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**Method development for quantification of prescription drugs  
Fluphenazine and Flupentixol in serum.**

**Bachelor Thesis**

by

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Publications: “An easy method for the quantification of cis-Flupentixol” Petra Gerhards, Luzia Schaaf, Agata Torzewska (see Attachment)

## **Abstract**

The LVR clinic in Viersen belongs to a group of nine psychiatric clinics in NRW, Germany. In the pharmacy laboratory, the therapeutic drug monitoring is offered for antiepileptic drugs (AED's), neuroleptics and antidepressants. Fluphenazine and Flupentixol are used to relieve the symptoms of schizophrenia and other similar mental health problems. The purpose of this work is the development and validation of methods for quantitation of the neuroleptics Fluphenazine and Flupentixol, contained in human serum. Therapeutic drug monitoring (TDM) is very important for a patient-matched therapy. TDM is applied to handle and direct the pharmacotherapy because of the pharmacokinetic differences between different individuals. The developed methods should be afterwards introduced into the daily routine of the laboratory. The analytical device used for this purpose is a Liquid Chromatography with a Mass Spectrometer (LCMS), with a Biphenyl Accucore separation column which is also used for other neuroleptics quantised. For Fluphenazine, an already existing in the laboratory sample precipitation method was used. Also, an already applied for other similar neuroleptics LCMS method, called "Biphenyl03\_new" (lasting for 11 minutes, with a high gradient between the elution agents) was taken on, and resulted in a good linearity. In case of Flupentixol, a completely new method had to be established, in order to achieve a baseline separation of cis-Z-Flupentixol and trans-E-Flupentixol. For the sample preparation, a solid phase extraction method was developed. The established methods were then applied for quantitation of patient samples, and the stability of the measurements was proved. Also, the accuracy and precision of obtained results was tested in the Proficiency Test. Limit of quantitation achieved for Fluphenazine was 0,5ng/ml, whereas for Flupentixol the LOQ was 0,25ng/ml. The Limit of detection found was 0,05ng/ml for both substances. The development was successful for both analytes and the established methods were integrated into the daily routine of the laboratory.

## Content

Abstract.....	I
1 Introduction.....	1
1.1 Description of the laboratory.....	1
1.2 Aim of the project.....	1
1.3 What is a psychosis, how do antipsychotics work?.....	2
1.4 Importance of Therapeutical Drug Monitoring (TDM).....	3
1.5 Description of the analytes .....	7
1.5.1 Key features of Fluphenazine .....	7
1.5.2 Key features of Flupentixol .....	8
2 Materials and Methods.....	10
2.1 Materials.....	10
2.1.1 LCMS.....	10
2.1.2 SOLA SPE Cartridges .....	16
2.1.3 TraceFinder Software .....	17
2.1.4 External control materials.....	17
2.1.5 Solutions, analytes and other materials.....	17
2.2 Methods.....	18
2.2.1 Method development of Fluphenazine .....	18
2.2.2 Method development of Flupentixol.....	20
3 Results.....	31
3.1 Selection of the solvent .....	31
3.2 Retention time determination .....	31
3.2.1 Measurement of methanolic solutions in defined concentrations using different methods.....	31
3.2.2 Selection of suitable Internal Standard Solution.....	33
3.3 Determination of measurement sensitivity .....	34
3.3.1 Limit of quantitation (LOQ) .....	34
3.3.2 Limit of detection (LOD).....	36
3.4 Linearity of the method .....	37
3.4.1 Five-point calibration Fluphenazine .....	38
3.4.2 Five-point calibration Flupentixol .....	38
3.5 In-day-variation .....	41
3.5.1 In-day-variation Fluphenazine.....	41
3.5.2 In -day-variation Flupentixol.....	42
3.6 Day-to-day-variation .....	43
3.6.1 Day-to-day-variation Fluphenazine .....	43

3.6.2	Day-to-day-variation Flupentixol .....	44
3.7	Participation in Proficiency Test (Round Robin Test) .....	45
4	Discussion .....	48
5	Conclusion .....	56
6	List of References .....	57
7.	Attachment.....	60
8.	Declaration of Authenticity .....	65

## List of abbreviations

<b>AGNP</b>	Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie
<b>EA</b>	Elution Agent “A”
<b>EB</b>	Elution Agent “B”
<b>EM</b>	Extensive Metabolizers
<b>FA</b>	Formic Acid
<b>GCMS</b>	Gas Chromatography with Mass Spectrometry
<b>GTFCH</b>	Society of Toxicological and Forensic Chemistry
<b>HESI</b>	Heated Electrospray Pressure Ionization
<b>HPLC</b>	High Performance Liquid Chromatography
<b>H<sub>2</sub>O</b>	Water
<b>IM</b>	Intermediate Metabolizers
<b>IS</b>	Internal Standard
<b>LOD</b>	Limit of detection
<b>LCMS</b>	Liquid Chromatography with Mass Spectrometry
<b>LOQ</b>	Limit of quantitation
<b>MeOH</b>	Methanol
<b>PM</b>	Poor Metabolizers
<b>RPC</b>	Reversed Phase Chromatography
<b>SPE</b>	Solid Phase Extraction
<b>TDM</b>	Therapeutic Drug Monitoring

## List of figures

Figure 1 Typical indications for measuring plasma concentrations of medications in psychiatry, for instance Elderly or adolescent patients and many more (Hiemke et al. 2011) .....	6
Figure 2 Structural formula of Fluphenazine (from: <a href="https://www.wikizero.com/de/Flupentixol">https://www.wikizero.com/de/Flupentixol</a> )	8
Figure 3 Two isomers of Flupentixol: upper - Z(cis)-form – active form of the substance, and lower - E(trans)-form – the spatially favoured form (from: <a href="https://de.wikipedia.org/wiki/Flupentixol">https://de.wikipedia.org/wiki/Flupentixol</a> ) .....	9
Figure 4 Schematic structure of a High Performance Liquid Chromatography analytical device with the elution agents, mixing chamber, injector, separating column, detector and the computer shown (from: <a href="https://www.youtube.com/watch?v=B1xGXSuAnmQ">https://www.youtube.com/watch?v=B1xGXSuAnmQ</a> ) .....	12
Figure 5 Calibration curve for Flupentixol on Hypersil Gold column, with only one signal for both isomers of Flupentixol .....	13
Figure 6 Calibration curve for Flupentixol on the Biphenyl column with a baseline separation of signals of Cis- and Trans-Flupentixol .....	13
Figure 7: Electrospray ionization-basic principle, showing the ESI needle, Taylor Cone, the mechanism of solvent evaporation and ion desolvation and the Ion transfer tube (from: “Exactive operations course” Unity Lab Services).....	14
Figure 8 Scheme of an Orbitrap mass spectrometer, beginning with the sprayer, through different lenses to deflector and secondary electron multiplier (Perry, Cooks and Noll 2008)...	15
Figure 9 Scheme of an Orbitrap, showing among other the split lens and the Ion transfer tube (Unity Lab Services “Exactive Operations Course”).....	16
Figure 10 LCMS Biphenyl method - 90% Elution agent A and 10% Elution agent B as starting concentration, 11minutes long – very steep curve, high percentage difference between solvents .....	19
Figure 11 Linearity of a serum sample mixed directly in the SPE cartridge, non-satisfactory results can be seen.....	21
Figure 12 Linearity of a serum sample mixed before the application into the SPE cartridge, a perfect linearity of the results can be seen.....	21
Figure 13 Sample preparation (five steps) method used for samples containing Flupentixol (Gerhards, Schaaf, Torzewska 2021) .....	24
Figure 14 Final method called "Flupentixol" with starting condition 64% EA and 36% EB, the percentage difference between the Elution agents smaller than in the initial method.....	25
Figure 15 LC method initial - not changed. In the graph it is visible that the curve is pretty steep, since the beginning condition is 90% Elution agent A and 10% Elution agent B; also, the method is 11minutes long. (optimal time for most samples). .....	27
Figure 16 LC method - first change trial. The gradient is smaller because the percentage of Elution agent A is set to 70% and of Elution agent B to 30% in the beginning conditions (the percentage between them gets smaller). .....	28
Figure 17 Second change trial. In the second trial the method is set longer. A single sample run takes now 14minutes instead of initial 11 minutes. ....	29
Figure 18 Third trial, the method is set to longer with the gradient 70% EA and 30% EB.....	30
Figure 19 Limit of quantitation for Fluphenazine 0,5ng/ml .....	35
Figure 20 Limit of quantitation for Flupentixol - 0,25ng/ml after adjustments of the method ...	36
Figure 21 Fluphenazine 0,025ng/ml in buffer solution, concentration too low .....	36
Figure 22 Fluphenazine 0,05ng/ml in buffer solution - limit of detection .....	37
Figure 23 Flupentixol 0,05ng/ml in buffer solution, limit of detection .....	37
Figure 24 Five-point calibration of Fluphenazine with R-value on top of the figure, indicating a very good linearity .....	38
Figure 25 Assumed rearrangement of Flupentixol taking place in Methanol and Formic acid. On the left-hand side, the Trans-Flupentixol can be seen, whereas on the right-hand side the Cis-Flupentixol (Gerhards, Schaaf and Torzewska 2021).....	39

