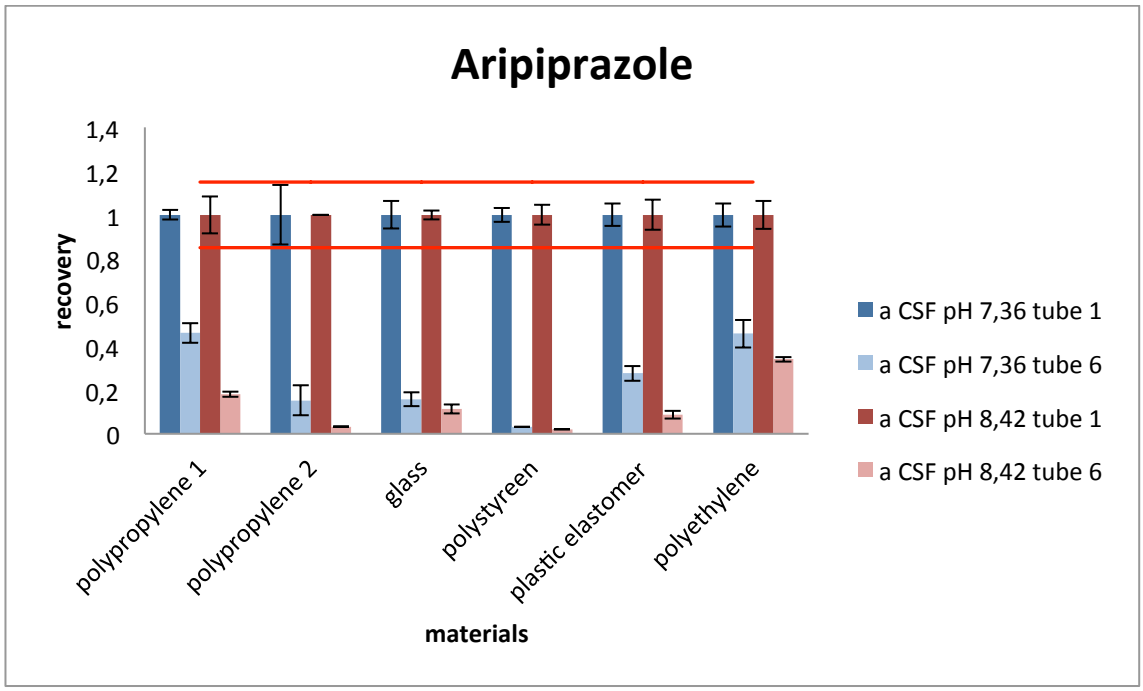


Figure 13: Influence of the pH of the matrix and the amount of recovery of compound Aripiprazole. The red lines are the 15% limits. Polypropylene 1= Fluidx tube, Polypropylene 2= Micronic tube.

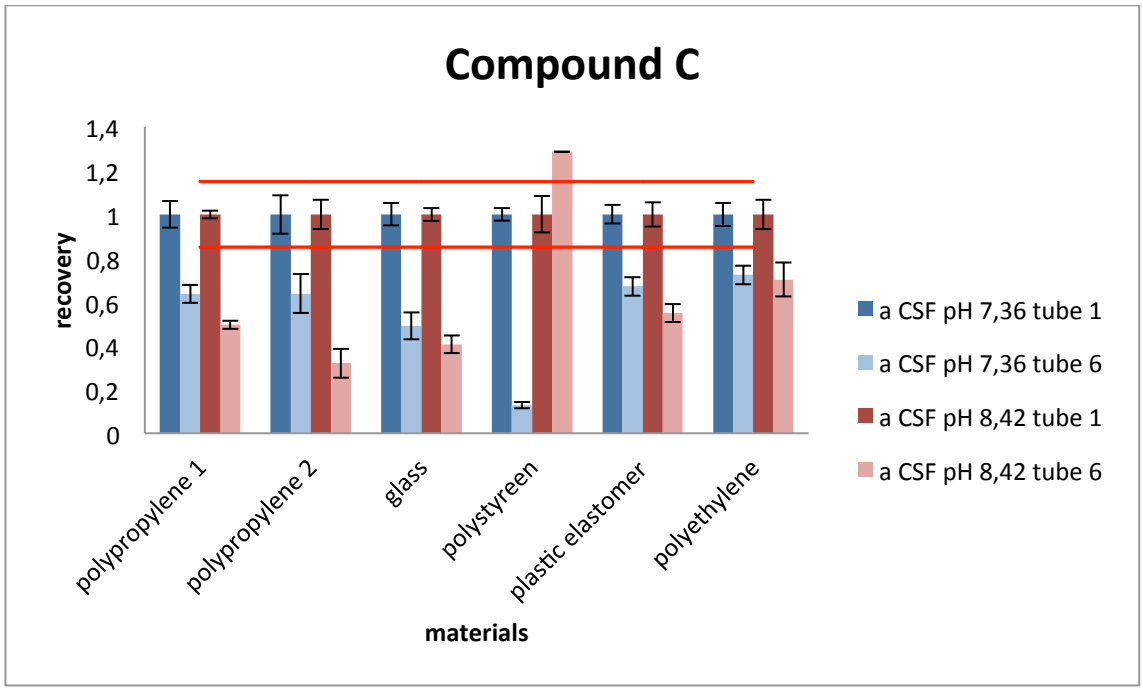


Figure 14: Influence of the pH of the matrix and the amount of recovery of compound C. The red lines are the 15% limits. Polypropylene 1= Fluidx tube, Polypropylene 2= Micronic tube.

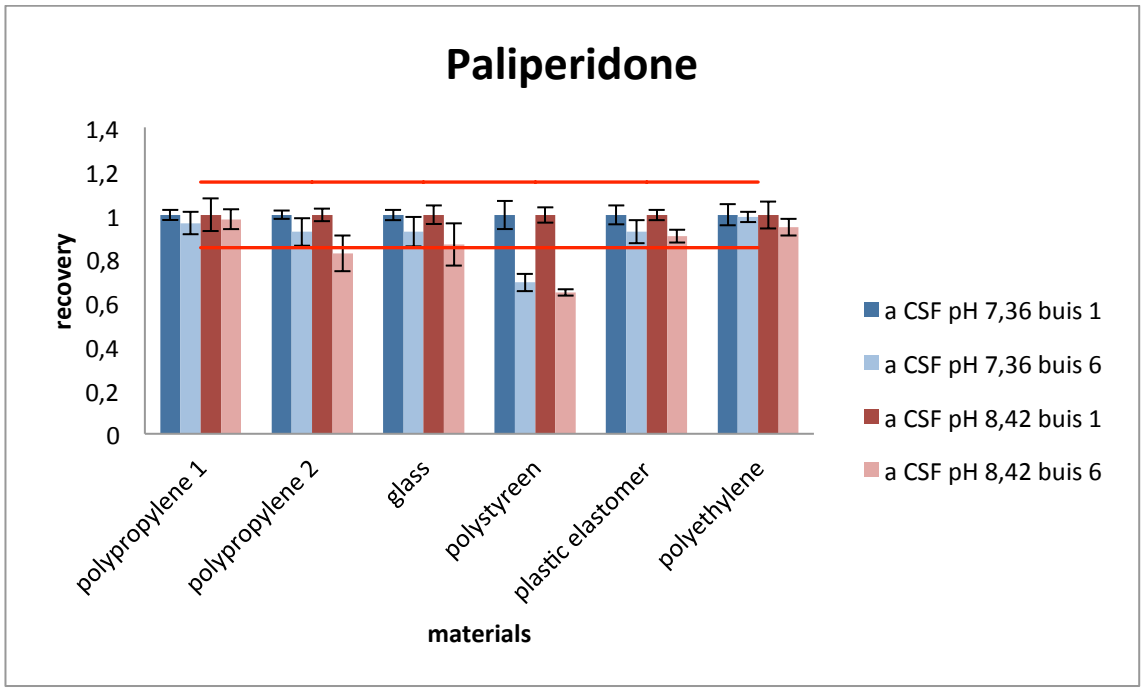


Figure 15: Influence of the pH of the matrix and the amount of recovery of compound Paliperidone. The red lines are the 15% limits. Polypropylene 1= Fluidx tube, Polypropylene 2= Micronic tube.

When the graphs are studied in more detail the conclusion of the influence of the pH is different. For compound A can be seen in figure 10 that there is no influence of the pH. The amount of adsorption in tube 6 with both pHs is almost the same. With Quetiapine figure 11, the pH is important when polypropylene is used. There can be seen that with pH 7,36 the adsorption is still between the limits but when the results of pH is 8,42 were evaluated 20% more adsorption can be found. Aripiprazole figure 13 has with every material a lot of adsorption, mostly at both pH's equally, but for Fluidx (polypropylene1) a significant different is seen. With pH 7,36 there is 20 % less adsorption compared to pH 8,42. With compound C figure 14 polypropylene gave an important difference of adsorption as well. PH 8,42 gave more adsorption as pH 7,36. And finally, Paliperidone figure 15 has an important difference with Micronic.

4.5.2 Selection of a surrogate matrix

To find the optimal surrogate different compositions of artificial CSF (different BSA concentrations) were used. The results of the compounds in real CSF were compared with the results in the different compositions of artificial CSF. The aim is to find a surrogate matrix wherein the compounds have the same amount of adsorption in different materials as in real CSF. Every matrix is tested in a few materials to see the influence of the materials with the adsorption in the different matrices. The materials that were tested were the same as used in the pH test. The different concentrations of BSA that were tested in aCSF were 0%, 0,25%, 0,5%, 1% and 2%.

The next tables 20-25 represent the results of the second test. This test was done with preparation method 3. In appendix 1 the corresponding graphs are represented.

Table 20: Recovery of compounds in different matrices in Micronic

	A	Quetiapine	B	Aripiprazole	C	Paliperidone
CSF	0.94	0.95	0.87	0.30	0.55	1.06
aCSF 0% BSA	0.79	0.68	0.87	0.68	0.44	0.87
aCSF 0,25% BSA	0.90	0.96	0.79	0.62	0.64	1.03
aCSF 0,5% BSA	0.97	0.86	0.69	0.68	0.75	1.00
aCSF 1%BSA	1.05	1.00	0.95	0.81	0.90	1.05
aCSF 2%BSA	1.03	0.97	1.05	0.89	0.94	1.02

Table 21: Recovery of compounds in different matrices in Fluidx

	A	Quetiapine	B	Aripiprazole	C	Paliperidone
CSF	0.94	0.93	0.87	0.98	0.68	0.76
aCSF 0% BSA	0.93	0.75	0.78	0.87	0.66	0.83
aCSF 0,25% BSA	1.02	0.91	1.01	0.73	0.83	1.00
aCSF0,5% BSA	1.04	0.98	0.94	0.87	0.89	1.00
aCSF 1%BSA	1.22	1.00	1.17	0.99	1.01	1.06
aCSF 2%BSA	1.15	0.97	0.96	0.96	0.93	1.06

Table 22: Recovery of compounds in different matrices in glass.

	A	Quetiapine	B	Aripiprazole	C	Paliperidone
CSF	0.96	1.06	0.92	1.44	1.01	0.98
aCSF 0% BSA	1.02	0.82	0.77	1.11	0.54	0.89
aCSF 0,25% BSA	1.00	1.00	1.04	1.01	0.95	1.03
aCSF 0,5% BSA	1.08	1.03	1.01	1.11	0.99	1.03
aCSF 1%BSA	1.18	1.10	1.07	1.20	1.15	1.12
aCSF 2%BSA	1.10	1.00	0.95	0.95	1.00	1.00

Table 23: Recovery of compounds in different matrices in polystyrene

	A	Quetiapine	B	Aripiprazole	C	Paliperidone
CSF	0.74	0.58	0.61	0.60	0.66	0.88
aCSF 0% BSA	0.53	0.21	0.70	0.77	0.25	0.83
aCSF 0,25% BSA	0.93	0.85	0.87	0.70	0.74	1.05
aCSF 0,5% BSA	0.99	0.86	0.90	0.77	0.76	1.01
aCSF 1%BSA	0.96	0.88	0.90	0.87	0.90	1.02
aCSF 2%BSA	1.04	0.93	0.91	0.91	0.98	0.99

Table 24: Recovery of compounds in different matrices in thermo plastic elastomer

	A	Quetiapine	B	Aripiprazole	C	Paliperidone
CSF	1.10	1.05	1.11	0.65	0.88	1.10
aCSF 0% BSA	1.00	1.13	1.17	0.94	1.04	0.96
aCSF 0,25% BSA	1.12	1.02	0.92	1.03	0.97	1.09
aCSF 0,5% BSA	1.13	1.12	0.97	0.94	1.03	1.03
aCSF 1%BSA	1.01	0.96	0.95	1.02	0.97	1.04
aCSF 2%BSA	1.04	1.01	0.91	1.00	0.98	0.95

Table 25: Recovery of compounds in different matrices in polyethylene

	A	Quetiapine	B	Aripiprazole	C	Paliperidone
CSF	0.96	0.91	1.05	1.00	1.01	1.52
aCSF 0% BSA	1.15	0.97	1.10	1.02	0.92	1.00
aCSF 0,25% BSA	1.06	1.00	0.89	1.07	0.98	1.00
aCSF0,5% BSA	1.00	1.01	0.94	1.02	0.99	1.01
aCSF 1%BSA	1.05	1.00	0.98	1.00	1.02	1.01
aCSF 2%BSA	1.02	1.04	0.97	1.06	0.97	1.00

The results shown in the tables above do not give a clear picture. Sometimes every aCSF composition can be used sometimes only one matrix is useful but for the different compounds another compositions are useful. Or sometimes no alternative corresponds to the results of CSF. No specific matrices can be selected that is a good match in all materials to all compound regard to CSF. The reason why no surrogate matrix did match is not understood.

The theory was that when the concentration of BSA increases, the adsorption of compounds in materials decreases. In this test for some compounds it remained unchanged which means that there is no adsorption. For other compounds this theory is right so this test confirmed the literature. Mostly there was a lot of adsorption in the matrices with 0% BSA and no more adsorption when adding 1% of BSA. The expectations were that the matrices with 0,25% BSA and 0,5 % BSA are the most likely matrices to be a possible surrogate because the protein concentration in CSF is about 0,35 %. But this cannot be concluded from the results.

In the further tests reals CSF was used if small amounts of CSF could be use. Otherwise aCSF with 0, 25% BSA was used as alternative, with this surrogate matrix an indication of the reaction of the compounds to the various materials could be obtained, although the absolute values may deviate from what would be found in real CSF.

4.6 Adsorption evaluation

The aims of these tests were to find out which materials provide the least adsorption. In all tests the first tube or first aliquot is the same as the reference. This will always be seen as 100% recovery. The other aliquots/tubes were compared with the reference.

4.6.1 Adsorption test for pipet-tips

Pipet tips are needed for the preparation and transfer of CSF solutions that are needed for the adsorption tests. Therefore it is important to select the best tips, those with the least adsorption, for the evaluation of the adsorption. In this test 2 different categories of pipets were tested; pipets that are used in the laboratory and pipets that are used in the clinic. Different aliquots were taken and compared to a standard point.

The follow table 26 showed the results of the adsorption in the pipet tips that are used in the laboratory. In this table the mean between the adsorption in the first aliquot and the second aliquot was taken. The mean of these two aliquots is taken because the second aliquot will be used and then an indication is given in worst case. This is worst case because the first aliquot has always the most adsorption and normally the first aliquot is thrown away. This test was done with preparation method 2. Table 23 presents the recovery of compounds of the mean of the first two aliquots. These results were compared to a standard point in this case.

Table 26: Evaluation of adsorption in pipet tips, p1= repeating pipet, p2= pos. displacement pipet of Gilson, p3= pos. displacement pipet of Eppendorf, p4= air column pipet of Eppendorf (reload), p5= low retention, p6= low bind. The green boxes are the tip with the best recovery, when a compound has 2 different green boxes this means the recovery is the same.

	p1	p2	p3	p4	p5	p6
Compound A	0.87	0.89	0.84	0.82	0.83	0.89
Quetiapine	1.03	1.04	0.98	0.98	1.02	1.04
Compound B	0.92	0.91	0.91	0.90	0.87	0.94
Aripiprazole	0.86	0.95	0.83	0.86	0.85	0.86
Compound C	0.88	0.91	0.89	0.86	0.88	0.89
Paliperidone	0.91	0.93	0.93	0.88	0.90	0.97

The results showed that none of the tested pipet tips showed adsorption of the compounds. Only for Aripiprazole the recovery in the pos. displacement pipet is about 10% higher than the other pipets. Therefore, this tip was selected for further use.

In Appendix 2 the corresponding graphs are shown. In those graphs the five aliquots are represented per pipet. There can be seen that the recovery included the error bars off all compound are within the limits so no significant adsorption was detect.

A second pipet test was done with the Sarstedt pipet that is used in the clinics. In this test the Sarstedt pipet was compared to the repeat pipet tip. With the repeated test the 2.5 ml tip was

used, 5 aliquots of 0.5 mL were made. With the Sarstedt pipet 7 aliquots of 0.5 mL were made. The test was done with aCSF because a large volume was needed. The preparation method in this test was method 3. The results are compared to a standard point. The results are represented in the table 27, a graph of these results are shown in appendix 3.

Table 27: Recovery in the different aliquots per compound (10 pipets, 5 or 7 aliquots and 3 preparations per aliquot), these results were compared to a standard point.

Compound	pipet	aliquots						
		1	2	3	4	5	6	7
Compound A	repeating pipet	0.78	0.80	0.82	0.78	0.77		
	Sarstedt pipet	0.83	0.82	0.82	0.83	0.83	0.83	0.82
Quetiapine	repeating pipet	0.80	0.80	0.82	0.81	0.78		
	Sarstedt pipet	0.82	0.82	0.81	0.82	0.80	0.82	0.82
Compound B	repeating pipet	0.76	0.74	0.77	0.74	0.74		
	Sarstedt pipet	0.76	0.75	0.73	0.75	0.75	0.75	0.74
Aripiprazole	repeating pipet	0.74	0.76	0.76	0.73	0.72		
	Sarstedt pipet	0.77	0.75	0.74	0.77	0.76	0.74	0.75
Compound C	repeating pipet	0.75	0.76	0.75	0.74	0.73		
	Sarstedt pipet	0.77	0.75	0.76	0.76	0.78	0.74	0.77
Paliperidone	repeating pipet	0.78	0.79	0.80	0.78	0.78		
	Sarstedt pipet	0.82	0.79	0.80	0.81	0.81	0.80	0.81

The results of the tips are compared to a standard point prepared in DMSO instead of aCSF. The reason for that is because in DMSO there is no adsorption. The first thing that can be seen in these results is that all results have a recovery around 80%. When the results per pipet were evaluated, table 28, the adsorption remains the same, this means that there is no saturation of the tip. But it could also be possible that there was made a mistake in making the compound solution in aCSF and that the concentration in the solution is circa 20% lower than expected. This could result in a conclusion that there is no adsorption of the tip.

If the results of the repeating tip are compared with the results of the previous test these are in contrast with each other, the results now have less recovery. It was suspected that the compound solution in aCSF 0,25% BSA was not well prepared, because the same calibration standard was used as in the test of the tips that are used in the laboratory and those results were good. Therefore, the test was repeated. The difference in the second test is that the results were compared to a reference in artificial CSF instead of a calibration standard and the amount of test samples of the test was reduced.

Because of the relative low recovery, an additional test was performed in order to try to identify the cause of concentration loss. The test of the pipet that is used in the clinic was repeated in a smaller design also with preparation method 3. Only aliquots 1 and 5 or 7 were analysed. The last aliquot is sufficient to give an indication of the loss. The results were compared with a reference

and a recovery was found of 100% in the first and last aliquot (graphs shown in appendix 4) this means the tips do not causes adsorption. In this test the standard point was measured as well and compared with the reference of this test. When those results where compared a loss of +/- 20 % was found. This means there was no human mistake in preparation the sample but probably adsorption occurred in a previous step, maybe the material where the sample was made in. Another explanation of the fact that 20% loss is found is not found yet.

4.6.2 Adsorption test for needles

Two different types of needles were investigated on adsorption: the Spinocath and Pencan needle. This test investigates if the compounds are adsorbed to the needles and after which volume the needle active adsorption sites are saturated.

Two tests were performed. In the first test 10 aliquots of 100 µl were used to rinse the needle. In a second test, 10 aliquots of 500 µl were used. The latter total volume of 5 ml corresponds more to the procedure in clinics, where total volumes are typically a few millilitres. Both tests were performed with preparation method 3. The following tables 28-33 represent the results of the test with the aliquots of 500 µl. Appendix 6 contains the corresponding graphs. The data of the 100 µl aliquots are not shown.

The tests showed an average recovery of 90% (100 µl aliquots) and 100% (500 µl) for all compounds. The variation of both tests was similar, but rather large (+/- 5 %). In general, the recovery with larger volumes was somewhat higher than with lower volumes for all compounds. These high recoveries indicate that adsorption to the needles is not significant. In the second test the difference between the Pencan needle is more pronounced than in the first test, the recovery in the Pencan needle is higher than in the Spinocath. The difference can be seen in the graphs in appendices 5 and 6.

The high recovery is in contrast what was seen in the first tests. This is probably because the composition of the needles, which are more metallic materials, is different than the other tested materials, which are polymers. Metallic materials could contain ions. In that case, the pKa of the compounds are very important because some ion interaction will occur.

Table 28: Recovery ratio of compound A in needles compared to a reference, (n=3 needles, 10 aliquots were taken and per aliquot 3 preparations)

	Aliquot									
Needle	1	2	3	4	5	6	7	8	9	10
Pencan	1.10	1.10	1.12	1.11	1.10	1.13	1.10	1.10	1.10	1.07
Spinocath	1.07	1.14	1.05	1.13	1.09	1.07	1.15	1.09	1.13	1.09

Table 29: Recovery ratio of Quetiapine in needles regard to a reference (n=3 needles, 10 aliquots were taken and per aliquot 3 preparations)

	Aliquot									
Needle	1	2	3	4	5	6	7	8	9	10
Pencan	1.11	1.12	1.15	1.14	1.13	1.14	1.12	1.12	1.10	1.09
Spinocath	1.09	1.11	1.12	1.15	1.10	1.07	1.13	1.09	1.15	1.09

Table 30: Recovery ratio of compound B in needles regard to a reference, (n=3 needles, 10 aliquots were taken and per aliquot 3 preparations)

	Aliquot									
Needle	1	2	3	4	5	6	7	8	9	10
Pencan	1.10	1.08	1.10	1.07	1.06	1.09	1.05	1.07	1.08	1.04
Spinocath	1.04	1.06	1.03	1.09	1.04	1.02	1.08	1.04	1.08	1.03

Table 31: Recovery ratio of Aripiprazole in needles regard to a reference, (n=3 needles, 10 aliquots were taken and per aliquot 3 preparations)

	Aliquot									
Needle	1	2	3	4	5	6	7	8	9	10
Pencan	1.02	0.99	1.00	1.02	1.06	1.08	1.05	1.05	1.06	1.06
Spinocath	1.03	1.03	1.07	1.07	1.05	1.02	1.09	1.08	1.13	1.08

Table 32: Recovery ratio of compound C in needles regard to a reference, (n=3 needles, 10 aliquots were taken and per aliquot 3 preparations)

	Aliquot									
Needle	1	2	3	4	5	6	7	8	9	10
Pencan	1.03	1.05	1.08	1.09	1.07	1.09	1.07	1.10	1.08	1.06
Spinocath	1.05	1.04	1.06	1.10	1.06	1.04	1.11	1.06	1.12	1.04

Table 33: Recovery ratio of Paliperidone in needles regard to a reference, (n=3 needles, 10 aliquots were taken and per aliquot 3 preparations)

	Aliquot									
Needle	1	2	3	4	5	6	7	8	9	10
Pencan	1.09	1.05	1.11	1.06	1.08	1.11	1.06	1.10	1.07	1.05
Spinocath	1.06	1.08	1.07	1.10	1.06	1.01	1.09	1.08	1.11	1.04

4.6.3 Adsorption test for collecting tubes

This test is the second stage in CSF sampling. In the clinics two types of tubes are used: Sarstedt 10 mL tube and a falcon 15 mL tube. The volumes used in the lab were 50% of the volume tubes. The test is done with a CSF with 0,25% BSA because a lot of sample volume was required and it is too much to use real CSF. Because 0,25% BSA artificial CSF was not representative for all compounds, this test is an indication of possible adsorption was given, and will provide a relative ranking of the adsorption per tube. In a later test, with a smaller design, less volume and less replicates, can be repeated using real CSF. In figure 16 the results of the adsorption in the collecting tubes for every compound are presented. The test is done with preparation method 3.

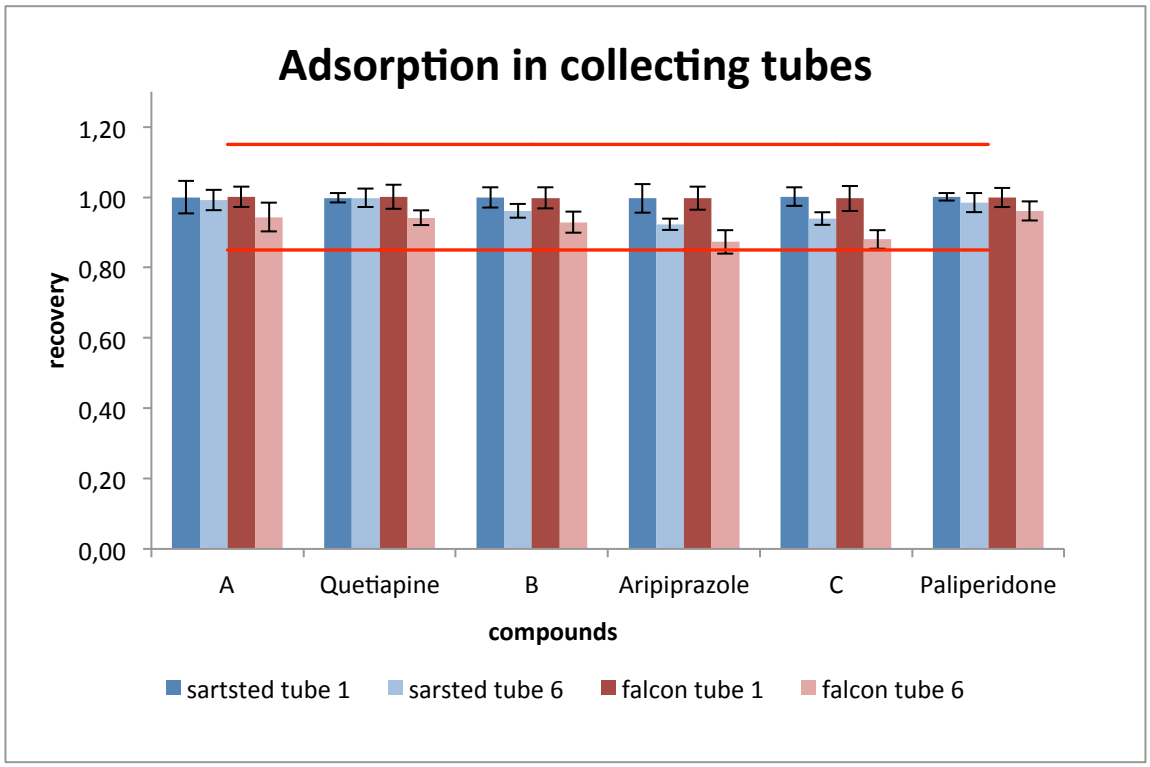


Figure 16: Adsorption of the compounds in the collecting tubes. The red lines are the 15% limits.

These results show that only Aripiprazole and compound C give adsorption in those tubes. If the Sarstedt tube and the Falcon were compared, the Sarstedt tube is the best tube and will be recommending to the clinics.

4.6.4 Adsorption test for storage tubes

The storage tubes have an important role in the sampling of CSF. In these tubes CSF remains a long time and it is important to find a tube with less adsorption in this stage. In this study only the amount of adsorption in the tube is investigated. The time related adsorption still need to be tested. In these tests not only the containers of the clinics are tested but also some containers that are used in the lab and they could be used as alternative when the containers of the clinic have too much adsorption. In the follow graphs (figures 17-22) the recovery per compound in the

different tubes are represented. This test is done with preparation method 3. Tube 1 is again the reference and was 100%.

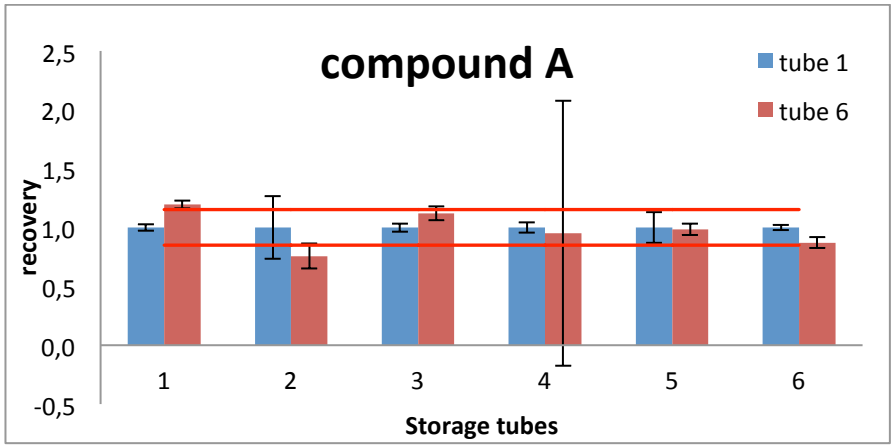


Figure 17: Recovery of compound A in storage tubes. 1= Fluidx; 2= Micronic; 3= 2ml microtubes (Sartstedt), 4= 0.5 mL tubes Sartstedt, 5= lo bind DNA (eppendorf), 6= lo bind protein (eppendorf). The red lines are the 15% limits.

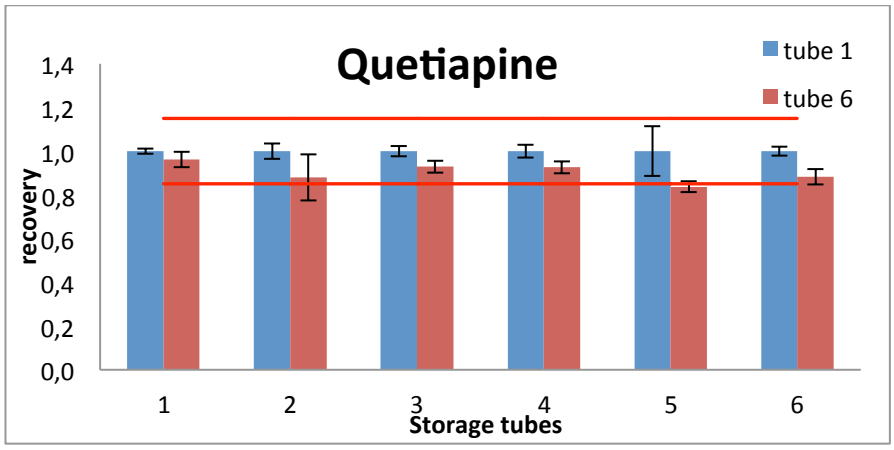


Figure 18: Recovery of Quetiapine in storage tubes. 1= Fluidx; 2= Micronic; 3= 2ml microtubes (Sartstedt), 4= 0.5 mL tubes Sartstedt, 5= lo bind DNA (eppendorf), 6= lo bind protein (eppendorf). The red lines are the 15% limits.

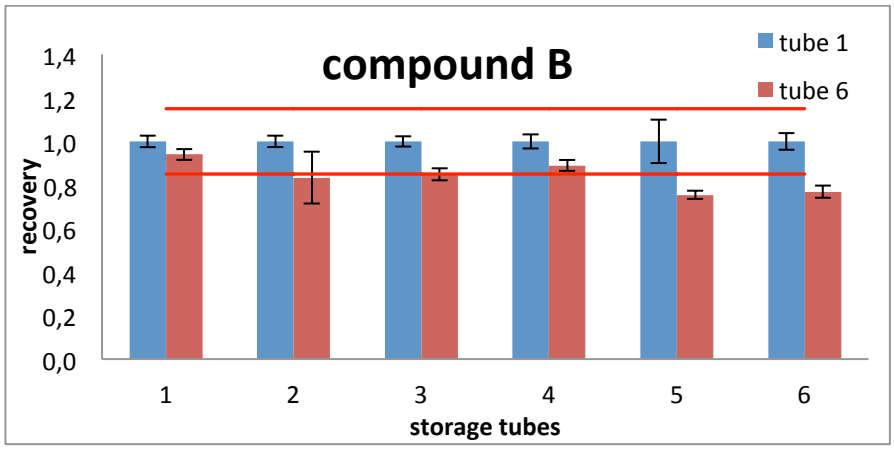


Figure 19: Recovery of compound B in storage tubes. 1= Fluidx; 2= Micronic; 3= 2ml microtubes (Sartstedt), 4= 0.5 mL tubes Sartstedt, 5= lo bind DNA (eppendorf), 6= lo bind protein (eppendorf). The red lines are the 15% limits.

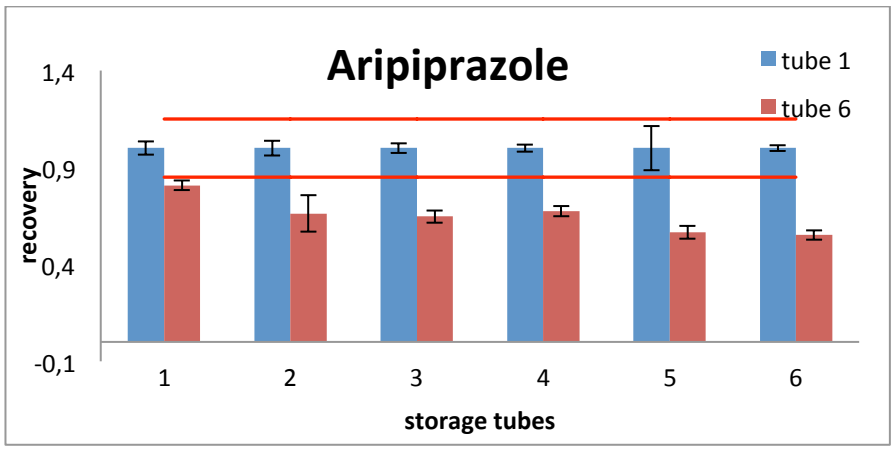


Figure 20: Recovery of Aripiprazole in storage tubes. 1= Fluidx; 2= Micronic; 3= 2ml microtubes (Sartstedt), 4= 0.5 mL tubes Sartstedt, 5= lo bind DNA (eppendorf), 6= lo bind protein (eppendorf). The red lines are the 15% limits.

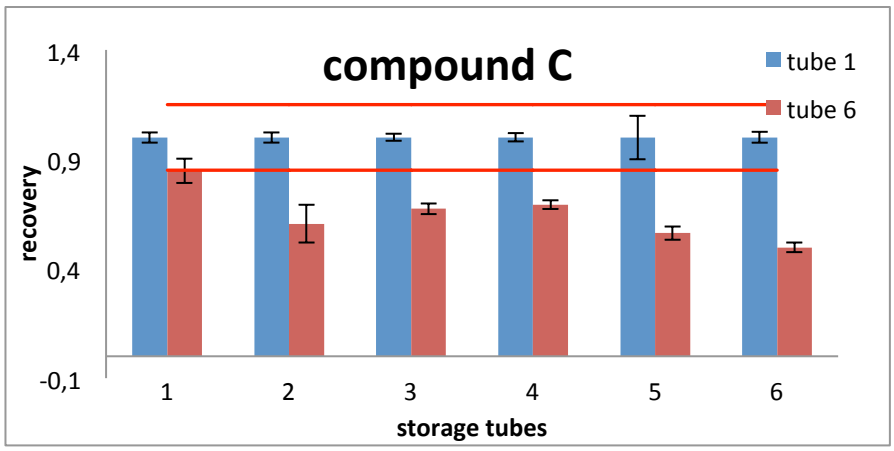


Figure 21: Recovery of compound C in storage tubes. 1= Fluidx; 2= Micronic; 3= 2ml microtubes (Sartstedt), 4= 0.5 mL tubes Sartstedt, 5= lo bind DNA (eppendorf), 6= lo bind protein (eppendorf). The red lines are the 15% limits.