Figure 26 Chromatogram of an external control material measured directly after sample preparation, only one signal of the active form of the substance (Z-Flupentixol) is visible in the chromatogram .....	40
Figure 27 Chromatogram of the same external control material (containing initially only Z-form) measured 48 hours after sample preparation, two signals showing both Flupentixol isomers are visible.....	40
Figure 28 Flupentixol 5-point calibration of five samples: K1 to K5, one run each, the results are stable and precise .....	41
Figure 29 Flupentixol 5-point calibration for ten samples: K1 to K5, run 10 times each, standard deviation between the results increasing with increasing time .....	41
Figure 30 Round Robin Test evaluation of two samples (A and B) containing Fluphenazine showing the result the laboratory got and a comparison among other laboratories, for the sample A, the result obtained was exactly the same as the target value, in case of sample B the result obtained was only slightly different from the target value (Society of toxicological and forensic chemistry) .....	46
Figure 31 Round Robin Test evaluation of two serum samples containing Flupentixol, both obtained by the laboratory results were very precise, with a slight difference from the target value, namely 0,14 (Society of toxicological and forensic chemistry) .....	47
Figure 32 Results of the Round Robin Test for Fluphenazine, values higher than allowed circled in red, number of results included for sample B much higher than for sample A (Society of toxicological and forensic chemistry) .....	60
Figure 33 Summary of the results of Round Robin Test, only 65% success rate for Fluphenazine, sample A, whereas 95% success rate for sample B, Fluphenazine (Society of toxicological and forensic chemistry) .....	60
Figure 34 Flupentixol - patient sample with a ratio almost like in a pill, around 60% to 40% trans- to cis.....	61
Figure 35 Flupentixol- patient sample with a high difference in ratio between two isomers, proportion of trans-form much higher than cis-form. ....	61
Figure 36 Example - Flupentixol one of the day-to-day measurements .....	62
Figure 37 Example - one of the day-to-day measurements for Fluphenazine.....	62
Figure 38 Example - the in-day measurement of Flupentixol.....	63
Figure 39 Package leaflet information - External Control Material - Neuroleptics 2, Level I ....	63
Figure 40 Package leaflet information - External Control Material - Neuroleptics 2, Level II...	64



## List of tables

Table 1 Summary of various solutions used for the second washing step .....	23
Table 2 Summary of different solutions used to fill the concentrated sample .....	24
Table 3 Method adjustment for Flupentixol samples - different settings with flow rates .....	30
Table 4 Concentrations of the calibration standards for both analytes Fluphenazine and Flupentixol.....	38
Table 5 Values for In-day-variation Fluphenazine, see Attachment for more detailed example of the results .....	42
Table 6 In-day-variation-Flupentixol, see Attachment for more detailed example of the results	43
Table 7 Day-to-day-measurements Fluphenazine, see Attachment for more detailed example of the results .....	44
Table 8 Day-to-day values - quantification of Flupentixol samples, see Attachment for more detailed example of the results .....	45

# **1 Introduction**

## **1.1 Description of the laboratory**

The method development is done at a pharmacy laboratory, which is part of the LVR-Clinic in Viersen. The LVR Clinic is a psychotherapeutic specialized clinic, that is aimed to help people with mental disorders, drug addictions etc. Therefore, this pharmacy laboratory deals mostly with pharmaceutical analytics, for instance: analysis of unknown samples, analysis for drugs of abuse in urine samples and quantitation of medical drugs in blood samples, using chromatographic procedures. Moreover, it checks the stability and best storage possibilities of drugs that are produced in the pharmacy for the patients of the hospital.

For these various purposes, different analytical instruments are used: GCMS (Gas Chromatography with a Mass Spectrometer), HPLC-PDA (High Pressure Liquid Chromatography (used for separation) Photometric Diode Array (for detection of substances)) and LCMS Orbitrap (Liquid Chromatography with a Mass Spectrometer and an Orbi-Trap).

## **1.2 Aim of the project**

The purpose of this work is to develop and validate a method for the quantitation of two pharmaceutical drugs: Flupentixol and Fluphenazine. The aim of the method development is achieved by analysing the chemical and physical features of both analytes, choosing an optimal solvent solution, determining the retention time and comparison of different methods, by measurements of methanolic solutions. The validation of the newly established method is done by the determination of measurement sensitivity, as well as linearity of the method and stability between different measurements, and participation in the Round Robin Test. For these purposes, a liquid chromatography with mass spectrometry (LCMS) analytical instrument is used. A high performance liquid chromatography (HPLC), which is also available in the laboratory, is not suitable, since the UV detector is not sensitive enough, to detect trace amounts of the substance. LCMS is able to detect a wider range of drugs and their metabolites, as well as simplify the sample preparation. (Pitt J. J. 2017)

Customer demands, time cost and economic reasons are crucial factors that lead to a

method development for quantitation of prescription drugs. The laboratory gains with time more clients and there are plenty of new drugs which are prescribed to patients. Furthermore, sending the samples to an external laboratory takes around 3 to 4 days up to one week to get the results of analysis for these two substances, because sometimes, the external laboratory does the quantification only once a week. Optimally, the results of an analysis should be available within 24 hours, one week between the day the sample was taken from the patient and examined is too long for an effective pharmacotherapy. For this reason, it is necessary for the laboratory to switch to a faster and cheaper measuring method since, with increasing number of new clients, the number of samples also increases. Additionally, the analytical laboratory at LVR Clinic cares a lot about their patients, highest quality and certainty of evaluation. For Fluphenazine, the external laboratory, where the samples were sent for analysis before a method development was done, has set the limit of quantitation for its measurements at 0,7ng/ml, but it is more professional to set it at 50% of the lower therapeutical range, so at 0,5ng/ml. This value was set in the very beginning as a target value for the limit of quantitation in the measurements done. Similarly, for Flupentixol, more precise data is desired. Since the external laboratory quantifies only the sum of E- and Z-isomer, but the active substance is only the Z-isomer, it is of high value to establish a method for exact measurement of cis-form separated from trans-form.

### **1.3 What is a psychosis, how do antipsychotics work?**

Psychosis is told to occur when a person loses some contact with reality, it might be for instance seeing or hearing things other people cannot see or hear, or believing things that are actually not true; severe behavioural abnormalities occurring. (NHS, 2019, *Overview Psychosis*) “Psychosis is a common response to wide variety of neurological insults, but also a common syndrome occurring in primary psychiatric disease.” (Cardinal RN, Bullmore ET, 2011) It can be caused by a psychological condition (mental illness) or by a medical condition, alcohol or drug misuse.

The two main symptoms of psychosis are hallucinations (a person hears and sees things that do not exist in the real world; sometimes even feel something very real) and delusions (a person has strong beliefs which are not shared by other people).