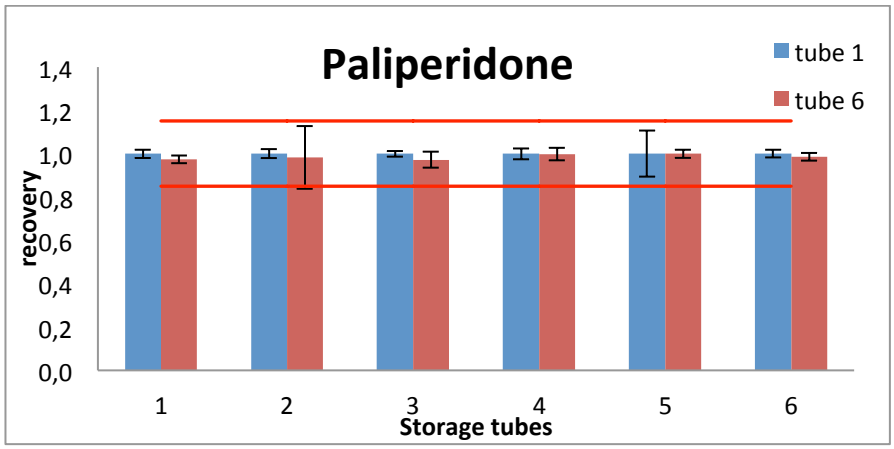


Figure 22: Recovery of Paliperidone in storage tubes. 1= Fluidx; 2= Micronic; 3= 2ml microtubes (Sartstedt), 4= 0.5 mL tubes Sartstedt, 5= lo bind DNA (eppendorf), 6= lo bind protein (eppendorf). The red lines are the 15% limits.

The results show that storage tubes give more adsorption than the materials at other sampling stages. Fluidx tubes give almost no adsorption, and even with the compounds that show adsorption, this tube is the most favorable tube. The lo bind tubes from Eppendorf are tubes in which the most adsorption is observed. Those tubes are the most unfavorable tubes. These will not be selected for use in clinics. The other tubes are not the best choice but for compounds without adsorption the results falls between the 15% limits. In most occasions the Sarstedt tubes are better than the Micronics.

5 Discussion

It is difficult to determine the actual contribution of each material to the overall adsorption because during the tests, additional adsorption occurs, related to the analysis procedure and preparation of the experiments. These sources of adsorption need to be minimized so only the adsorption of the relevant materials is determined. This is done by using the same materials and same actions in every step during sample preparation.

Many materials were available to test, but only those that are used for this purpose in the clinics, were selected. The adsorption of the selected materials was determined in separate tests, for each category of materials, to know the contribution in the corresponding sampling step. Unfortunately, the adsorption of the entire sampling procedure could not be tested within the limited time period.

The compounds were selected based on their physicochemical properties to have compounds in a large range of log P values and pKa values. The compounds were chosen from a list of compounds from which is known that they pass through the blood brain barrier. Another criterion was that it should be possible to analyse the compounds within one HPLC-MS/MS method.

The HPLC-MS/MS method that was set up to analyse the current mix was tested on its reproducibility and sensitivity for each compound in the relevant concentration range. The results showed that the analysis method was not equally sensitive for every compound. The variation was too high with the lower concentrations of some compounds. For Aripiprazole it was therefore decided to increase the concentration 5 times.

5.1 Optimal surrogate matrix

Before starting the experiments it was necessary to find a suitable surrogate matrix to work with instead of CSF. First, the **pH** of the available CSF had to be defined, because a pH change can affect the adsorption for some compounds if their pKa is too close to the pH of the CSF. The pH of CSF was more alkaline than described in the literature, 8,43 instead of 7,36. For the compound Quetiapine (pKa = 5,87) the difference is 2 log units so it is a borderline. The results showed that pH indeed has an influence on the adsorption especially for Aripiprazole in Fluidx and with Quetiapine in Micronic. This influence, however, cannot only be explained by pKa. The pKa of Quetiapine is 5,87 and the pKa of Aripiprazole is 4,29. The pKa of Aripiprazole is not close to the pH of the matrix and still an influence can be seen. It is possible that when the pH changes, the pH may influence the properties of the materials of which the tubes, pipet tips, etc. are made from. This could have an influence on the adsorption of the compounds. The observation that the pH has an influence on the adsorption is an important statement. This means the storage tubes need to be evaluated on adsorption at different pH values. This is important because the adsorption can increase or decrease during storage depending on the properties of the compounds. Since the selected compounds are basic (pKb was converted to pKa to facilitate reading on the pH scale), the log P of such a compound increases when the fluids become more alkaline and the adsorption will

increase. E.g. Quetiapine, in chapter '1.6.2 log P' there can be seen in the figure that when the pH of the matrix increases the log D increases as well and that means there is more adsorption. A potential solution could be to find an additive/ buffer that can decrease the pH back to 7,36 in the storage tubes so the adsorption can be minimalized. It is also important that the pH will be tested before analysing the stored CSF. In this investigation there was not enough time to do some tests were the influence of the pH was taking into account. The tests with aCSF were done with pH 7.36 the tests with CSF were done with the pH of the stored CSF ca 8,43. In the literature nothing was found of the influence of the pH so a lot of investigation is needed to know more about this influence.

By **selecting an optimal surrogate matrix** it is important that all compounds have almost the same amount of adsorption as in CSF otherwise comparing will be difficult. For this test the composition of real CSF was important. No optimal artificial CSF could be found, most compounds gave less adsorption in all the matrices compared to CSF; only in the matrix with 0% BSA, a lot more adsorption of the compounds compared with CSF was observed. The reason why the results of a surrogate matrix did not match is not understood. The influence of the amount of BSA that was added to the surrogate matrix was tested as well. Those results were also compared to the article [12]. In this article they made the hypothesis that when the amount of BSA increase, the adsorption decreases. This hypothesis is confirmed in the results of this test. This can be interesting, since maybe BSA could be added to avoid adsorption.

The results from the tests of selecting an optimal surrogate matrix already showed which compounds gave adsorption and which not. There can be seen that compound C and Aripiprazole, the compounds with the highest log P value, have the lowest recovery. When the adsorption was compared between the various materials, a lot of the compounds did not adsorb to glass. This can be explained by the fact that glass is a polar material and the compounds are more non polar and have not much affinity towards polar surfaces. When the results in polystyrene were studied, it was observed that the compounds with a higher log P value had more adsorption than the other compounds. This could be explained based on the polarity of the materials, the log P of styrene is 2.71 and of propylene is 1.49, this means polystyrene is a more non-polar material. The compounds with a higher log P value are more attracted to the non-polar materials than to the matrix, the compound is leaving the matrix to stick to the non-polar material. The results in Micronic and Fluidx are curious. Both tubes are made of polypropylene, so it was expected that the recovery in both tubes is comparable. However, it was concluded that Micronic tubes give more adsorption (25-50%) with Aripiprazole and compound C than Fluidx to which those compounds adsorb only minimally (0-20%). This means that the compositions of the tubes are actually different. The reason why Fluidx has less adsorption was already discussed in '1.7 Materials'. And these tests confirm that the composition of Fluidx has less adsorption than other polypropylene tubes.

5.2 Adsorption tests

For the **pipet tips** that were tested, the difference of adsorption between the tips was limited. The compounds almost did not adsorb to the tips. The results showed that, to have less adsorption, the pipet needs to be pre-wetted. The first aliquots need to be thrown away. For Aripiprazole the recovery was a bit better with the Gilson pos. displacement tip compared to the other tips so this tip was selected as best tip for further experiments.

The tests also showed no adsorption to the Sarstedt pipet which is used in the clinic. However, this was in contrast with what was expected based on the composition of the tip. The tips are made of lower density polymer, because of less ordered chains and therefore more adsorption was expected. This is a hypothesis based on the fact that when the chains are less ordered, the compounds could settle between the chains and this could result in more adsorption. Because of the low adsorption to the tip, it can be concluded that the tip can still be used in clinics.

From the tests with the **needles** it could be concluded that the adsorption in the needles for all compounds is limited.

The **tubes** were divided into two groups depending on their function: collecting or storage. In both tested collecting tubes a bit of adsorption was seen with Aripiprazole and compound C. But the amount of adsorption remained between the limits after transferring 5 times. When the adsorption per tube was evaluated it can be seen that these do not have an important contribution to the adsorption in the total procedure. The tube that had the highest recovery is the Sarstedt tube and is chosen as best test.

For many storage tubes there is adsorption, especially for Aripiprazole and compound C. With the lo binding tubes all compounds give adsorption, Paliperidone gave the least adsorption and Aripiprazole the most. Fluidx showed the lowest amount of adsorption with the chosen compounds, which was expected because of the special composition of the tube [23]. The results of lo binding tubes were rather unexpected because they are called lo binding. The composition of the tube/ coating on the inside is not known so it cannot be explained why the results were unexpected. For most compounds there was no adsorption seen. It is important to keep in mind that the recovery that is found was after transferring the fluid 5 times. The other tubes had some adsorption with the compounds but they were not the best tubes to choose with these compounds. These tubes will probably have the most impact of adsorption in the whole sampling procedure.

If the results of the storage tubes were compared with the results that were found in the literature [11] the conclusion of the Fluidx tubes can be confirmed. In the literature it was found that Fluidx tubes have less adsorption than Eppendorf and Sarstedt tubes. In this investigation the same conclusion was made. In the literature [11] the Sarstedt tubes have the highest adsorption, which is in contrast with this investigation because lo binding protein tubes of Eppendorf showed more adsorption than Sarstedt tubes. However, in the article tests were performed with proteins instead of organic compounds, which might explain the difference.

For most sampling steps and most compounds the adsorption was lower than the limit of 15 %. However, the results of each step need to be combined to know the potential adsorption of an entire procedure. A first estimation can be made by putting all results together. For this, from all tests, the most optimal materials with the least adsorption were selected and the result of each step was summed. Table 35 presents the total sum of the best materials. For the needles and the pipet tips a mean of all aliquots was taken. For the tubes the adsorption to each tube was calculated. Because the results above were given for 5 transfers there can be see that with only one transfer the results are better than expected. The adsorption in the whole procedure is now within the 15% limit. The variation on this result was not calculated. This is an indication of what the adsorption could be in a whole procedure. The different materials still need to be tested after each other to know the real adsorption in the procedure.

The influence of the adsorption of a freeze- thaw cycle is not investigated. When the adsorption of the whole procedure needs to be known more exact, this needs to be investigated as well. This is because the freeze-thaw cycle can affect the pH of the matrix and the test of the pH showed that the pH of the matrix has an influence on the adsorption of compounds in some materials. This is a realistic situation because the samples are never analysed the same day as they are sampled so there is always one freeze- thaw cycle.

The table below shows the adsorption in a whole procedure when the different sampling steps are put after each other. The compounds were ranked from low log P to the highest log P. From this table some conclusions can be made. There can be seen that the amount of adsorption increased when the log P increase, except for Quetiapine. Quetiapine and compound B have almost the same log P but the amount of adsorption differs a lot. This means that only log P cannot be used to make predictions about the amount of adsorption. If the pKa of these compounds are compared there can be seen that these value differs a lot. But the amount of adsorption can be explained with the pKa and the structure of Quetiapine. The pH of the matrices that were used was lower than the pKa of compound B, so there the compound is mostly in the ionized form present (because this is a basic compound), this means a lot of ion-interactions may have taken place. This is a hypothesis that is made but because less information of the composition of the materials this cannot be confirmed. With Quetiapine the pH was higher than the pKa so the more non-ionized form is available, less ion interaction will have taken place. In the structure of Quetiapine there can be seen that the molecule has a polar side (see ‘4.2 Compounds’).

Table 34: The estimated adsorption of an entire procedure.

	Paliperidone	A	B	Quetiapine	C	Aripiprazole
Pencan needle	0.00	0.00	0.00	0.00	0.00	0.00
Sarstedt collecting tube	0.00	0.00	0.01	0.00	0.01	0.02
Sarstedt pipet clinic	0.00	0.00	0.04	0.00	0.01	0.03
Fluidx storage tube	0.01	0.00	0.01	0.01	0.03	0.04
Gilson pos. disp. pipet lab	0.03	0.07	0.05	0.00	0.06	0.03
Total adsorption	0.04	0.07	0.11	0.01	0.11	0.12
log P	1.07	1.27	2.98	2.99	4.86	5.31
pKa	4.41	19.4	11.02	5.87	12.85	4.29

In the table five steps where shown but the sampling procedure contains only four steps. The fifth step is needed in the lab and is not included in the sampling procedure. There can be seen that the materials used in the sampling procedure in the clinic has the least impact on the adsorption and that the adsorption is a problem when the samples are send to the lab. Also can be seen that the compounds have more adsorption in the storage tubes than in the collecting tubes. This can be explained with the amount of volumes that was tested in the tubes. The collecting tubes have more volume so the impact of the adsorption remains limited. The storage tubes have less volume, the compounds will be attracted to the active sites of the containers and in less volume the impact will be more pronounced. When the collecting tubes will be used as storage tubes the impact of adsorption will be the same. Although the Sarstedt pipet from the clinic has lower adsorption than the Gilson pos. displacement tip from the lab, it cannot be used in the lab because the Sarstedt tip is not accurate enough and cannot be used for small volumes.

The influence of adsorption could be minimized. This could be done if the calibration curve and the QC's, that are needed to measure the concentrations in the sample, were made with the same material as in the clinics. The needle will be more difficult. But if the curve and the QC's are made in a collecting tube and are aliquoted with the same pipet and in the same storage tubes as those used in the clinics, this will compensate the adsorption in the sampling procedure.

6 Conclusion

The aim of this investigation was to see if a generic sampling process could be set up with materials used in the clinic and with less than 15% adsorption in the whole sampling procedure. Sub goals to realise the aim were to find an optimal surrogate and investigate the influence of the physicochemical properties of compounds on the amount of adsorption.

Finding an optimal surrogate matrix was not as easy as first thought. A matrix was searched in which all compounds reacts the same as in real CSF but that was not found. In all tested matrices the compounds did react different than in CSF. Because of the time the adsorption test were done with real CSF.

In the evaluation of the physicochemical properties and the adsorption a trend may be visible. But because not all compounds reacted as hypothesized the adsorption cannot be predicted based only on log P, but the pKa is needed as well. The link between the pKa and the adsorption still needs to be evaluated. More research is needed to evaluate a relation between different physicochemical properties of the compound and the adsorption in order to predict the degree of adsorption.

The impact of some tests (tubes) were exaggerated to evaluate the adsorption better, otherwise the adsorption could be missed because of the variation. This could also catch the variations of the clinic: difference in temperature, the time that samples remain at room temperature, the different ways of sampling… The problem of enlarging the problem is that is introduces new variation on the results of analysing. Now the use of small volumes in larger tubes gives more variation. In clinic the tubes are more filled as was done in the tests.

The variation that was part of the sampling processing procedures was quite high (approx. 10%) As a consequence, it became difficult to assess differences in the order of 15 % that are relevant for the tests. Using more replicates was helpful, but further improvements to the methodology are needed to better assess the adsorption.

Setting up a generic procedure with the best materials is not done yet but an estimate was made of the possible adsorption with the materials what did have the less adsorption in the individual test. When this estimation is compared with the desirable limit, all tested compounds fall between the desirable limit but when log P increases more than 6 the adsorption will be probably higher than the limit. To set up a generic test more materials need to be tested because there are compounds with a higher log P than tested. Another option is to investigate the influence of adding an additive. First the question was made if it has an advantage of using additives from working with the storage tubes. The results showed that the most adsorption is present when CSF is transferred into the storage tubes.

This investigation is not finished, more researched is needed. The first step is that some tests need to be repeated with real CSF to know the adsorption in CSF. This can be done in a smaller design,

using only the best materials that were selected from the test with aCSF. Another test that needs to be done is testing the different best materials of the sampling steps all in one experiment mimicking the entire sampling procedure. Besides that the use of additives to release the compound from the container wall can be tested. Finally, it is also important to investigate a freeze thaw cycle.

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Appendices

Appendix 1: Results of optimal surrogate.

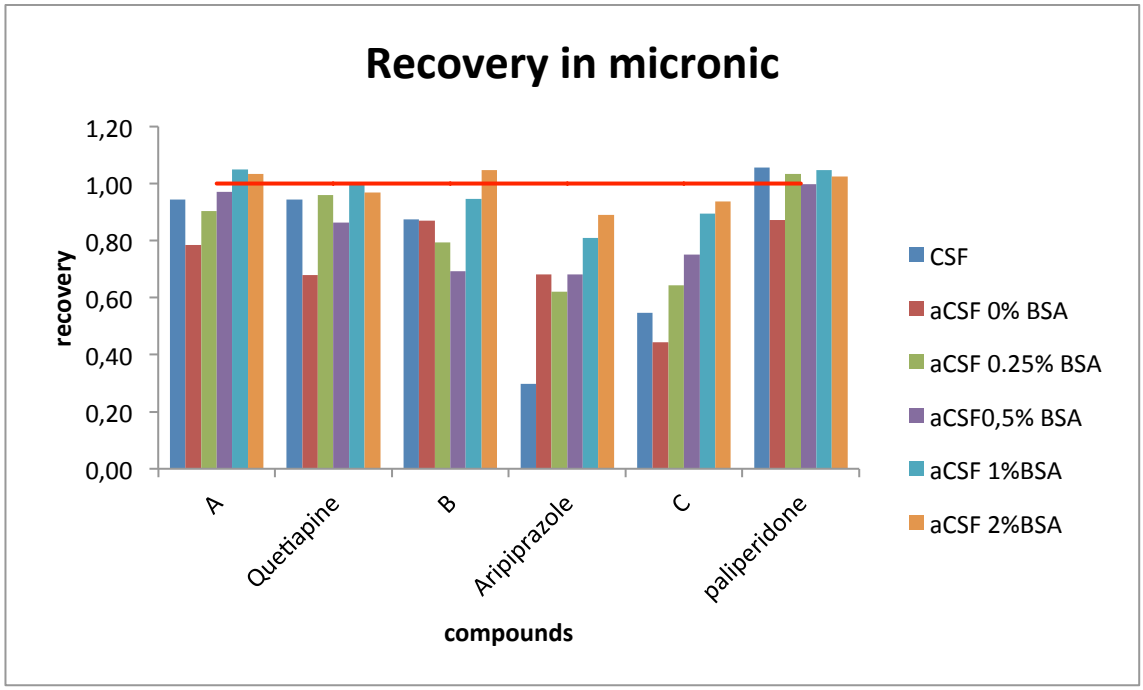

Figure 23: Recovery of compounds in different matrices in Micronics. The red line is 100% recovery.