Psychological causes of this disease, which can influence the type of psychotic occurrence are:

- Schizophrenia – a mental health condition which causes hallucination and delusions
- Bipolar disorder – possibility of episodes of low mood and highs
- Stress or anxiety
- Depression – persistent feeling of sadness
- Lack of sleep

Antipsychotic medicines are most commonly used and recommended as first treatment. These are a mainstay in the illness treatment, since it is proven that they reduce psychotic symptoms and relapse rates among schizophrenia patients. Antipsychotic drugs work by blocking the effect of dopamine – a neurotransmitter, one of chemicals that brain uses to transmit messages from cell to cell. (NHS, 2019, *Causes - Psychosis*) Dopamine transmits the impulses between different nerve cells. The antipsychotics block dopamine receiving points (D-receptors) and so reduce the activity of those nerve cells, which use dopamine to transmit the information. Newer drugs can also block other receptors (for instance serotonin receptors). (Neurologen und Psychiater im Netz, *Psychopharmaka: Antipsychotika*) Lots of patients receiving these drugs, benefit from it as the symptoms are reducing. However, there is a high percentage of patients suffering from side effects, negative symptoms and a progressive brain tissue volume loss. Possible side effects are: drowsiness, shaking, weight gain, restlessness, muscle twitches, blurred vision, dizziness, loss of sex drive. (van Haren et al. 2008)

Antipsychotics may be taken by mouth (orally) or as an injection, which can be either - in case of acute psychosis - injected intravenous (in order for a fast effect) or in case of chronic disease – intramuscular (in order for a long- time effect), called depot. The depot contains so called “slow-release” medicines, which allow the patient to take the injection every 1 to 4 weeks.

#### **1.4 Importance of Therapeutical Drug Monitoring (TDM)**

Therapeutic drug monitoring (TDM), so assessment of the concentration of the medication dose for instance in serum, is very important for a therapy, that is patient-

matched. It may be successfully employed in order to guide drug therapy in clinical routine, since it provides all the information from laboratory results about the drug concentration in patient's blood specimen. (Haen E et al. 2008) TDM is based on the assumption, that there is a relationship between plasma concentrations and clinical effects. The "therapeutic window" (also called "therapeutic reference ranges") is defined as the range of drug concentration in plasma of maximal effectiveness and safety. These ranges specify the lower limit, below which a response caused by the drug is pretty unlikely to occur, and upper limit above which the probability of enhancing therapeutic improvement is relatively low.

The most important reason to use TDM for handling and directing the psychopharmacotherapy, is the interindividual variability of pharmacokinetic properties of patients, since the drugs can be easily under- or overdosed. Furthermore, psychopharmaceuticals have often strong side effects, which can be excluded or minimised with help of TDM. This valuable tool uses the quantification of drug concentrations in the sample to define the dose of individual patients in order to obtain a low risk of toxicity and highest tolerability and probability of response.

Psychopharmacotherapy requires a special patient dosage, which is connected to individual characteristics. Non-response at therapeutic doses, uncertain drug adherence, suboptimal tolerability or pharmacokinetic drug-drug interactions are just few examples of situations where TDM is helpful. Moreover, TDM could also improve the cost-effectiveness of psychopharmacotherapy. Groups of patients that can primarily benefit from TDM are for example children, pregnant women or elderly patients. However, in order to obtain the potential benefits of TDM the method has to be appropriately combined with the therapy. In 2004, Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie (AGNP) has published guidelines for TDM. There are 4 levels of recommendation for using TDM:

- *Level 1: Strongly recommended*

Controlled clinical trials have shown beneficial effects of TDM, reports on decreased tolerability or intoxications.

TDM is strongly recommended for dose titration and for special indications.

- *Level 2: Recommended*

Reports on decreased tolerability or intoxications at "supratherapeutic"

plasma concentrations.

TDM is recommended for dose titration and for special indications or problem solving, it will increase probability of response in “non-responders”.

- *Level 3: Useful*

Plasma concentration related to pharmacodynamic effects are either not yet available or based on retrospective analysis of TDM data.

TDM is useful for special indications or problem solving.

- *Level 4: Potentially useful*

Plasma concentrations do not correlate with clinical effects due to unique pharmacology of the drug.

TDM is not recommended for dose titration, but may be potentially useful for special indications or problem solving.

TDM has enormous advantages in psychopharmacotherapy, because it is a valuable tool for adapting the dosage of prescribed medications to individual characteristics of the patient, since the pharmacokinetic properties change from patient to patient. Due to age, disease, genetic peculiarities etc. the ability to metabolize, absorb, distribute or excrete drugs may differ between individuals. Many psychotropic drugs are used as racemic compounds, where their enantiomers differ significantly in their pharmacokinetics, metabolism and pharmacology. Most of the drugs are metabolised by the liver, only some of them in extrahepatic tissues like intestinal mucosa or the brain. According to Hiemke (2011) “Most psychotropic drugs undergo Phase-I metabolism by oxidative, reductive or hydrolytic reactions. [...] The Phase-I reactions are predominantly catalysed by cytochrome 450 (CYP) enzymes which comprise more than 200 isoenzymes.” However, due to genetic peculiarities and considering the pharmacokinetics, there are also so-called poor metabolizers (PM) – they lack functional alleles (which determine how much of enzyme is being expressed), intermediate metabolisers (IM) - are either genetically heterozygous, carrying an active and an inactive allele (or an allele with reduced activity) or have 2 alleles with reduced activity, extensive metabolisers (EM) are wild type with 2 active alleles, and ultra-rapid metabolisers (UM) have an amplification of functional alleles. (Hiemke C. et al. 2011), (Evans WE, Relling MV 1999), (Antwerpes S, Blaschke J. 2018)

Poor metabolizers practically cannot metabolize the drug which potentially leads to

strong side effects or even toxicity, whereas extensive metabolizers, metabolize the drug quickly so that given a normal dosage, there are no detectable levels of the drug in serum sample and no effects. Both examined substances, Fluphenazine and Flupentixol involve the enzyme CYP2D6 in their metabolism.

In the LVR Clinic, if a patient is given a normal dose of the drug and the results of chromatography are abnormal (no impact of the drug can be seen, or higher than recommended concentration of the substance is present in the serum) then a so-called genotyping is done. Genotyping means a process of determining the differences in genotype of a person, which is done by examining the DNA material and comparing it to reference sequences. (Mayo Clinic 2018) This is of high importance for pharmacists and enables prescription of optimal drugs for every individual.

Furthermore, in a psychotherapy, compliance plays important role. Compliance is defined as a cooperative behaviour in terms of therapy and is in many cases hard to achieve since patients with mental disorders often do not notice they are sick. TDM helps to improve it. Nonetheless, inappropriate use of TDM may lead to waste of laboratory resources and brings the risk that misleading results could negatively influence the clinical decision making which has to be taken into consideration. (Hiemke et al. 2011)

- Dose optimization after initial prescription or after dose change
- Drugs, for which TDM is mandatory for safety reasons (e. g., lithium)
- Suspected complete or partial non-adherence (non-compliance) to medication
- Lack of clinical improvement under recommended doses
- Adverse effects and clinical improvement under recommended doses
- Combination treatment with a drug known for its interaction potential or suspected drug interaction
- TDM in pharmacovigilance programs
- Relapse prevention under maintenance treatment
- Recurrence under adequate doses
- Presence of a genetic particularity concerning drug metabolism (genetic deficiency, gene multiplication)
- Pregnant or breast feeding patient
- Children and adolescent patient
- Elderly patient (> 65 y)
- Individuals with intellectual disabilities
- Patients with pharmacokinetically relevant comorbidities (hepatic or renal insufficiency, cardiovascular disease)
- Forensic patient
- Problems occurring after switching from an original preparation to a generic form (and vice versa)

Figure 1 Typical indications for measuring plasma concentrations of medications in psychiatry, for instance Elderly or adolescent patients and many more (Hiemke et al. 2011)

## 1.5 Description of the analytes

### 1.5.1 Key features of Fluphenazine

Fluphenazine is a pharmaceutical drug belonging to the group Phenothiazines. This tricyclic neuroleptic contains a piperazinyl side chain. Fluphenazine works through blocking of dopamine receptors and decreases thereby the effect of dopamine as a transmitter substance, it shows a high affinity for D2-receptors.