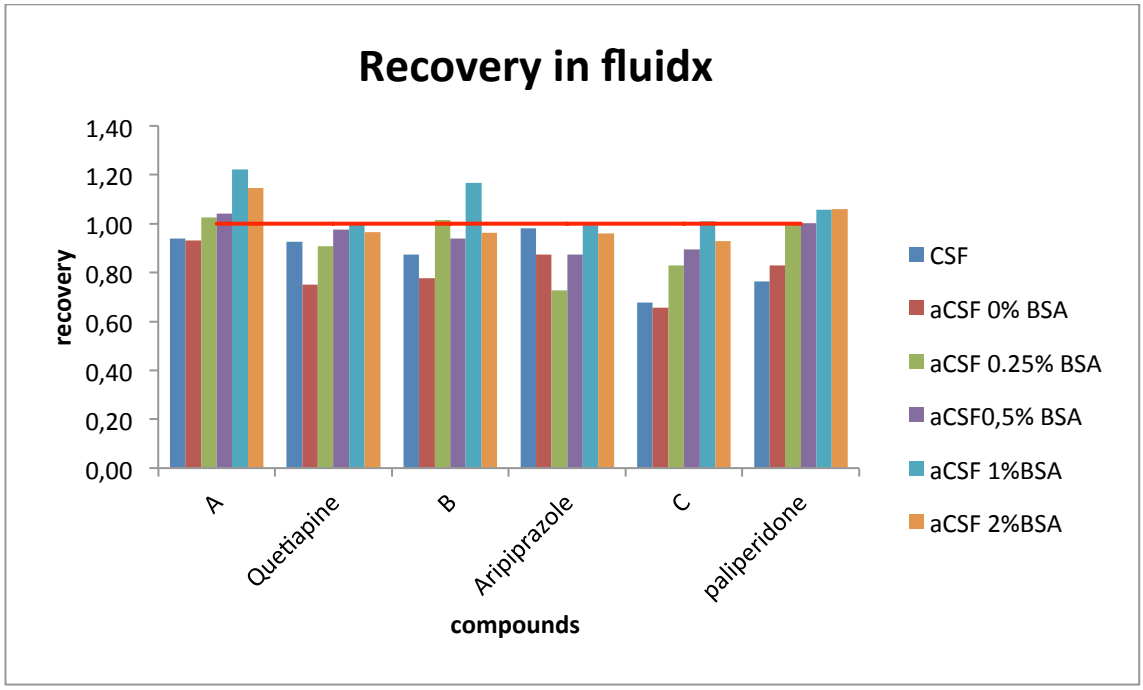

Figure 24: Recovery of compounds in different matrices in Fluidx. The red line is 100% recovery.

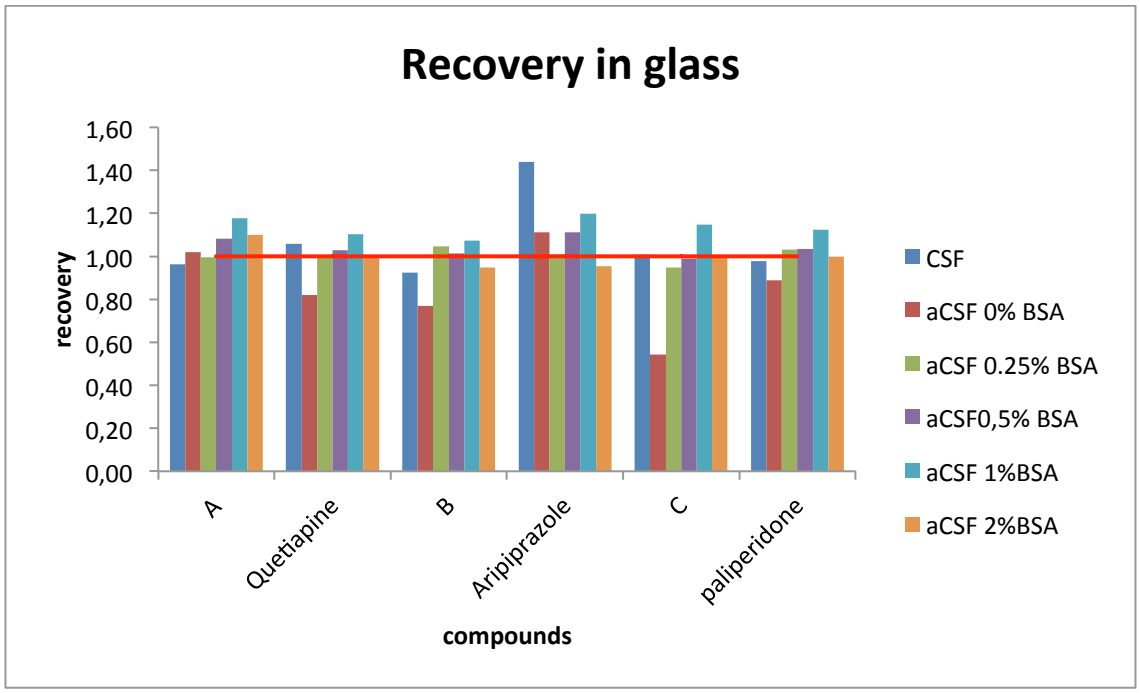

Figure 25: Recovery of compounds in different matrices in glass. The red line is 100% recovery.

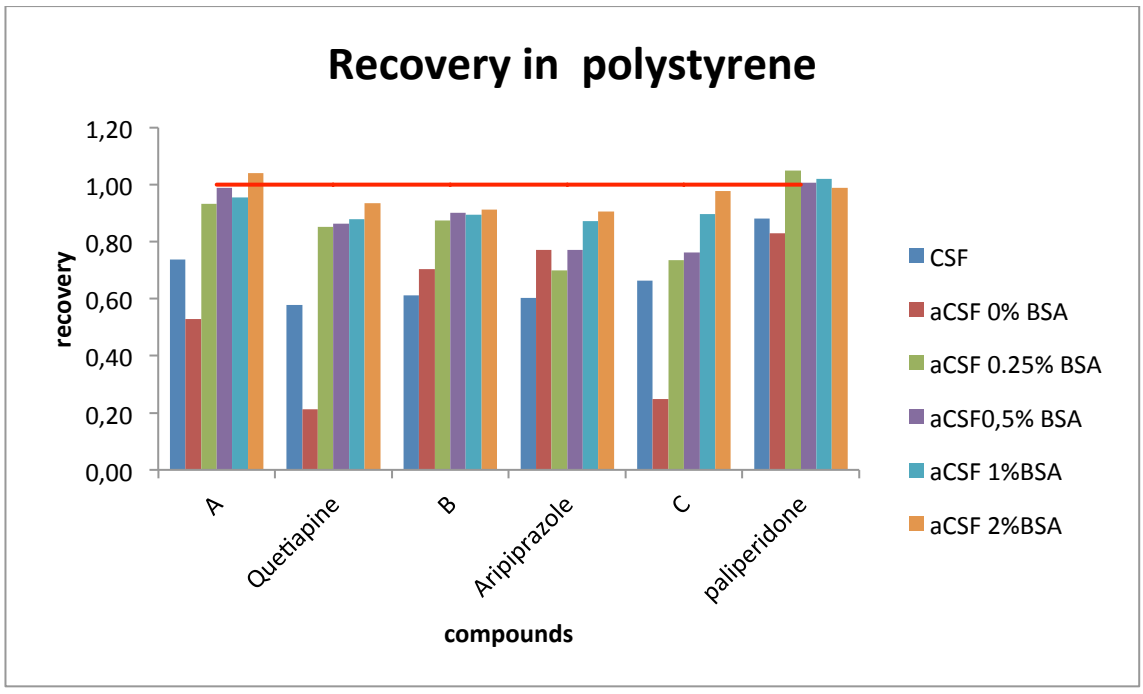

Figure 26: Recovery of compounds in different matrices in polystyrene. The red line is 100% recovery.

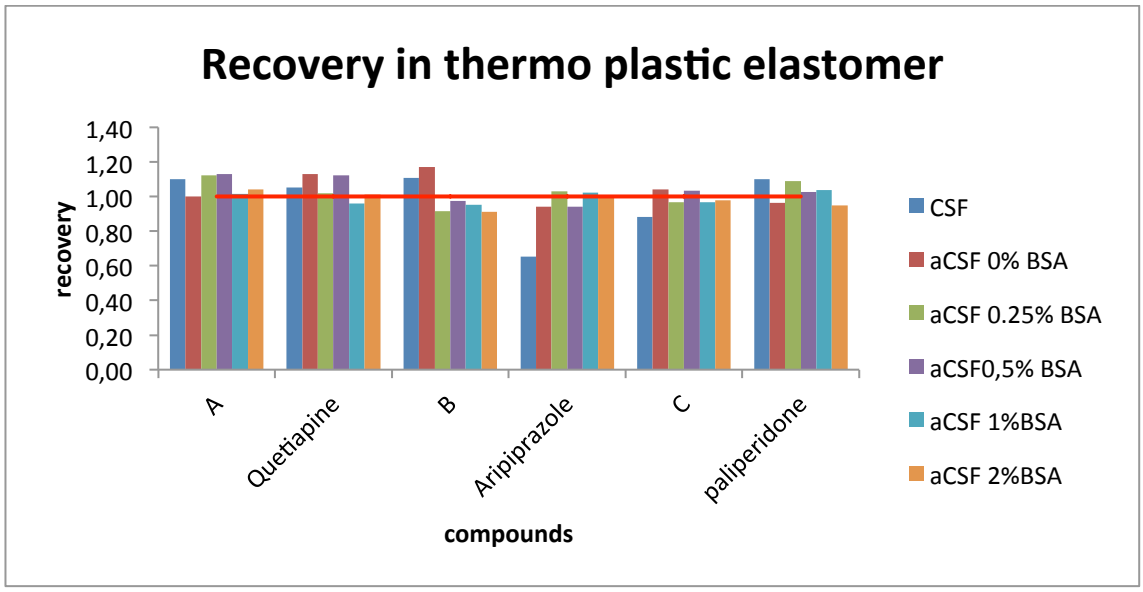

Figure 27: Recovery of compounds in different matrices in thermo plastic elastomer. The red line is 100% recovery.

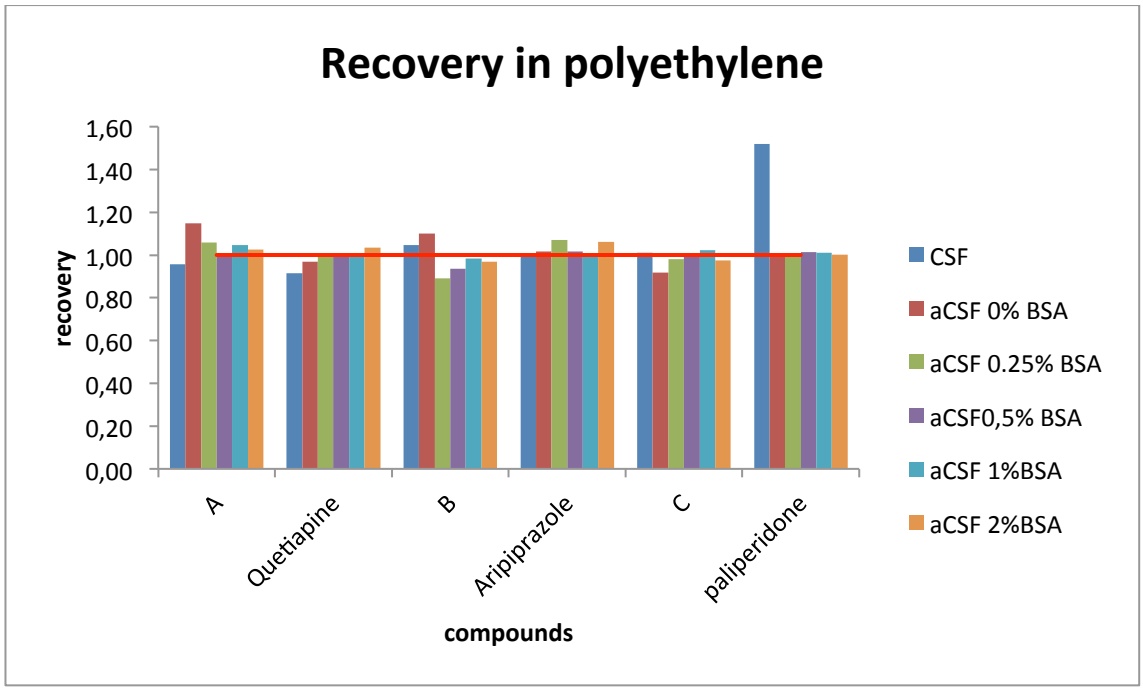

Figure 28: Recovery of compounds in different matrices in polyethylene. The red line is 100% recovery.

Appendix 2: Evaluation of the adsorption in pipet tips.

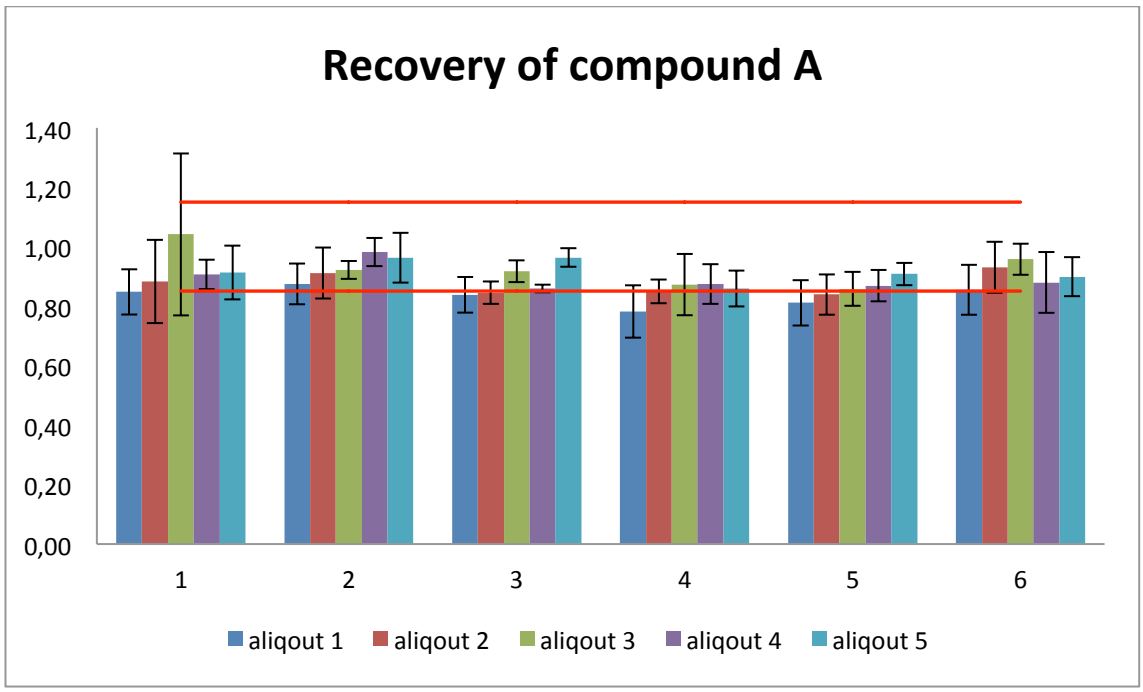

Figure 29: Recovery of compound A in pipet-tips. 1= repeating pipet, 2= pos. displacement pipet of Gilson, 3= pos. displacement pipet of Eppendorf, 4= air column pipet of Eppendorf (reload), 5= low retention, 6= low bind. The red lines are the 15% limits.

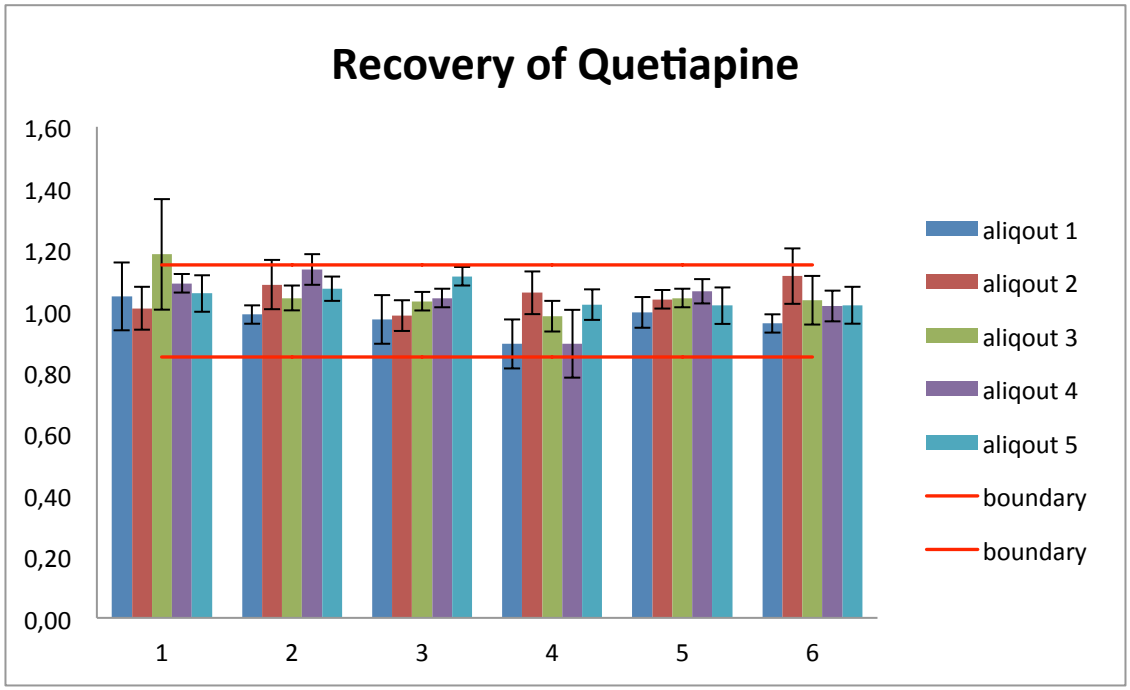

Figure 30: Recovery of Quetiapine in pipet-tips. 1= repeating pipet, 2= pos. displacement pipet of Gilson, 3= pos. displacement pipet of Eppendorf, 4= air column pipet of Eppendorf (reload), 5= low retention, 6= low bind. The red lines are the 15% limits.