It is applied for long-term treatment and for relapse prevention of certain mental diseases, for instance schizophrenic psychosis. Fluphenazine has a sum formula  $C_{22}H_{26}F_3N_2OS$ , with the exact mass of 438.18214g/mol. It's therapeutic reference range lies between 1ng/ml to 10ng/ml (Hiemke et al. 2011). This drug is available as salt, Fluphenazine dihydrochloride, sum formula  $C_{22}H_{28}Cl_2F_3N_2OS$  and the exact mass of 510.44g/mol. The substance is easily soluble in water, poorly soluble in Ethanol 96% and Dichloromethane. The solutions should be protected from light. (Siebert 2018)

The drug is produced in Germany under the name Fluphenazine-neuraxpharm by Neuraxpharm Arzneimittel GmbH.

Very common side effects of this drug are: dry mouth, increased heart rate, low blood pressure, tiredness, tongue and throat cramps, jaw muscle cramps, difficulty seeing objects clearly, eye cramps, decreased facial expressions, inability to move, inability to remain seated, urge to move, circulatory regulation disorders when sitting up, standing up. Common side effects: weight gain, increased liver values, muscle stiffness, kidney failure and liver dysfunction, Parkinson's symptoms, epilepsy attacks, unusual facial expressions, breast formation in men, increased sensitivity to light. (Bristol-Myers Squibb Srl 2017), (Siebert 2018)



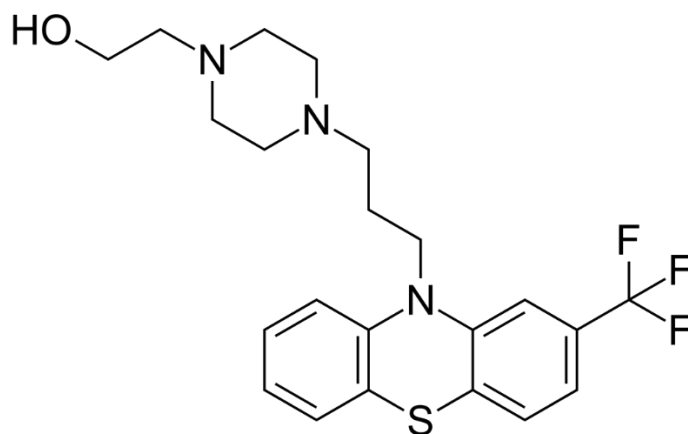


Figure 2 Structural formula of Flupentixol (from: <https://www.wikizero.com/de/Flupentixol>)

### 1.5.2 Key features of Flupentixol

Flupentixol is an alkene with an asymmetrically substituted double bond. There are two isomeric forms: Z-isomer (cis-isomer) and E-isomer (trans-isomer). Both isomers have different physical, chemical and pharmacological properties. The Z-isomer is the more active form of Flupentixol than the E-isomer, and this is the reason why it is more often applied in medicine; however, tablets include both isomer forms. The Z-isomer blocks the D1- and D2- receptors.

Flupentixol has a sum formula  $C_{23}H_{25}F_3N_2OS$ . Its therapeutic reference range lies between 1ng/ml to 10ng/ml for both isomers, however for cis-form it is 0,5 to 5ng/ml. (Hiemke et al. 2011). Flupentixol has exact mass of 435.17125g/mol. (Siebert 2018)

It is available as a salt, Flupentixol dihydrochloride salt (of chemical formula  $C_{23}H_{27}Cl_2F_3N_2OS$  and exact mass of 507,44g/mol) which is easily soluble in water, soluble in Ethanol 96% and almost not soluble in Dichloromethane. The drug is sensitive to UV light, so every solution of the substance should be stored in a brown-glass bottle. (Siebert 2018)

Flupentixol is available in form of tablets produced in Germany under the name Fluanxol, produced by Bayer AG; and also in form of a Depot (as an intra-muscular injection).

Fluanxol is a neuroleptic drug, a derivation of Thioxanthene. It is used for treatment of certain diseases of central nervous system and mental disorders, for patients with a schizophrenic psychosis. The active ingredient of the drug is Flupentixol as dihydrochloride. There are three dosage ranges available for the conducted

measurements, 0.5mg, 1.0mg and 5.0mg. Side effects depend on the dosage height as well as other accompanying diseases. (Beipackzettel - Flupentixol)

A Depot is another form of medication in form of an intramuscular injection. It has many advantages in psychiatry, for instance: injection is used because of easier application, since not all patients are able to take their medicines in oral way regularly, depot as a long term-medication with injection intervals between 1 and 4 weeks is a better option. Also, the risk of relapse is smaller compared to oral medication. Depot contains only the active form, so 100% of the Z-form.

The antipsychotic effect of neuroleptics is believed to be related to their dopamine receptor blocking effect. The thioxanthenes have high affinity for D1 and D2 receptors.

Very common side effects of this drug are: tongue and throat spasms, jaw muscle spasms, torticollis, back muscle stiffening, gaze cramps, reduced facial expressions, muscle stiffness, sedentary lifestyle, urge to move, circulatory regulation disorders, drop in blood pressure, palpitations, tiredness. Frequent side effects are: dry mouth, visual disturbances, diarrhoea, loss of appetite, vomiting, increased pressure in the eye.

(Beipackzettel - Flupentixol)

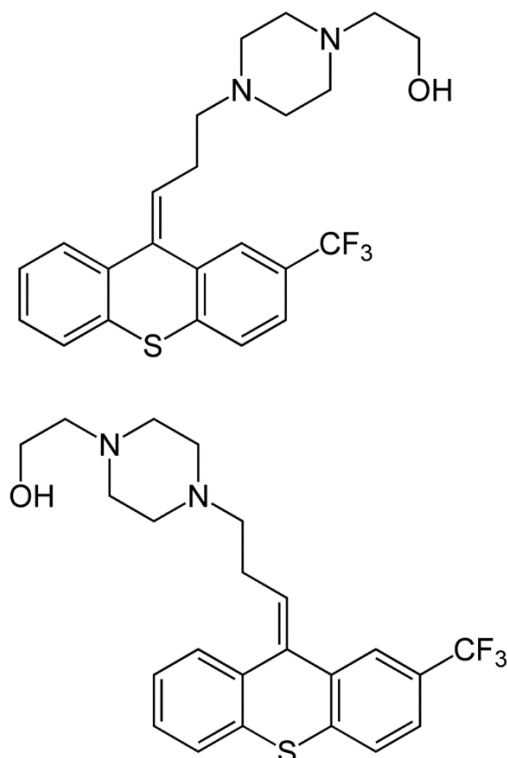


Figure 3 Two isomers of Flupentixol: upper - Z(cis)-form – active form of the substance, and lower - E(trans)-form – the spatially favoured form (from: <https://de.wikipedia.org/wiki/Flupentixol>)

## **2 Materials and Methods**

### **2.1 Materials**

For a Therapeutical Drug Monitoring of psychotropic drugs, chromatographic analytical instruments such as High-Performance Liquid Chromatography, Gas Chromatography and Liquid Chromatography with Mass Spectrometry (optionally with an Orbitrap) are used. These are precise, accurate and robust enough for the analysis.

Chromatography describes processes that aim to separate mixtures of substances and detect and quantitatively determine the individual components. All chromatographic processes require two immiscible substances, which are known as phases. One of them, called “Stationary phase” is often located in the separation column. With help of the second, “Mobile phase” the mixture to be separated will be transported through the Stationary phase. (Rücker, Neugebauer, Willems 2001)

#### **2.1.1 LCMS**

The Exactive Plus mass spectrometer with an Orbitrap detector with an ionisation method called Heated Electrospray Pressure Ionization (HESI) source for liquid chromatography (LC) mass spectrometry (MS) with high throughput applications, which brings a powerful capability of a wide range of many applications. For instance, it allows analysis of complex substances with various components. Furthermore, it offers highest levels of accuracy and precision. (Makarov 2012)

#### **Liquid chromatography part (LC)**

Liquid chromatography is a special form of column chromatography where pressure is applied. It serves to separate mixtures of substances as well as their qualitative and quantitative analysis. With help of pumps, the eluent flows through the separation column (which is filled with the stationary phase). A sufficient flow speed of the mobile phase (obtained through gravitational forces) is here required. The separation efficiency of the column depends on the particle size and the particle size distribution of the stationary phase. The smaller and more evenly shaped the particles, the greater the separation performance. (Rücker, Neugebauer, Willems 2001) The time that a substance needs to flow through the column longer than a mobile phase is called the retention time. The retention time is characteristic for the substance since the strength of

interactions depends on the properties of examined substance, the elution agent and stationary phase. Thus, the weaker the adsorption to the stationary phase and the greater the affinity of the substance to the mobile phase, the sooner the substance will leave the column, so the earlier a signal in the detector will occur. In this way single components of a mixture of more unknown substances can be distinguished from another according to their properties like for instance molar mass or polarity.

This analytical instrument consists of: two elution agents (a polar and nonpolar), pumping system with two pumps, a gradient-mixer, a sample inlet system, separating column, detector, recorder, integrator. (Rücker, Neugebauer, Willems 2001)

There are high requirements about the eluents. These must be very pure, of high quality and must not cause any interference and any disturbing signals in the detector. The first polar elution agent, called simply “Elution agent A” consists of 1L H<sub>2</sub>O, 0,5ml Formic acid and 100ml MeOH. It is mixed with the second, nonpolar “Elution agent B” which contains 0,1% Formic acid in MeOH, the proportion of agent A and B is changed over time. These solutions must be first degassed before they can be used by the instrument and are exchanged every three days. The mixture of these two agents improves the separation and the added Formic acid protonates the analytes before they enter the separating column. Interference signals may also be caused by the air in the eluents or in the tubes. Air can get in the tubes while changing to a new bottle of eluent. This difficulty can be overcome for example by putting the affected parts into an ultrasonic device. The system used for this study includes a degasser.

In the figure shown below, a scheme of a High Performance Liquid Chromatography analytical device can be seen. The graphic shows two elution agents (polar and nonpolar), a mixing chamber, injector, the separating column, detector and a computer.

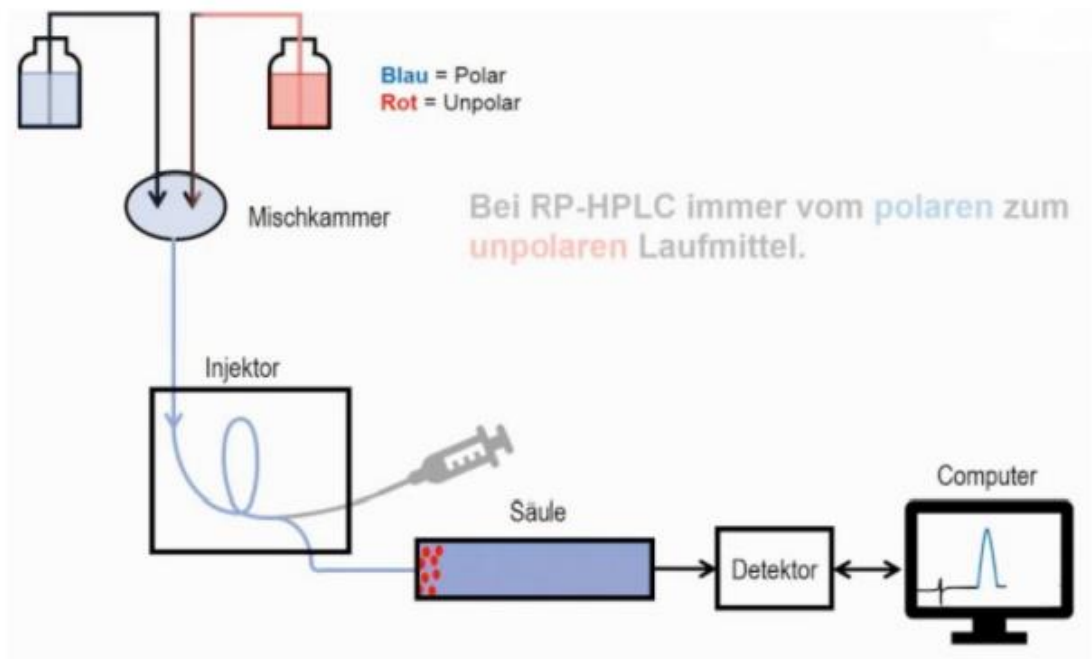


Figure 4 Schematic structure of a High Performance Liquid Chromatography analytical device with the elution agents, mixing chamber, injector, separating column, detector and the computer shown (from: <https://www.youtube.com/watch?v=B1xGXSuAnmQ>)

The pumps have to deliver pressures up to 300 or even 400 bar and enable constant, continuous flow rates of 0,1 to 10ml/min without any pulsations which could again affect the signal. (Rücker, Neugebauer, Willems 2001)

An optimal separation of substances cannot always be achieved with a fixed mixture of eluents, in most cases the gradient between elution agents A and B has to be changed during the process in order to for best results.

As stationary phase, so called “Accucore Biphenyl Column” was used. Since both analysed substances contain aromatic rings as well as sulphur groups, analysis of these compounds could be problematic using a traditional alkyl column (C18 carbon chains). A biphenyl chemistry offers complementary selectivity to a C18 column, and the ability to resolve isomeric compounds, such as drugs of abuse and steroids by taking advantage of  $\pi$ - $\pi$  and polar interactions between the analyte and stationary phase (Makarov 2012). Because of these properties the biphenyl is the column of choice for this application, while mechanism based on C18 does not provide the necessary separation capacity. The length of the column is 100mm, the diameter is 2,1mm and the particle size 2,6 $\mu$ m. The flow rate for this column is 0,3ml/min and the sample injection volume 5 $\mu$ l. Mixtures of

water or aqueous buffer and organic solvents are used to elute the analytes from the column. (Mehta 2012), (Molnár, Horváth 1976), (Thermofischer Scientific, *When to use biphenyl selectivity?*)

Most laboratories use the C18 column in Reversed Phase Chromatography (RPC). However, using the same sample preparation method and the same LC method on this column, the isomers of Flupentixol sample eluted at the same retention time (sum of both Z and E forms), so as a result there was only one signal in the chromatography, whereas with the biphenyl column two signals occurred (separate signal for each isomer). This observation has proven that there is an isometry occurring at the double bond since the phenyl rings of the column interact with the  $\pi$ -bonds of the analyte. Because the laboratory aims to deliver as accurate data as possible to the doctors and furthermore to the patients, this column was chosen.

For comparison, graphs of calibration curves of Flupentixol using a Biphenyl column and a C18 column are shown below. As it can be observed, the biphenyl column provides a basis line separation between the two peaks for Z and E-isomer.

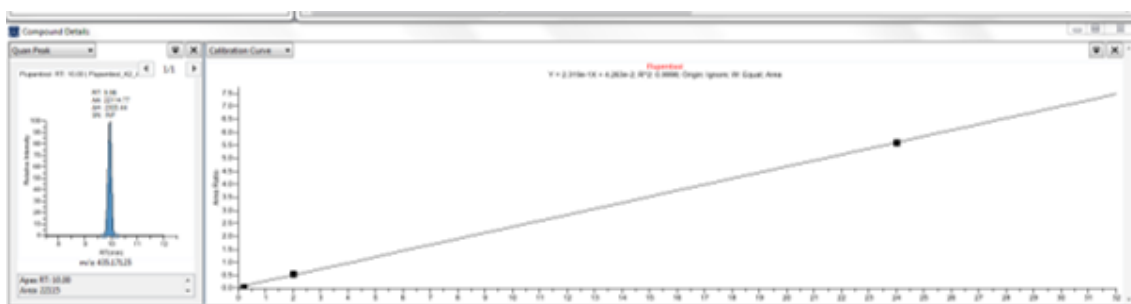


Figure 5 Calibration curve for Flupentixol on Hypersil Gold column, with only one signal for both isomers of Flupentixol

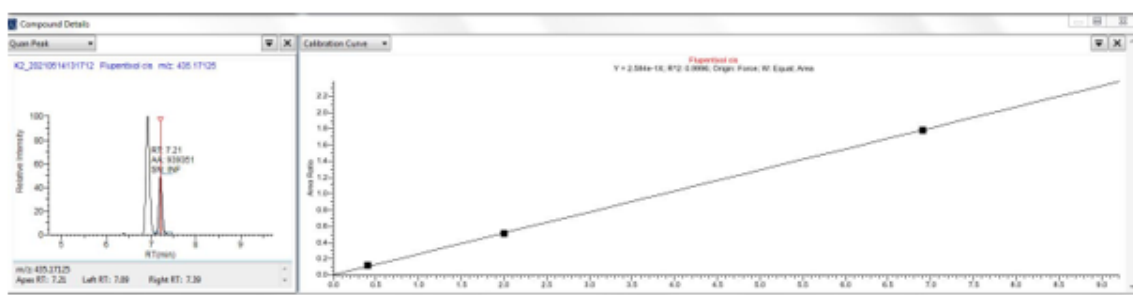


Figure 6 Calibration curve for Flupentixol on the Biphenyl column with a baseline separation of signals of Cis- and Trans-Flupentixol

## HESI Source of ions

After leaving the separation column the substances must be detected to get signals in the chromatogram. The Heated Electro Spray Ionisation (HESI), in which ions are transferred from liquid to gas phase, is the source of ions. Higher temperatures achieved through heating give the ability of higher flow rates and higher sensitivity. Samples can be introduced to the ESI source at the front side by a variety of methods, for example direct infusion via a syringe pump.