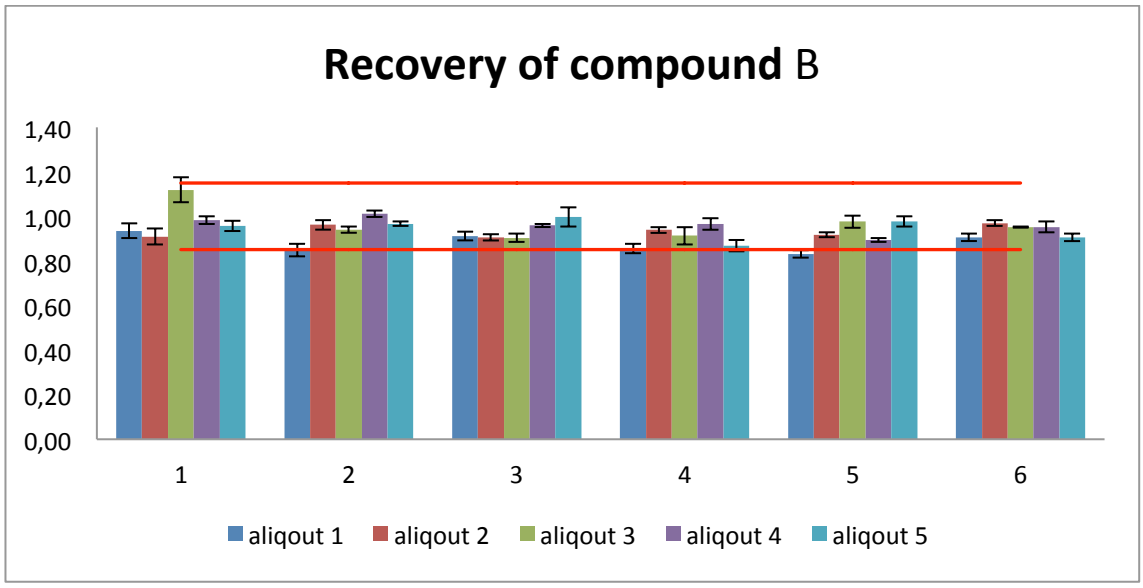

Figure 31: Recovery of compound B in pipet-tips. 1= repeating pipet, 2= pos. displacement pipet of Gilson, 3= pos. displacement pipet of Eppendorf, 4= air column pipet of Eppendorf (reload), 5= low retention, 6= low bind. The red lines are the 15% limits.

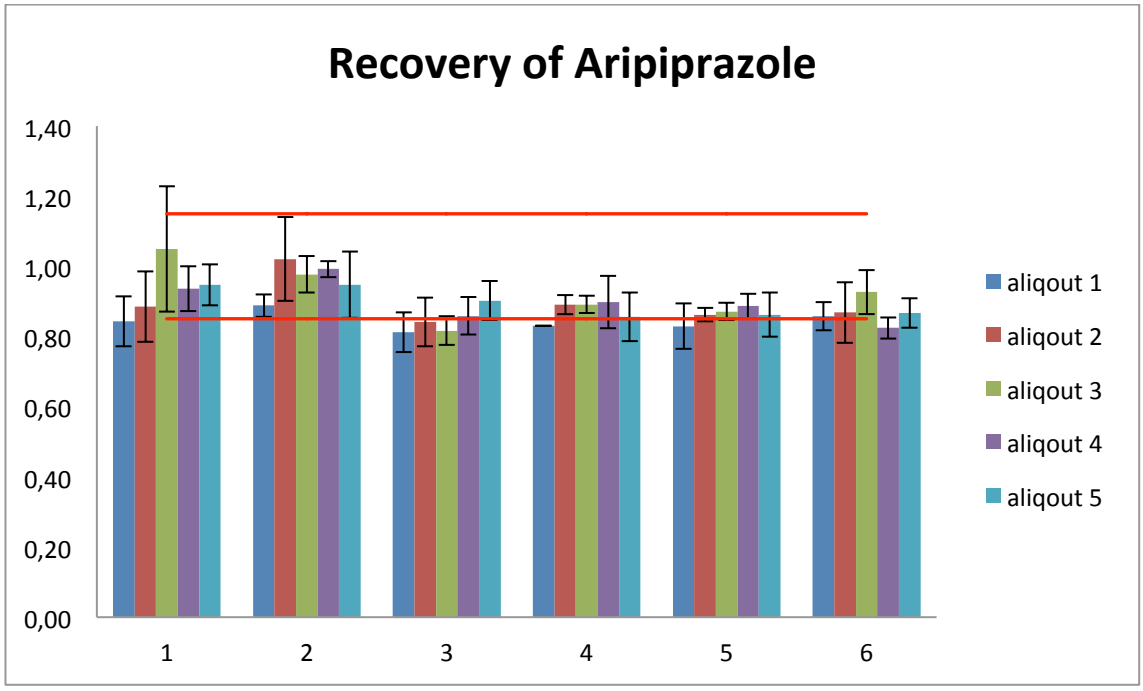

Figure 32: Recovery of Paliperidone in pipet-tips. 1= repeating pipet, 2= pos. displacement pipet of Gilson, 3= pos. displacement pipet of Eppendorf, 4= air column pipet of Eppendorf (reload), 5= low retention, 6= low bind. The red lines are the 15% limits.

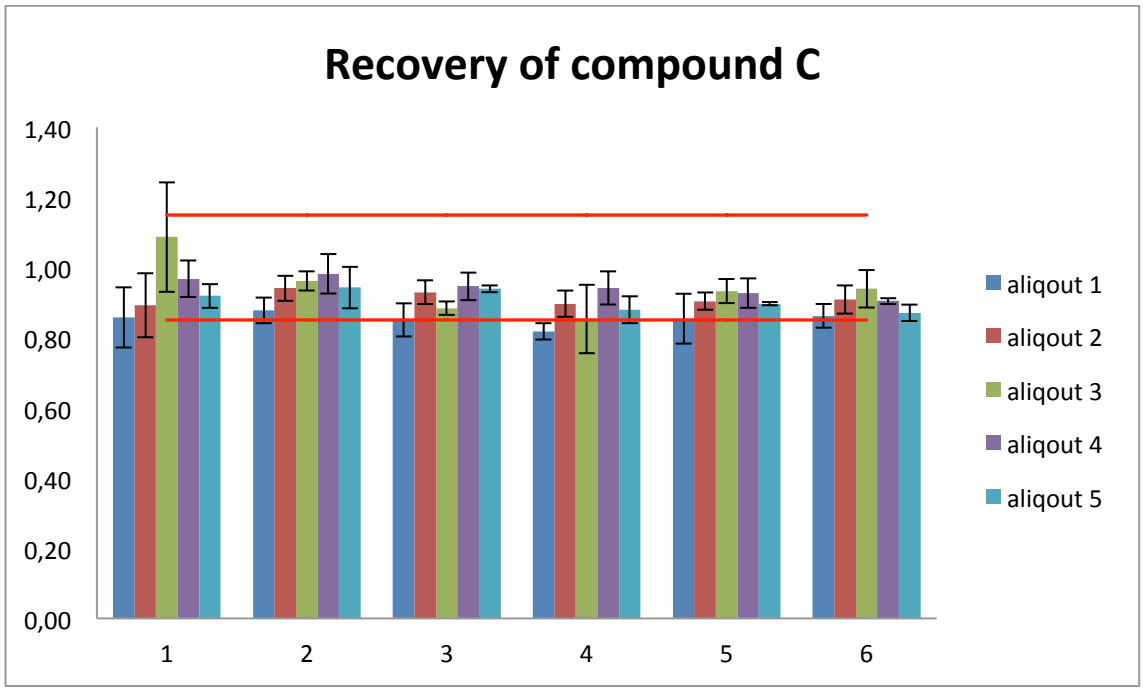

Figure 33: Recovery of compound C in pipet-tips. 1= repeating pipet, 2= pos. displacement pipet of Gilson, 3= pos. displacement pipet of Eppendorf, 4= air column pipet of Eppendorf (reload), 5= low retention, 6= low bind. The red lines are the 15% limits.

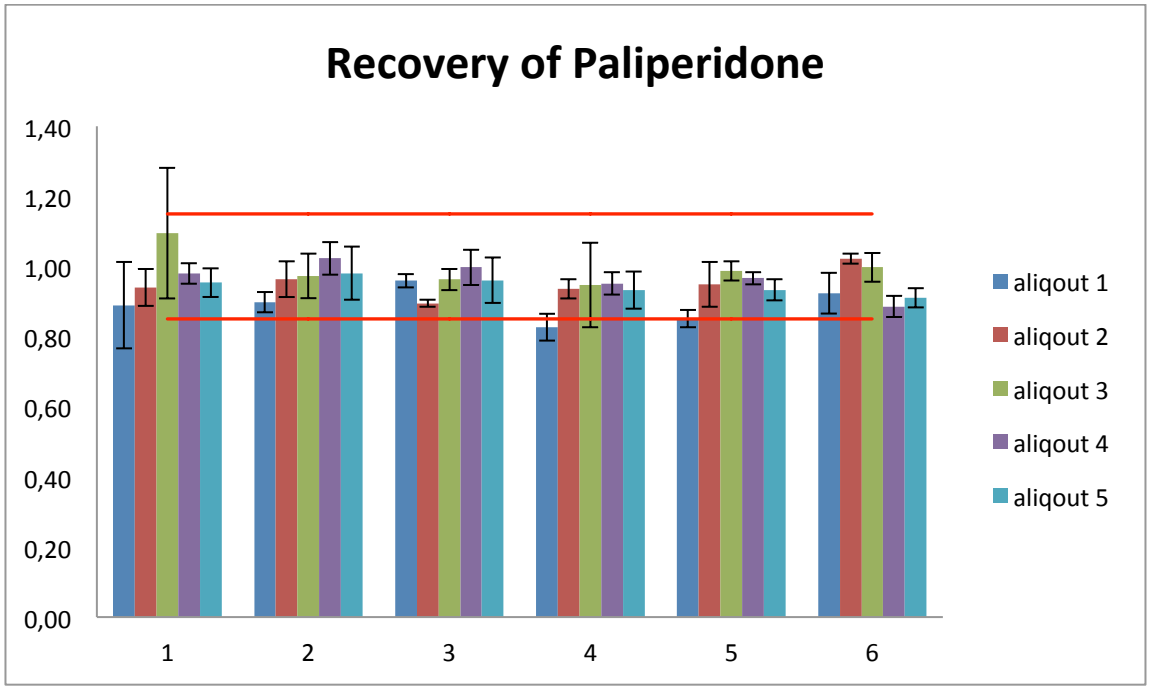

Figure 34: Recovery of Paliperidone in pipet-tips. 1= repeating pipet, 2= pos. displacement pipet of Gilson, 3= pos. displacement pipet of Eppendorf, 4= air column pipet of Eppendorf (reload), 5= low retention, 6= low bind. The red lines are the 15% limits.

Appendix 3: Evaluation of adsorption in pipet tips of stage 3

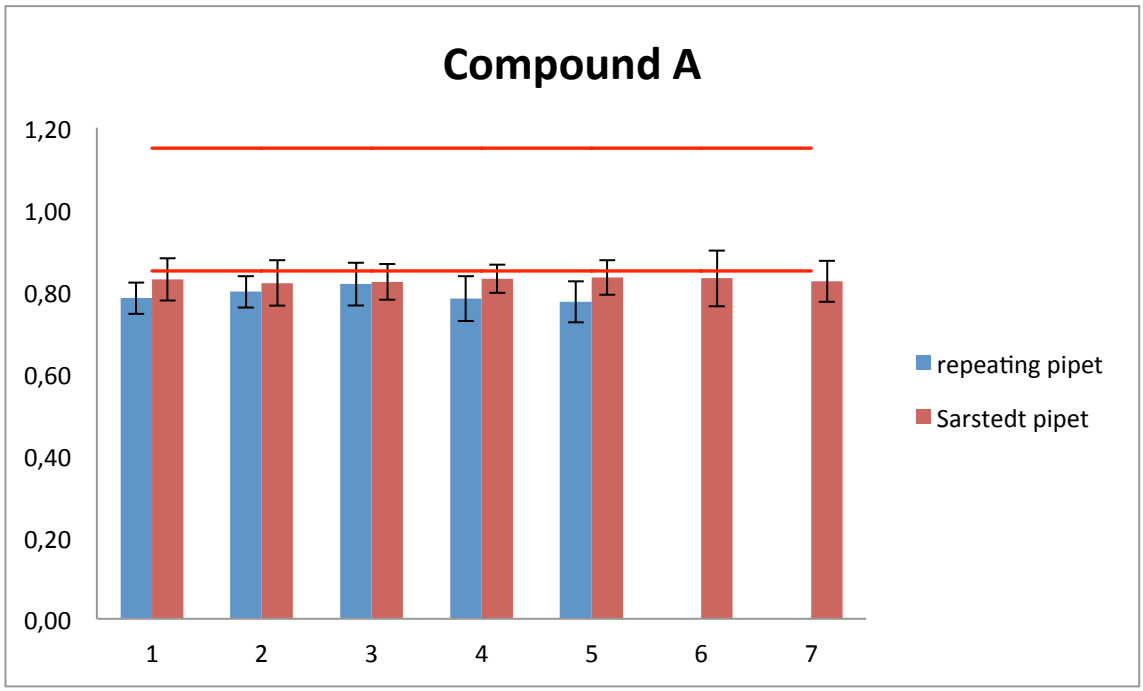

Figure 35: Recovery of compound A in pipet tips used in the clinics, test 1. The red lines are the 15% limits.

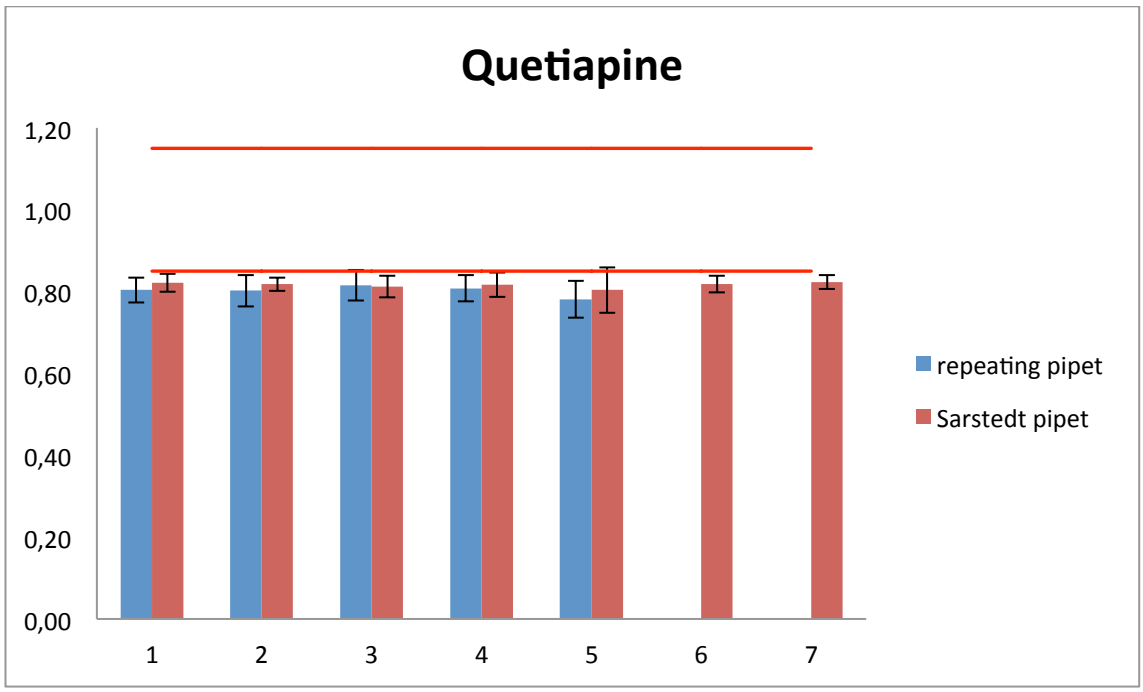

Figure 36: Recovery of Quetiapine in pipet tips used in the clinics, test 1. The red lines are the 15% limits.

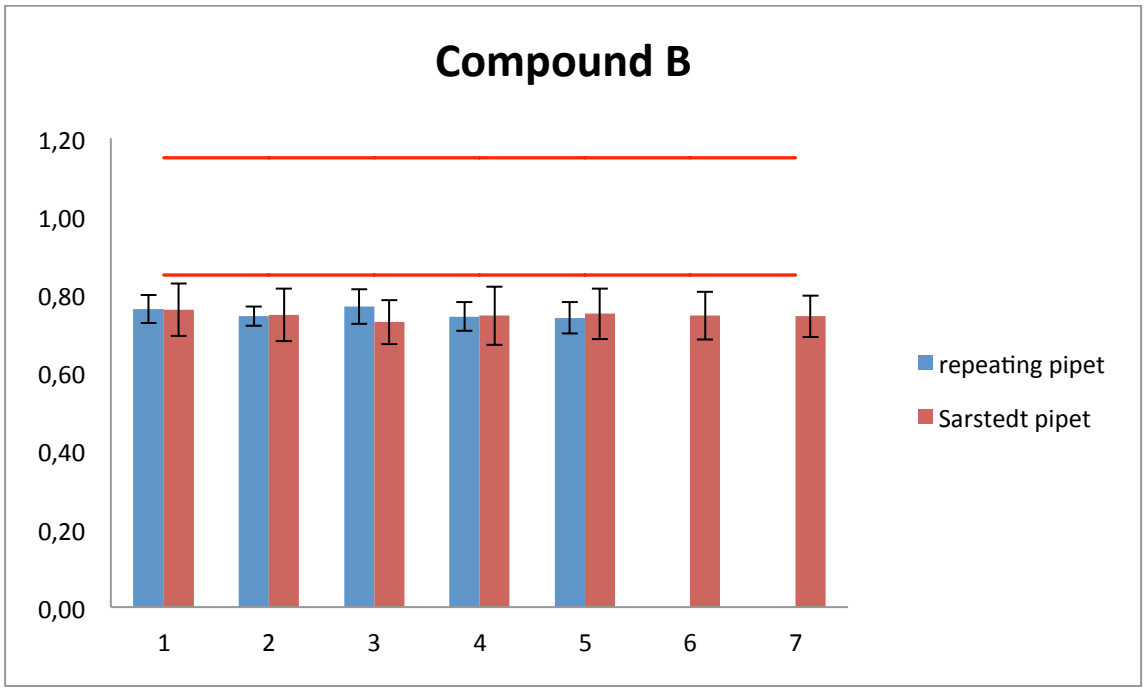

Figure 37: Recovery of compound B in pipet tips used in the clinics, test 1. The red lines are the 15% limits.