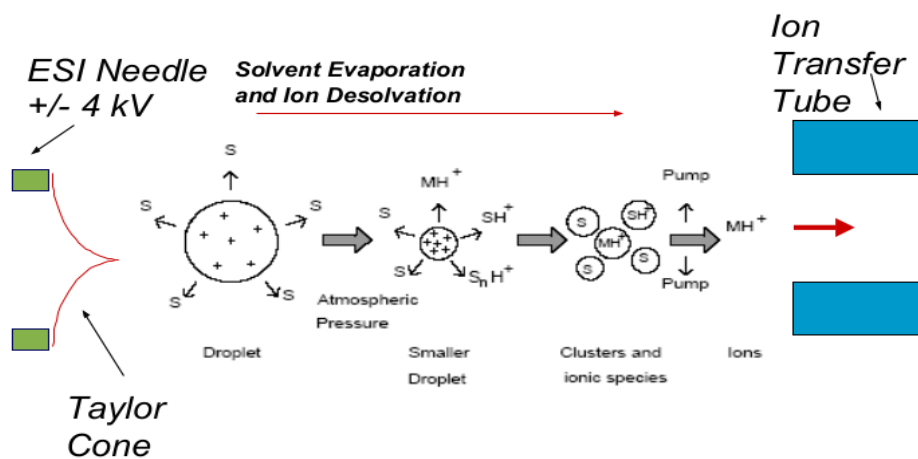


Figure 7: Electrospray ionization-basic principle, showing the ESI needle, Taylor Cone, the mechanism of solvent evaporation and ion desolvation and the Ion transfer tube (from: "Exactive operations course" Unity Lab Services)

Electrospray is a soft ionization process used to transfer ionized species from liquid solutions into the gas phase. The sample solution is sprayed from a region where it is contact with high voltage (+/- 3 to 5 kV typically), where excess charges are imparted upon droplets which emerge at the end of the sample tube.

In ESI ions are produced and analysed as follows: Sample solution enters the ESI needle, to which a voltage is applied. Then, the ESI needle sprays the sample solution into a fine mist of droplets that are electrically charged on their surface. The electrical charge density at the surface of the droplets increases as solvent evaporates from the droplets, and it increases to a critical point (known as Rayleigh stability limit). At this point, drops divide into even smaller droplets, because the electrostatic repulsion is greater than the surface tension. The process is repeated many times to form very small droplets. Last, but not least from the very small, highly charged droplets, sample ions

are ejected into the gas phase by electrostatic repulsion. The sample ions pass through an ion transfer capillary and enter the MS detector. (Unity Lab Services)

## Mass spectrometry (MS)

After the ions have left the Heated Electro Spray Ionization source, they are transferred into the C-Trap through four stages of differential pumping. In the C-Trap, ions are accumulated and their energy dampened using a bath-gas (nitrogen). The ions are then injected through three further stages of differential pumping using a lens system (Z-lens) into the orbitrap analyser where mass spectra are acquired via image current detection. This can be seen in a following figure. (ThermoFischer Scientific 2012)

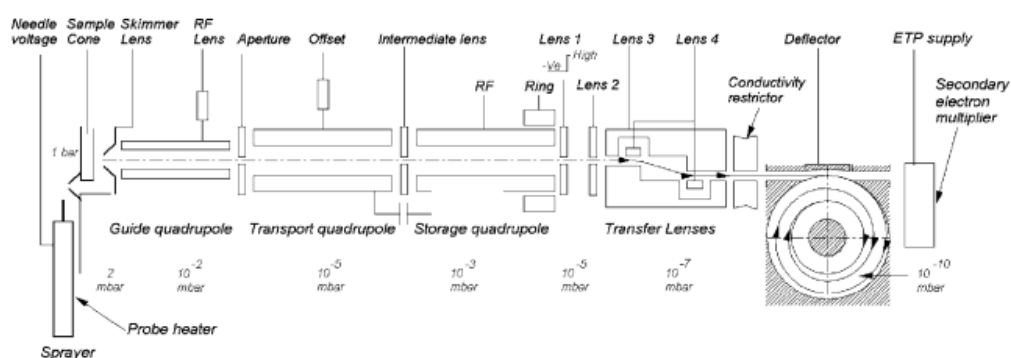


Figure 8 Scheme of an Orbitrap mass spectrometer, beginning with the sprayer, through different lenses to deflector and secondary electron multiplier (Perry, Cooks and Noll 2008)

The outer electrodes create a potential barrier so that the ions cannot leave the trap. Due to the decentralised inlet these oscillate laterally along the electrode axis. This movement is measured as a current by the outer electrodes, which also serve as detectors. The oscillation is inversely proportional to the square root of the  $m/z$  ratio. These multiple ions in the Orbitrap generate a complex signal whose frequencies are determined using a Fourier Transformation

$$\omega = \sqrt{\frac{k}{m/z}}$$

Using this formula, mass ( $m$ ) of the analyte can be calculated. The vibration frequency ( $\omega$ ), charge of the particles ( $z$ ) and the field curvature constant ( $k$ ) are used for this calculation. (Rücker, Neugebauer and Willems 2001)



## “Gate Lens” Open/Closed

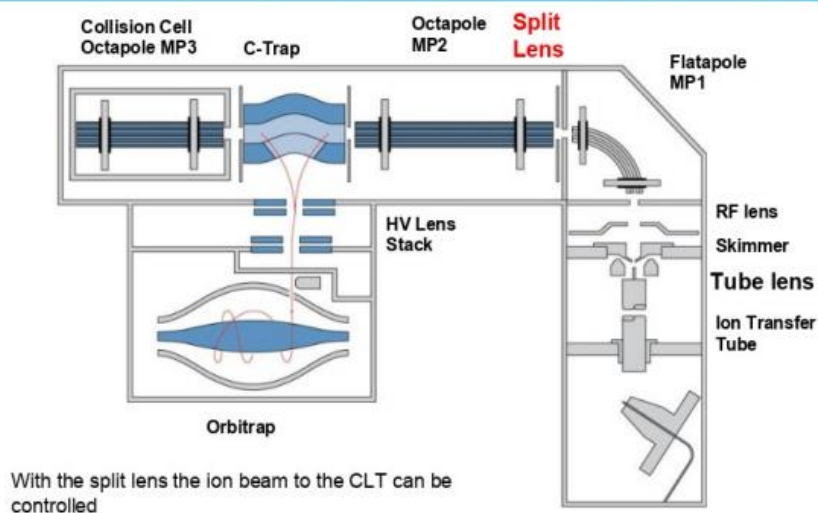


Figure 9 Scheme of an Orbitrap, showing among other the split lens and the Ion transfer tube (Unity Lab Services “Exactive Operations Course”)

This analytical instrument has actually relatively simple design and high space charge capacity, which makes it suitable for tackling complex scientific problems in which high performance is required. The Orbitrap has proven to be a robust mass analyser that can routinely deliver high resolving power and mass accuracy. Because Orbitrap technology provides quantitative and qualitative high-resolution and accurate-mass data analytical capability in a single platform and often in a single run, it has been increasingly applied towards a range of challenging applications requiring high selectivity and sensitivity. (Perry, Cooks and Noll 2008)

### 2.1.2 SOLA SPE Cartridges

For solid phase extraction (SPE) cartridges designed and produced by Thermo Scientific company are used. These have unique fritless structure (no need for ceramic frits to hold the sorbent material) which brings many advantages, for instance: significantly increased reproducibility, higher levels of extract cleanliness, reduced solvent requirements and increased sensitivity as well as reduced failure rates and prevention of sample failures. (ThermoFischer Scientific 2020) SPE cartridges are made of polymer material with a cationic ion exchanger.

### **2.1.3 TraceFinder Software**

To quantify the measurements and acquired data, a TraceFinder software is used. Its' relatively simple operation, flexible data review and report generation make it very efficient, and simplify the sample analysis. TraceFinder software enables control of LC-MS systems. For every measurement, a calibration curve is created, which is allowing to find a concentration of unknown samples by comparing it to a set of standard solutions (called here K1, K2 etc.) with known concentration.

### **2.1.4 External control materials**

To be absolutely sure and to check if the values quantified in the laboratory are completely right and correspond to the actual content of the samples, external control materials, which are available on the market are also run additionally to calibration solutions. For both substances, an external plasma control material "Neuroleptics 2" Level I and Level II were used. These contain a table with target values listed and the range in which the obtained result is acceptable. For Flupentixol, the lower (Lev. I) target value is 1,84ng/ml and the Level II value it is 14,3ng/ml. For Fluphenazine it is 1,85ng/ml and 14,4ng/ml respectively. (see Attachment, Figure 39 and 40)

### **2.1.5 Solutions, analytes and other materials**

Analytes examined are Flupentixol and Fluphenazine. Other substances and solutions used are: Methanol, Acetonitrile, Formic acid, preserved water, Ammonia, Internal Standard used for Fluphenazine "IS Orbitrap" – (40ng/ml Fluperlapin and 40ng/ml Bromperidol in Acetonitrile), Buffer solution used for Fluphenazine ( 500ml Aqua LC-MS Grade, 0,25ml Formic Acid, 50ml MeOH LC-MS Grade), Internal Standard used for Flupentixol – (Fluperlapin 6ng/ml, Remoxiprid 4ng/ml).

Other laboratory equipment used is: pipettes (for instance: Hamilton pipette, automatic pipettes), mortars, volumetric flasks, „Lab dancer“ etc.

## **2.2 Methods**

### **2.2.1 Method development of Fluphenazine**

#### **Sample preparation**

Serum samples containing Fluphenazine need to be precipitated before the measurement can be done. Precipitation means elimination of a dissolved substance from a solution, so of the proteins in serum. It is done by outreaching its solubility by changing the environment. First, 100µl of the sample is taken and using Eppendorf pipette filled in an Eppendorf tube, then, 400µl of an Internal Standard solution (40ng/ml Fluperlapin and 40ng/ml Bromperidol in Acetonitrile) is added. This is done in order to denaturize the proteins. After these two steps the probe is shaken on a „Lab dancer“ device for 1min and centrifuged for 4mins. Then, 400µl of a Buffer solution (LCMS quality water, 0,05% Formic Acid, 100ml LCMS quality MeOH) is put in a vial, 400µl of the centrifuged fluid is taken carefully (without taking the solid which has settled at the bottom), put into the vial and shaken with the Buffer.

This sample preparation method has been already successfully applied in the laboratory for quantification of 15 other neuroleptics and antidepressants. Since the desired limit of quantitation and limit of detection can be easily reached using this method and it occurs to work well for Fluphenazine, it is taken on. Therefore, the existing analytical instrument method can also be taken on from previous experience.

This is again a big advantage for the laboratory because Fluphenazine quantification can be introduced into the daily routine and prepared and run together with 15 other analytes, leading to time and resources savings.

#### **LCMS Method**

To run the samples at the LCMS measuring device, an already existing method (called „Biphenyl03\_new“) is used, since it works perfectly for this substance. The injection volume is 5µl. Considering the fact, that there are a lot of patient samples that need to be quantised every day, the samples used for method development are always run at night after more important samples were run.

Careful programming is also crucial, because it should be possible for every co-worker

of the laboratory to easily trace back and find the corresponding files. Programming is always done corresponding to the date and the analyte. Patient samples are given a number (for instance starting from 600 for serum) and the calibrations are called simply K followed by a number (usually 1, 2 or 3 since there are normally three levels of calibrations). This is a simple way saving a lot of time and effort.

Shown below figure illustrates the LC method used for Fluphenazine samples. This method lasts for 11 minutes. The method starts with gradient of 90% of Elution agent A (EA) and 10% Elution agent B (EB), and then after 6 minutes changes to 20% EA and 80% EB to finally get to 10% EA and 90% EB at 6,10 minutes. This condition is kept for two more minutes and then the method goes back to the initial conditions in order to prepare the same environment for the next serum sample.

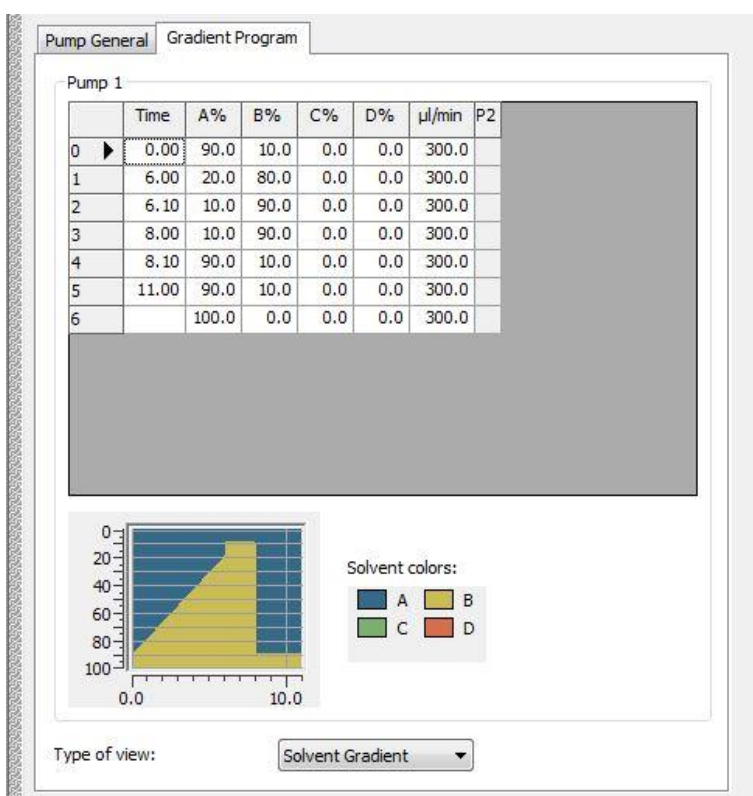


Figure 10 LCMS Biphenyl method - 90% Elution agent A and 10% Elution agent B as starting concentration, 11 minutes long – very steep curve, high percentage difference between solvents

## 2.2.2 Method development of Flupentixol

### Sample preparation

For Flupentixol, the precipitation method occurred not to be suitable. Because the precipitation results in a dilution of the analyte, the desired limit of quantitation (0,25ng/ml), was unfortunately not reachable using this method. For this reason, a different sample preparation method is chosen.

In the very beginning, precipitation was used for Flupentixol samples because it is cheaper and faster method than other ones, however, the results were not satisfying, the solution with concentration 0,25ng/ml could not be quantised. Therefore, a sample preparation method (solid phase extraction) which is used in the laboratory also for another analyte – Olanzapin, is taken as a template and then changed and improved in order to get best results possible for Flupentixol. This extraction was developed in order to get a highly concentrated and pure extract of the substance from the patient's serum.