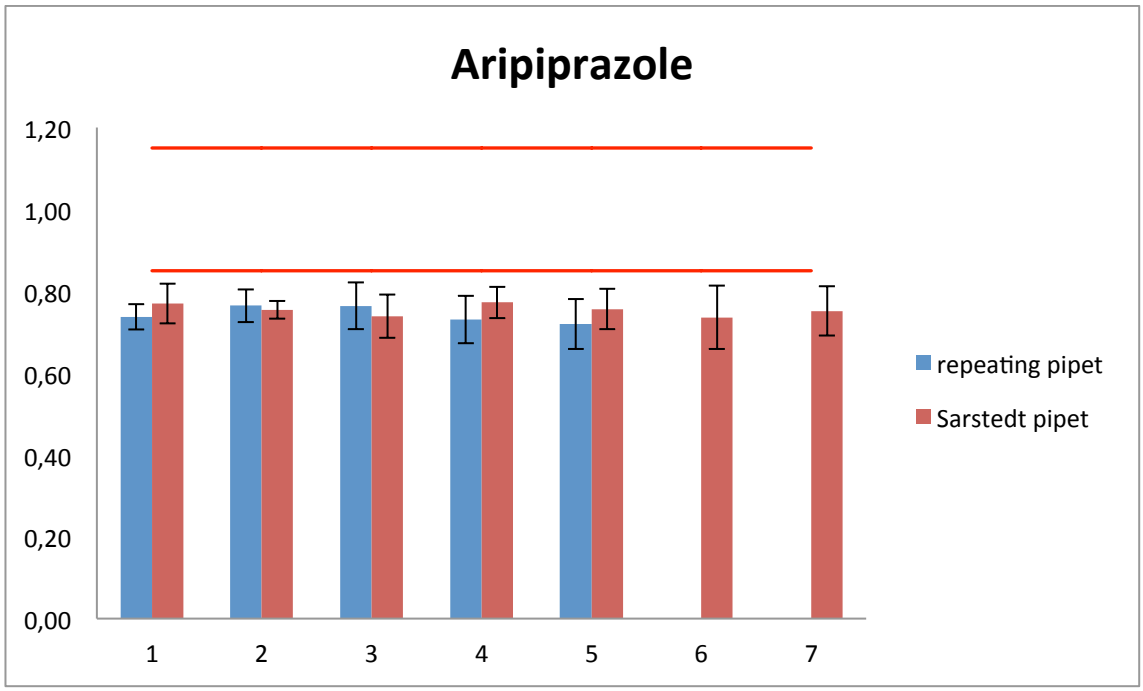

Figure 38: Recovery of Aripiprazole in pipet tips used in the clinics, test 1. The red lines are the 15% limits.

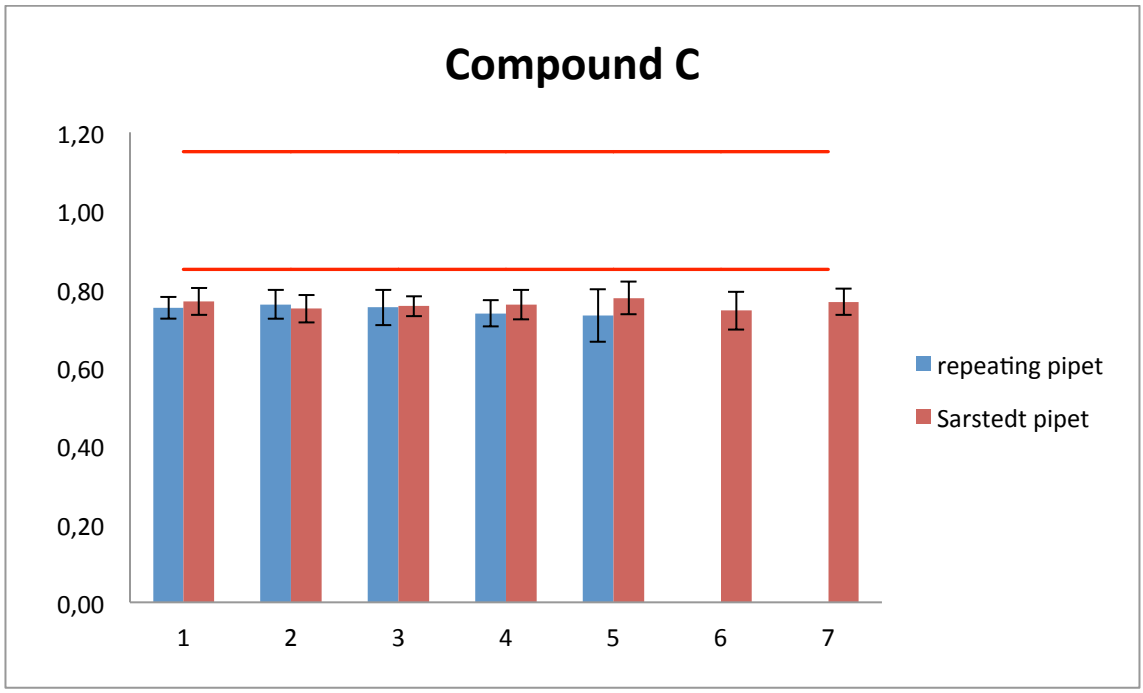

Figure 39: Recovery of compound C in pipet tips used in the clinics, test 1. The red lines are the 15% limits.

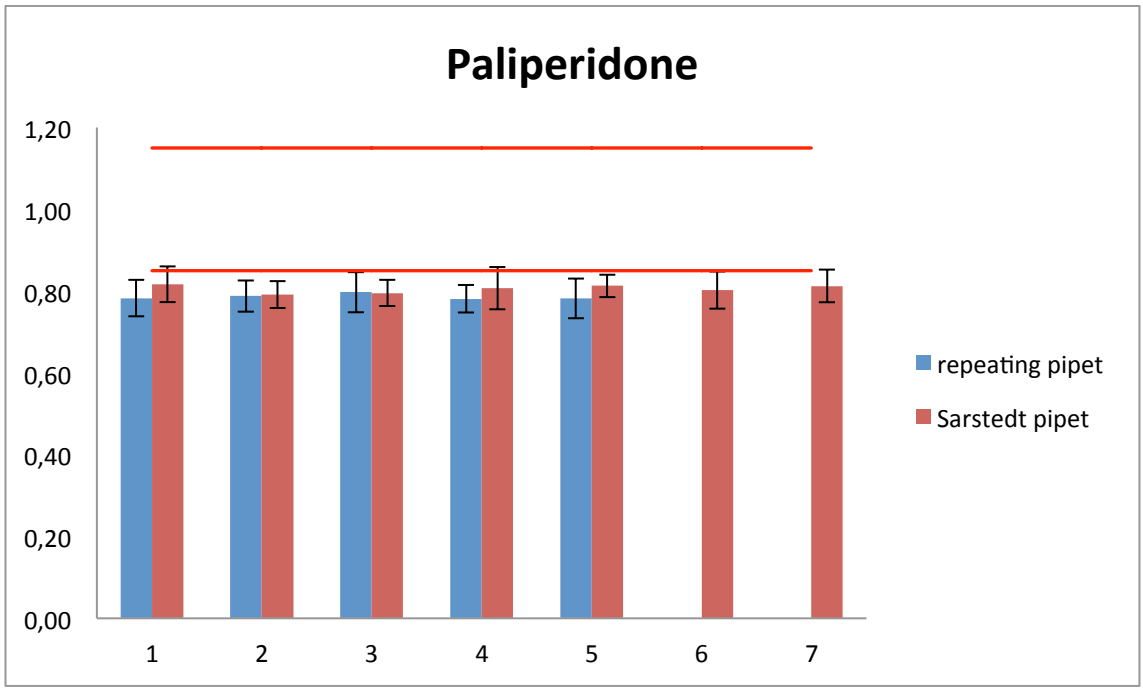

Figure 40: Recovery of Paliperidone in pipet tips used in the clinics, test 1. The red lines are the 15% limits.

Appendix 4: Evaluation of adsorption in pipet tips of stage 3, test 2.

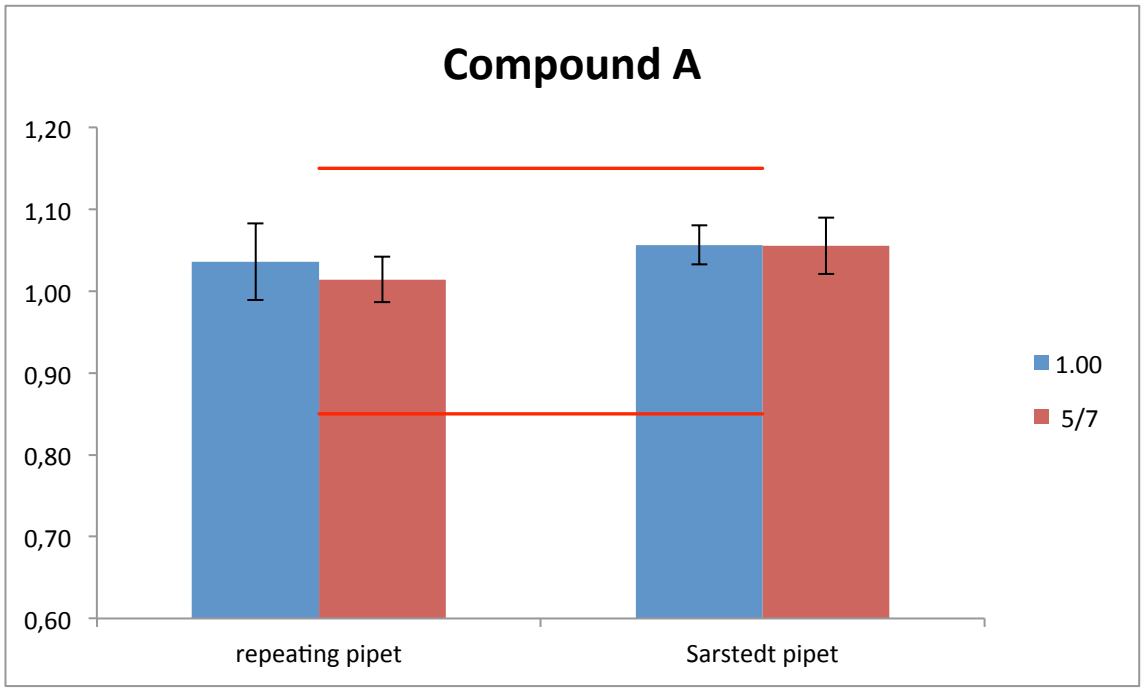

Figure 41: Recovery of compound A in pipet tips used in the clinics, test 2. The Blue box is the first aliquot and the red box is aliquot 5 for the repeating pipet and aliquot 7 for the Sarstedt pipet. The red lines are the 15% limits.

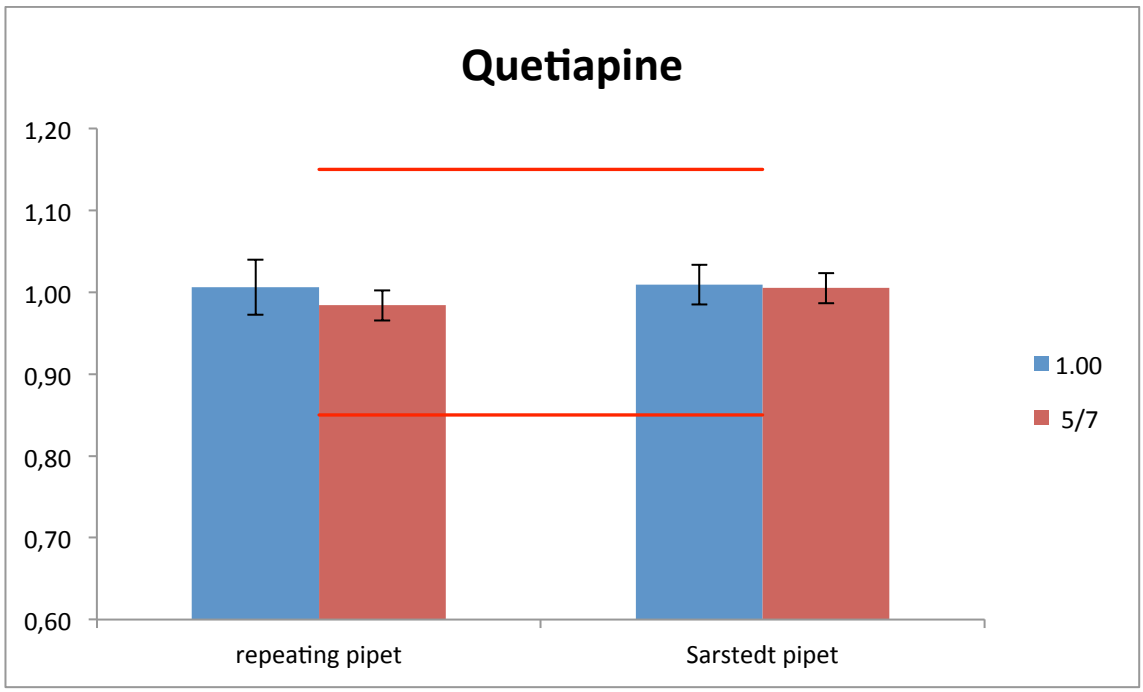

Figure 42: Recovery of Quetiapine in pipet tips used in the clinics, test 2. The Blue box is the first aliquot and the red box is aliquot 5 for the repeating pipet and aliquot 7 for the Sarstedt pipet. The red lines are the 15% limits.

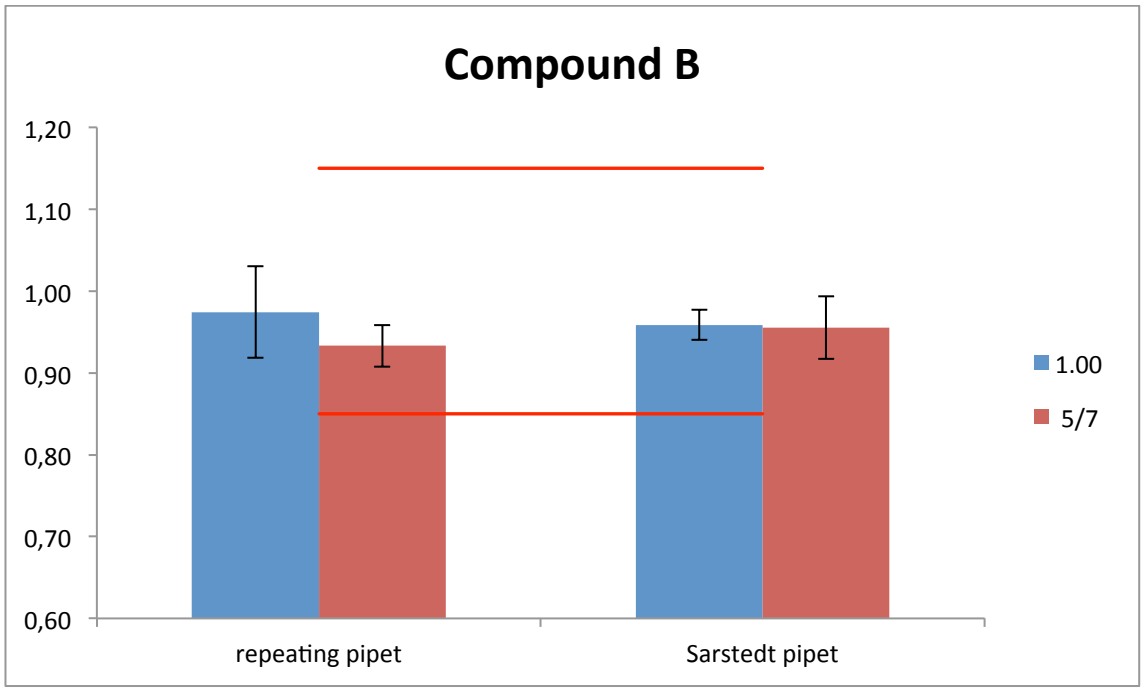

Figure 43: Recovery of compound B in pipet tips used in the clinics, test 2. The Blue box is the first aliquot and the red box is aliquot 5 for the repeating pipet and aliquot 7 for the Sarstedt pipet. The red lines are the 15% limits.

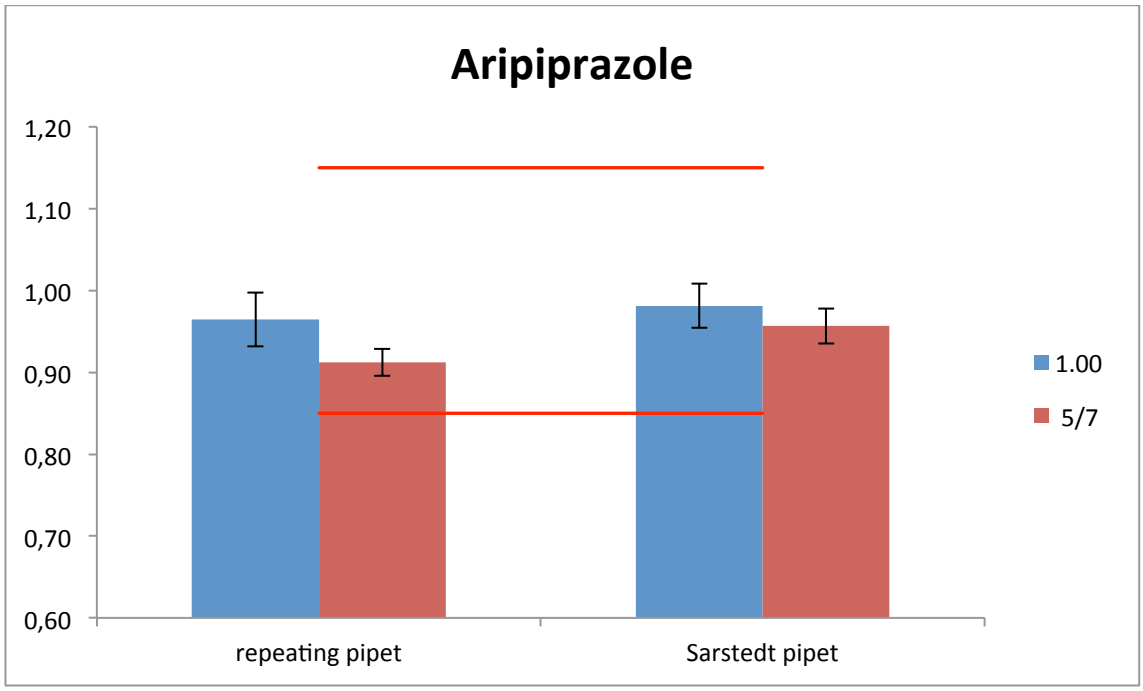

Figure 44: Recovery of Aripiprazole in pipet tips used in the clinics, test 2. The Blue box is the first aliquot and the red box is aliquot 5 for the repeating pipet and aliquot 7 for the Sarstedt pipet. The red lines are the 15% limits.

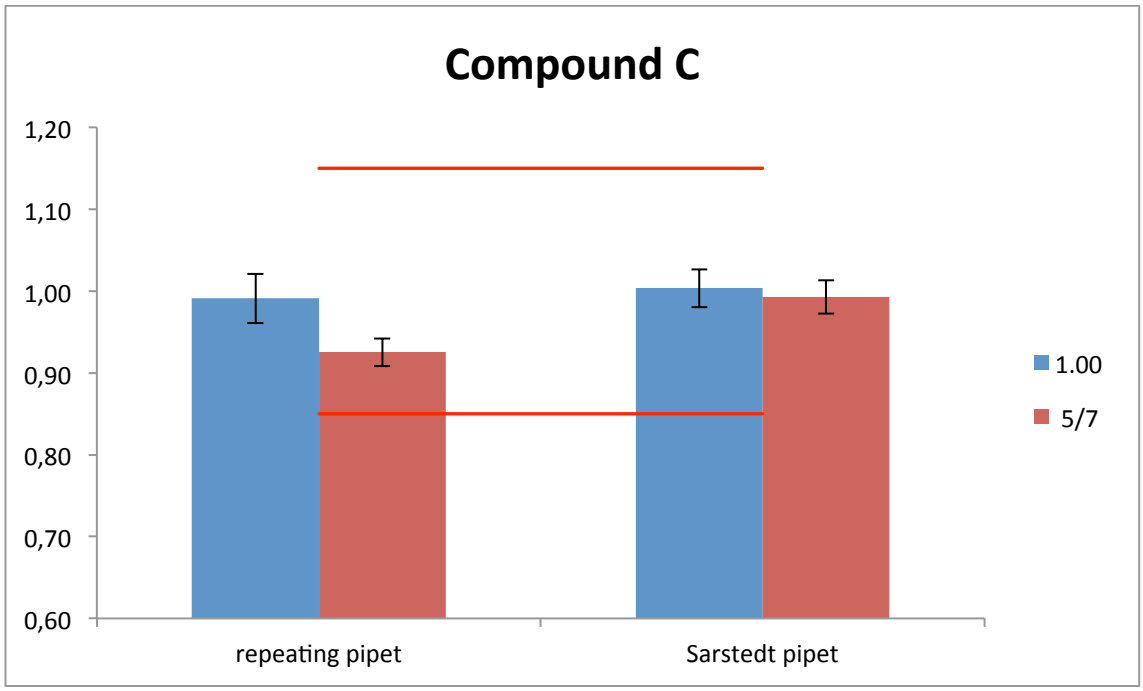

Figure 45: Recovery of compound C in pipet tips used in the clinics, test 2. The Blue box is the first aliquot and the red box is aliquot 5 for the repeating pipet and aliquot 7 for the Sarstedt pipet. The red lines are the 15% limits.

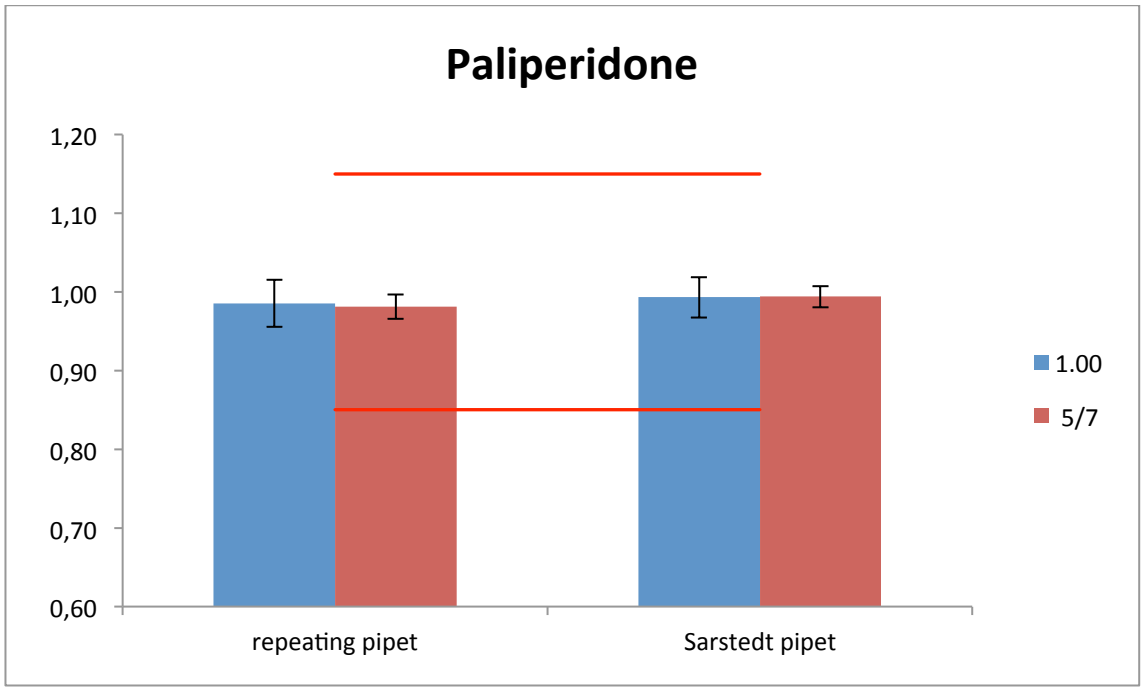

Figure 46: Recovery of Paliperidone in pipet tips used in the clinics, test 2. The Blue box is the first aliquot and the red box is aliquot 5 for the repeating pipet and aliquot 7 for the Sarstedt pipet. The red lines are the 15% limits.

Appendix 5: Recovery of compounds in the needles with aliquots of 100µL.

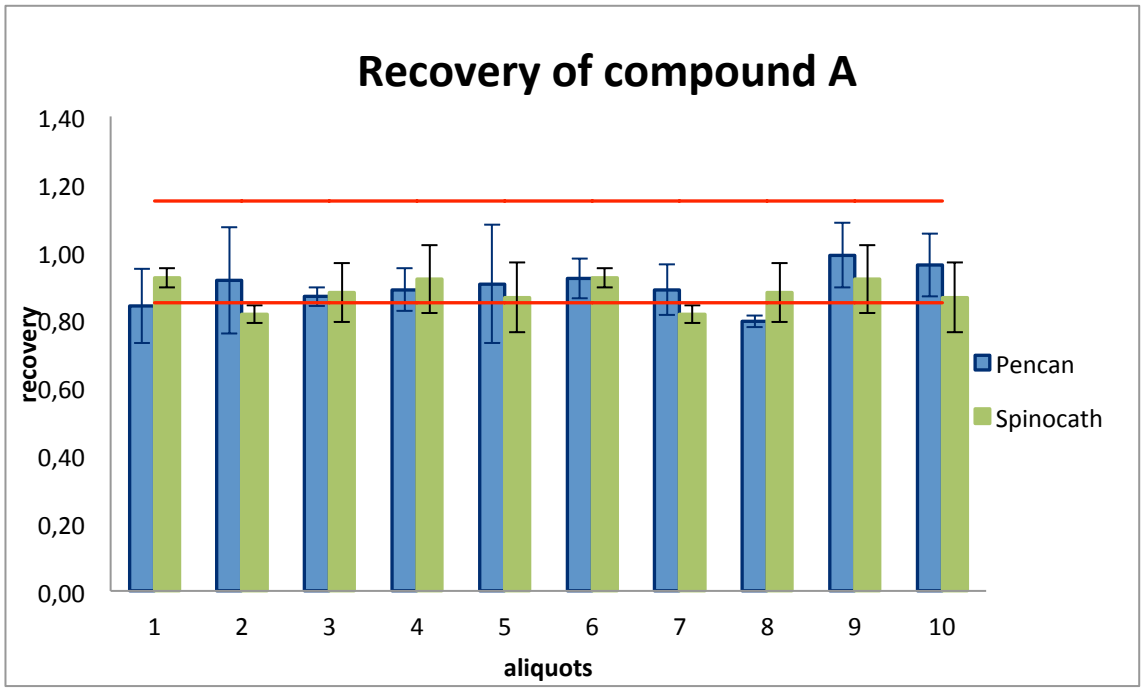

Figure 47: Recovery of compound A in needles, aliquots of 100 µL. The red lines are the 15% limits.

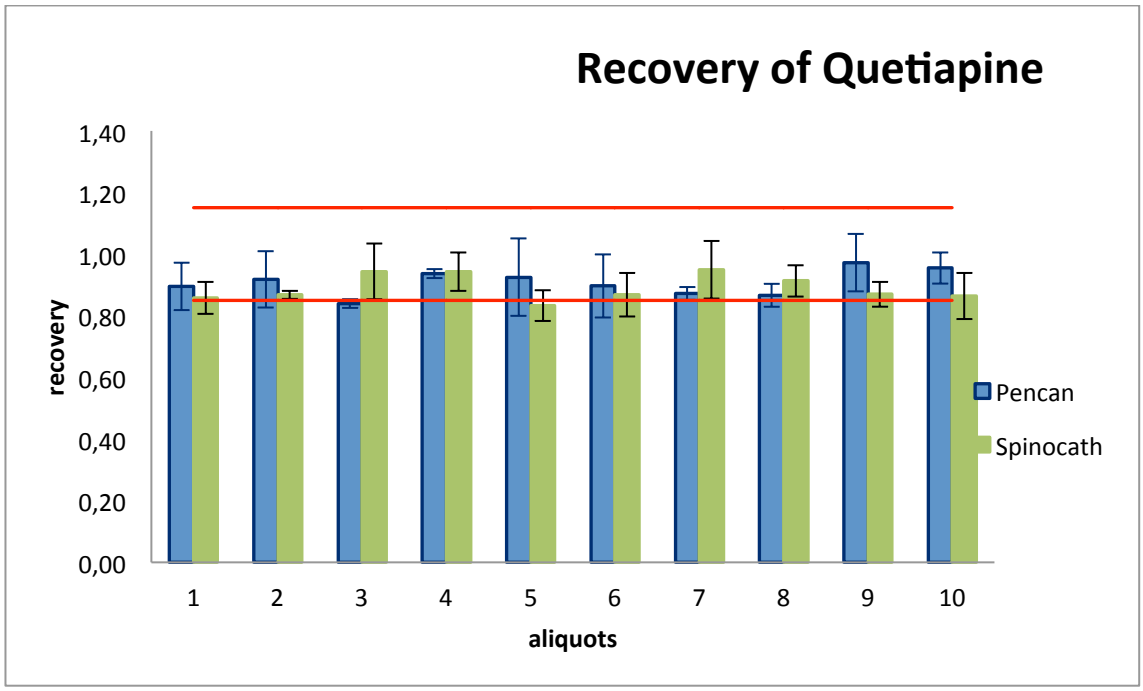

Figure 48: Recovery of Quetiapine in needles, aliquots of 500 µL. The red lines are the 15% limits.

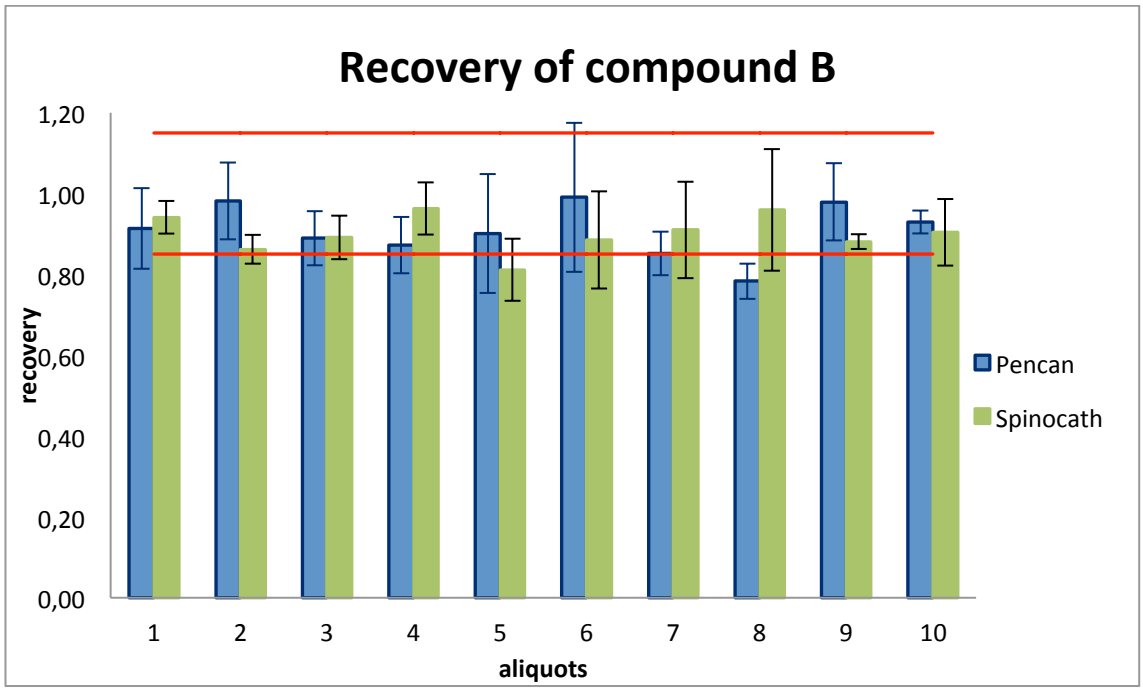

Figure 49: Recovery of compound C in needles, aliquots of 500 µL. The red lines are the 15% limits.

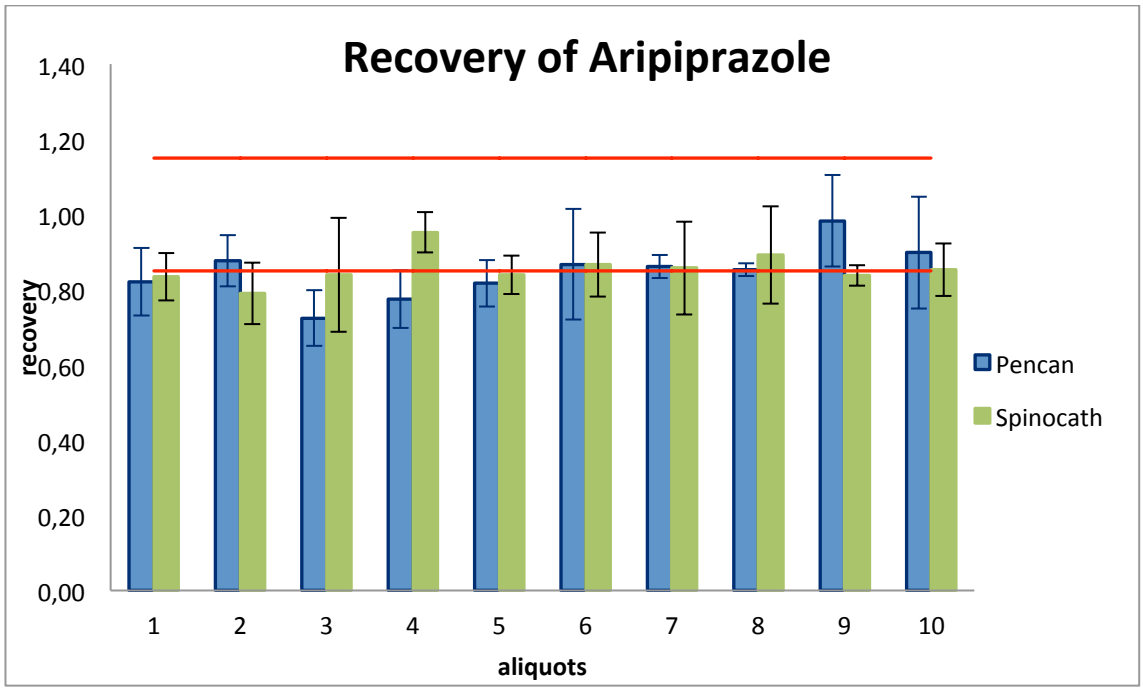

Figure 50: Recovery of Aripiprazole in needles, aliquots of 500 µL. The red lines are the 15% limits.

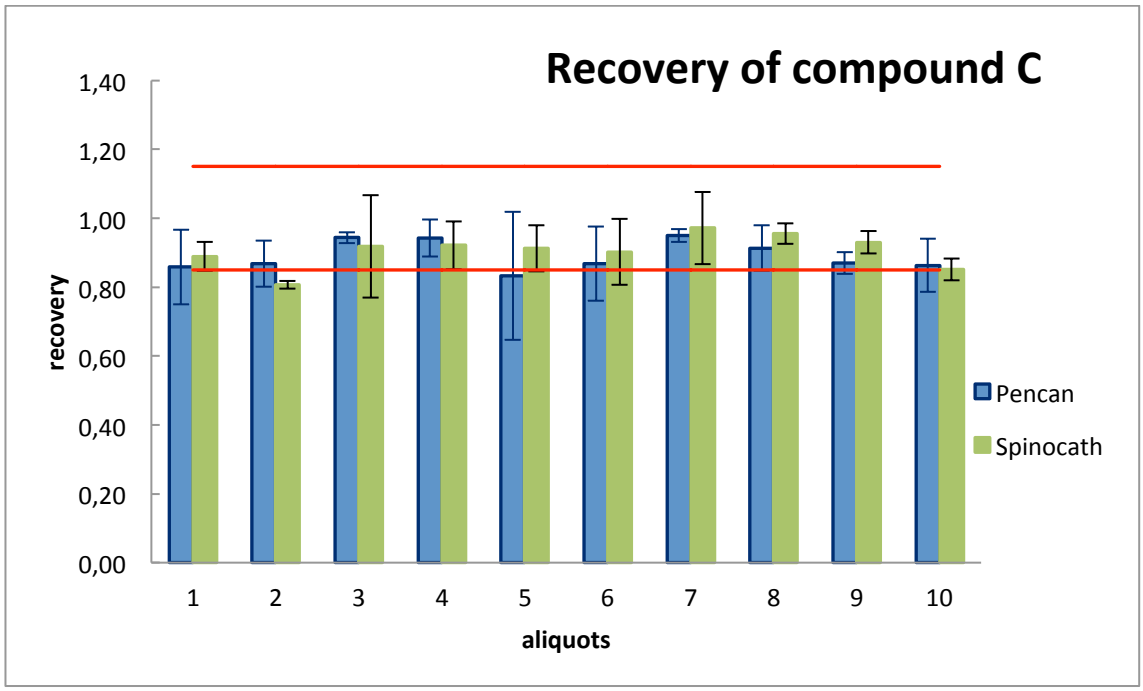

Figure 51: Recovery of compound C in needles, aliquots of 100 µL. The red lines are the 15% limits.

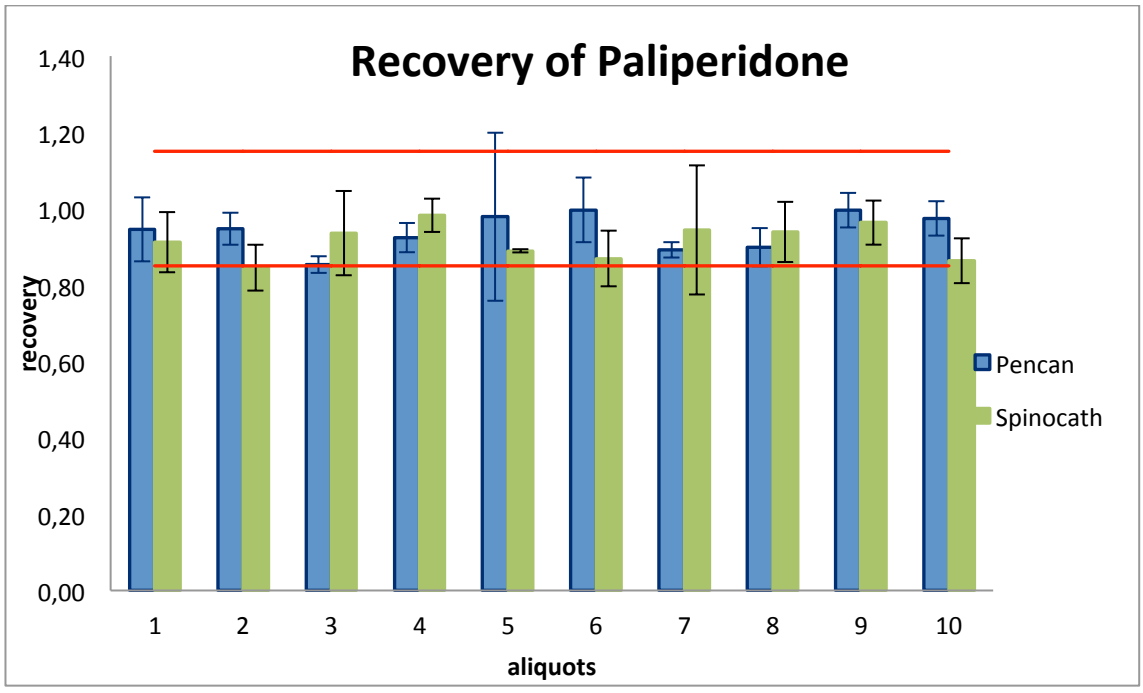

Figure 52: Recovery of Paliperidone in needles, aliquots of 100 µL. The red lines are the 15% limits.

Appendix 6: Recovery of compounds in the needles with aliquots of 500µL.

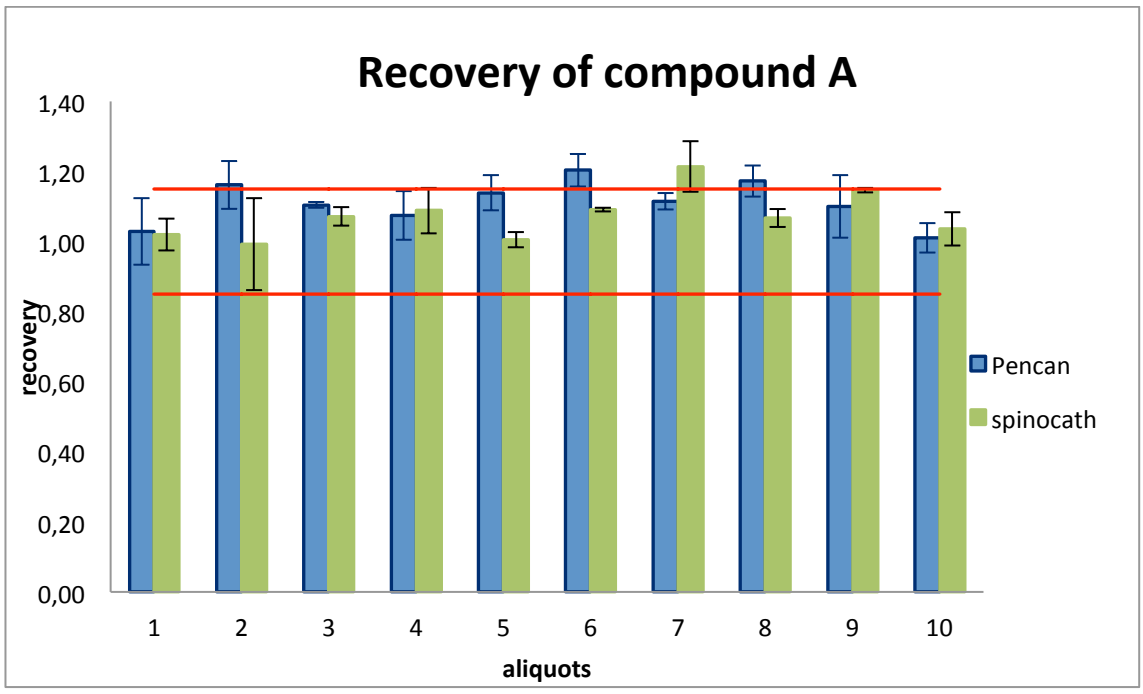

Figure 53: Recovery of compound A in needles, aliquots of 500 µL. The red lines are the 15% limits.

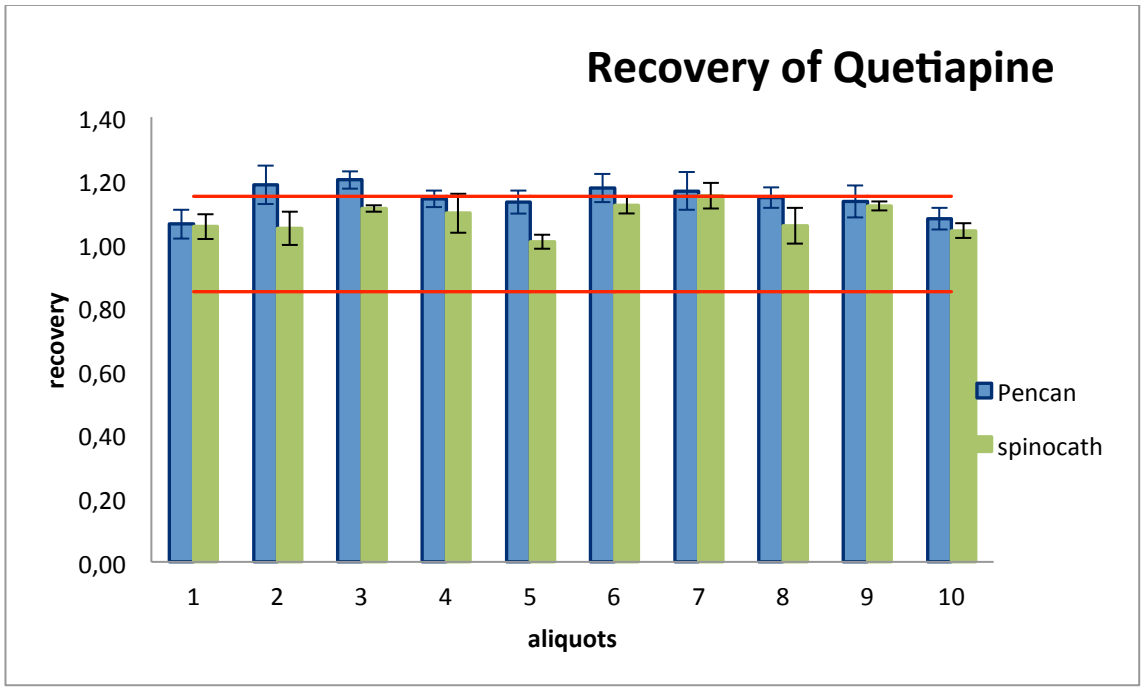

Figure 54: Recovery of Quetiapine in needles, aliquots of 500 µL. The red lines are the 15% limits.

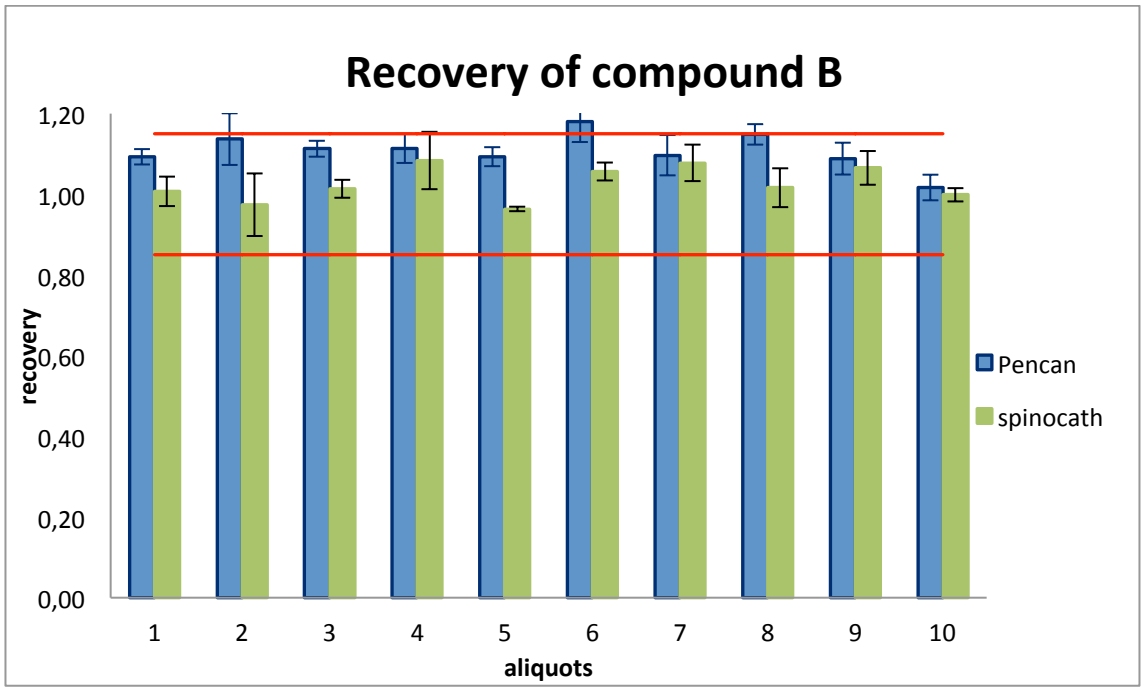

Figure 55: Recovery of compound B in needles, aliquots of 500 µL. The red lines are the 15% limits.

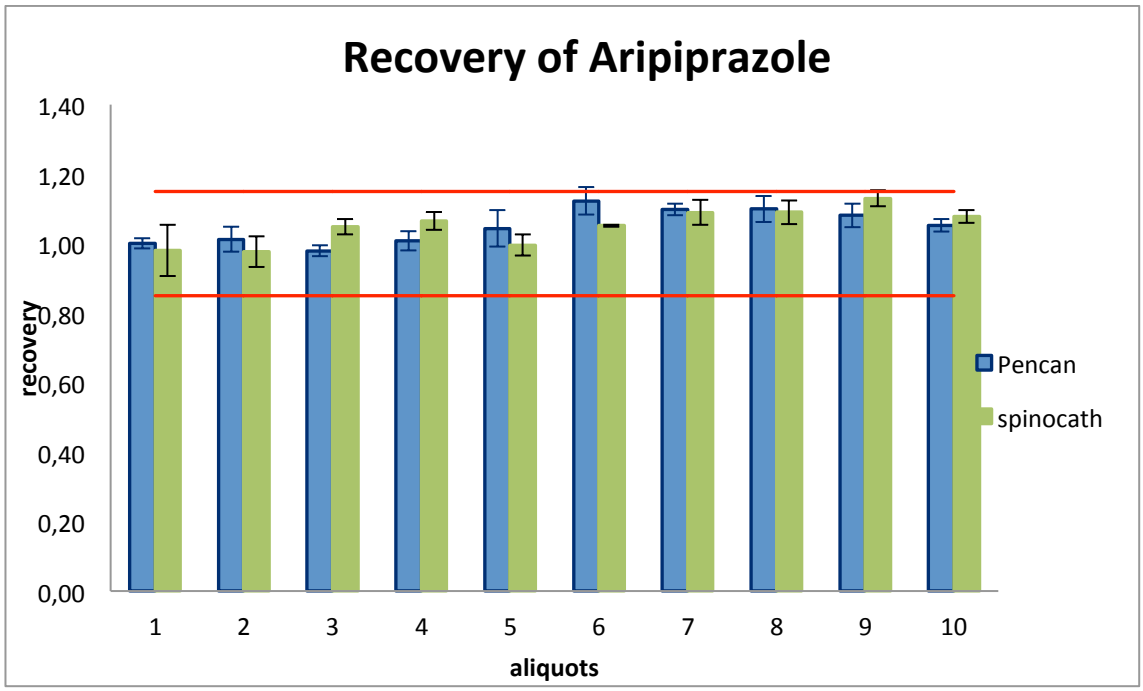

Figure 56: Recovery of Aripiprazole in needles, aliquots of 500 µL. The red lines are the 15% limits.

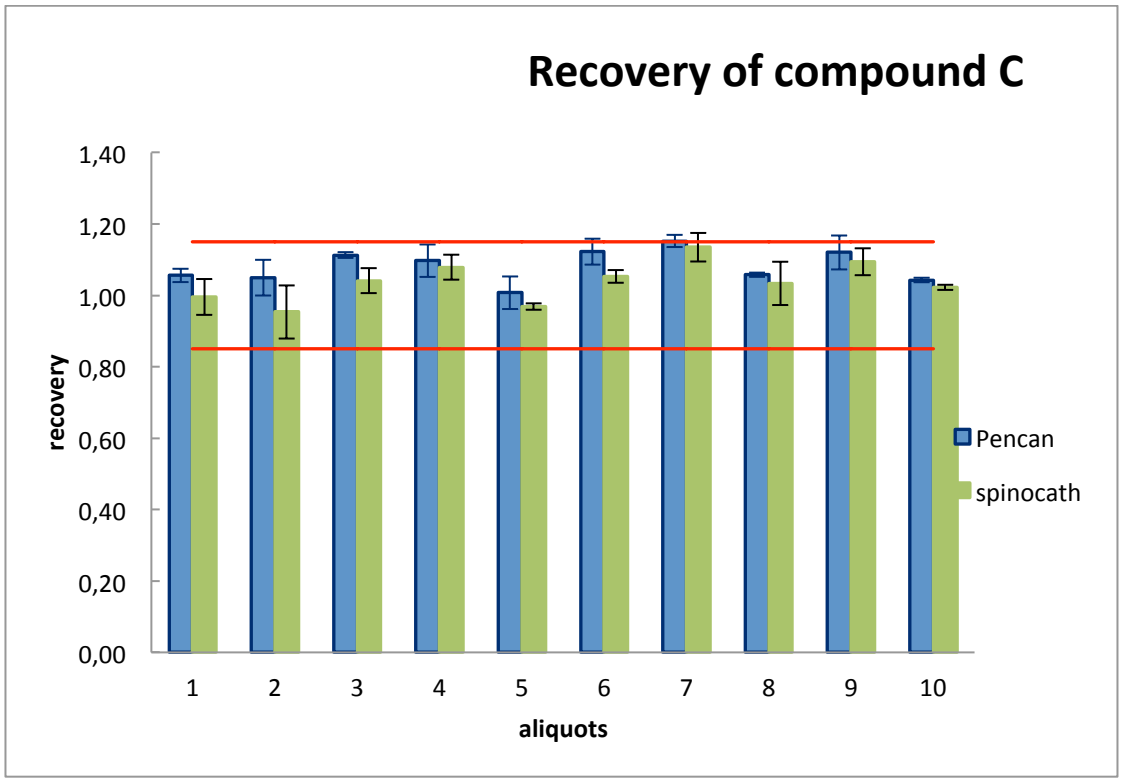

Figure 57: Recovery of compound C in needles, aliquots of 500 µL. The red lines are the 15% limits.

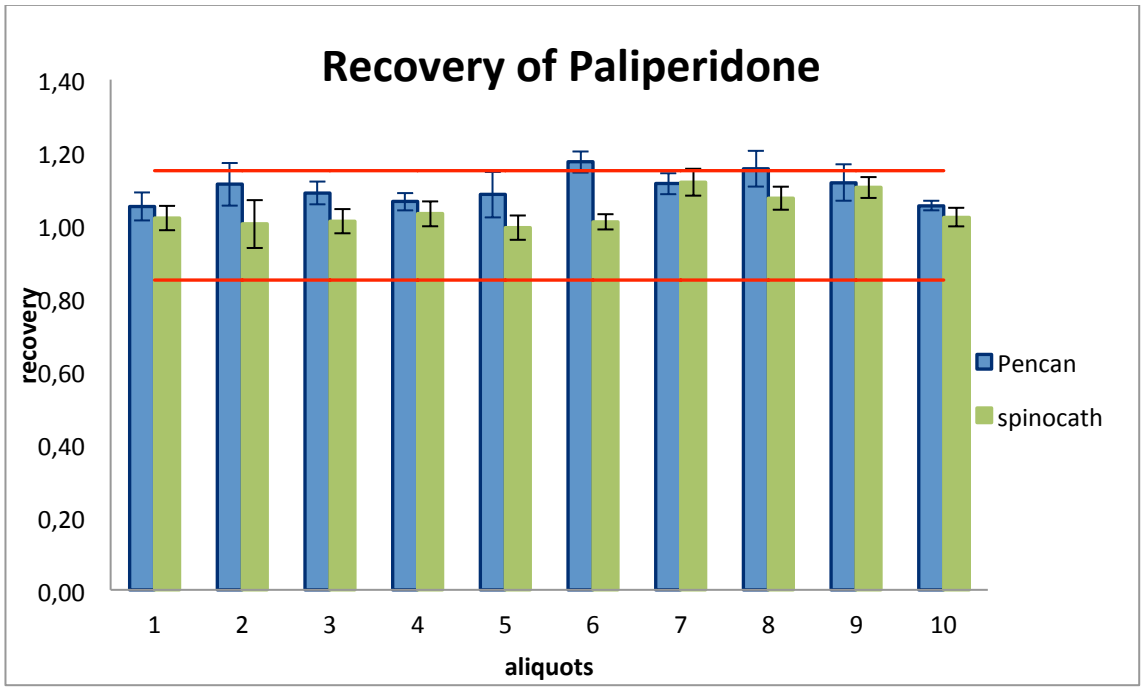

Figure 58: Recovery of Paliperidone in needles, aliquots of 500 µL. The red lines are the 15% limits.

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Richting: **master in de industriële wetenschappen: biochemie**
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Verbeke, Delphine

Datum: **13/06/2016**