Solid Phase Extraction (SPE) is one of various techniques which is available to analysts for the sample treatment after it was collected. This technique is applied in order to get the sample into a form that is compatible with the analytical instrument that is used. (ThermoFischer Scientific 2020)

In the very first step, before beginning with the SPE, the sample is prepared. It is done by mixing thoroughly 200µl of serum containing the analyte with 1000µl of solution 0,1% Formic Acid in Water and 100µl of Internal Standard (Fluperlapin 6ng/ml, Remoxiprid 4ng/ml) in an extra 5ml vial.

This step is done, in order to provide enough time for the analyte to get protonated by the formic acid. If the sample could be mixed directly in the cartridge, instead of an additional glass, savings in time and resources would occur. Thus, measurements with the sample mixed in the cartridge were done. However, as it can be clearly seen after comparison of the following chromatograms, mixing of the sample before putting it onto the cartridge needs to be done.

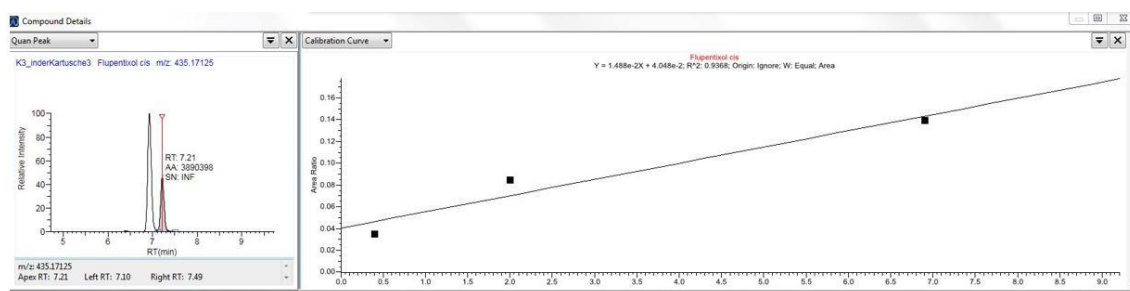


Figure 11 Linearity of a serum sample mixed directly in the SPE cartridge, non-satisfactory results can be seen

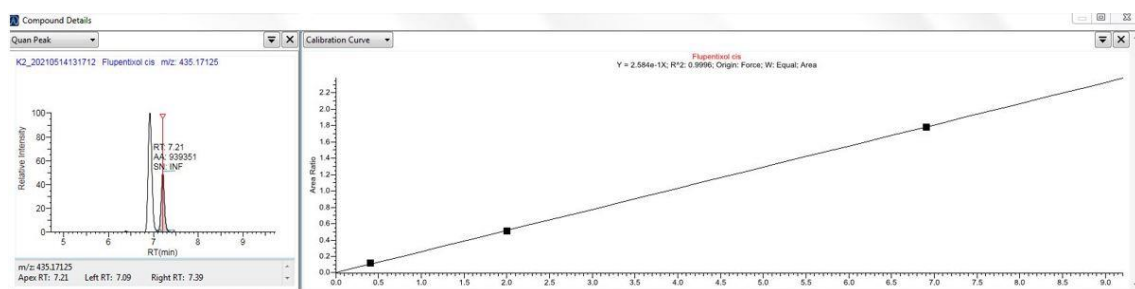


Figure 12 Linearity of a serum sample mixed before the application into the SPE cartridge, a perfect linearity of the results can be seen

Furthermore, the volume of analyte taken to mix with the IS and FA in H<sub>2</sub>O was improved. Since sometimes there is a small amount of patient's serum available, and it is important to save the resources (like external control material), resulting in saving money, it is always better to take as small amount of the serum as possible. For this reason, trials with 200 $\mu$ l and 300 $\mu$ l sample were done. Because the Limit of quantitation (LOQ) was reachable also taking less analyte (200 $\mu$ l), and the calibration curves were satisfying, this volume was chosen for the final method.

In the first step of sample preparation, the SOLA Cartridge is activated with a solution of Methanol and 0,1% Formic acid. Methanol serves as a moisturiser for the dry polymer cartridge, whereas the small percentage of Formic acid protonates and activates the ion-exchanger. In this step, it is important to let it slowly drip through, also that the cartridge does not run dry, nonetheless only a tiny amount of Methanol and 0,1% Formic acid should stay in it. Letting the solution slowly drip is important, since it takes some time, until the solvent has gone into all pores. If the flow rate is too fast, the entire surface cannot be reached, and so the capacity of the cartridge can decrease. If it runs dry, wetting is no longer complete so the capacity and interaction get lower, in result the

recovery rates get smaller as well. This procedure is same for all sample preparation methods.

In the second step, a solution of 0,1% Formic Acid in Water is poured onto the cartridge to achieve the environment that is needed for the next step. In this stage there also must be some liquid left in the cartridge, it cannot run dry. The surface must be completely covered by the aqueous solution. It is very important that there is some liquid left in the cartridge after conditioning, otherwise it has to be conditioned once again (starting from step 1). This procedure stays unchanged as well.

Next, the analyte (the prepared sample) is added onto the watery solution in the cartridge. Then, the cartridge is filled up to 3ml with 0,1% Formic acid in water and then the sample is let through. In this step it is crucial to let the sample drop slowly through the cartridge, with a tiny pressure, so that the substance has enough time to interact with the material and as much as possible of the searched analyte stays in there ("sticks" on the ion exchanger). The correct flow rate obviously depends on the analyte and its affinity. Strong or fast interactions allow a faster flow, whereas weak or slow interactions require slower flowrates. The ion exchangers result in strong interactions, and nonpolar polymers result in weaker van der Waal forces.

After the analyte is added onto the cartridge, two washing steps are done in order for the analyte to stay at the ion exchanger and to remove left matrix components from the material. These would be otherwise carried with the analyte while elution. First, the sample is washed with 1ml of 0,1% Formic acid in water solution. The second washing step is done with 1ml of mixture of solutions of 0,1% Formic acid in water and 0,1% Formic acid in methanol in proportion 70% to 30%. This has to be run dry.

This proceeding has been chosen as the most suitable for this substance since it resulted in the best chromatography outcome and best recovery. For the second washing step, some trials were done, the solutions were altered to get the most suitable one. The quantification was done also using other washing solutions for the second wash, these

are represented in the table below.

*Table 1 Summary of various solutions used for the second washing step*

Solution number	Components
1	50% of (0,1% Formic Acid in H <sub>2</sub> O) 50% of (0,1% Formic Acid in MeOH)
2	30% of (0,1% Formic Acid in H <sub>2</sub> O) 70% of (0,1% Formic Acid in MeOH)
3	50% of (0,1% Formic Acid in H <sub>2</sub> O) 50% of (0,1% Formic Acid in MeOH)
4 (final, the most suitable solution)	70% of (0,1% Formic Acid in H <sub>2</sub> O) 30% of (0,1% Formic Acid in MeOH)

Last but not least, a small glass is put under the cartridges, and 0,5ml of Methanol 5% in Ammoniac is poured onto the sample twice to elute the analyte (detach the substance from adsorbent). In this step, the pH value is switched, so that the molecules can get deprotonated and become released from the ion exchanger. It is done twice and both are done under low pressure in order to provide the substance with enough time for interaction with the basic eluent.

The sample with the extracted liquid, containing the analyte, is now put into an evaporator (Barkey Vapotherm) which is set to 65°C and the liquid part is being evaporated under vacuum. After 15minutes, the eluent is evaporated. The concentrated sample is taken out to cool to the room temperature and is then filled with 100µl solution of 0,1 % Formic acid in MeOH. This mixture is added to protonate the molecules before they are injected in the analytical instrument.

Nevertheless, the formic acid provides an environment in which rearrangement of the molecule is favourable. In order to avoid this, other three solutions were checked for suitability. The table below lists other solutions with which the sample was filled.



Table 2 Summary of different solutions used to fill the concentrated sample

Solution	Result
0,1% Acetic acid in MeOH	The percentage of rearrangement reaction from cis- to trans-form after 48hours was lower with the tested reagents, however the obtained values were not linear, so the first method was kept.
0,1% Formic acid in H <sub>2</sub> O	
Drops of Phosphoric acid in 10ml MeOH (pH 3.5 in H <sub>2</sub> O)	
0,05% Formic acid in MeOH	

To achieve a better overview of the sample preparation method, a graphic illustrating every step, which can be seen below (Figure 13), was established.

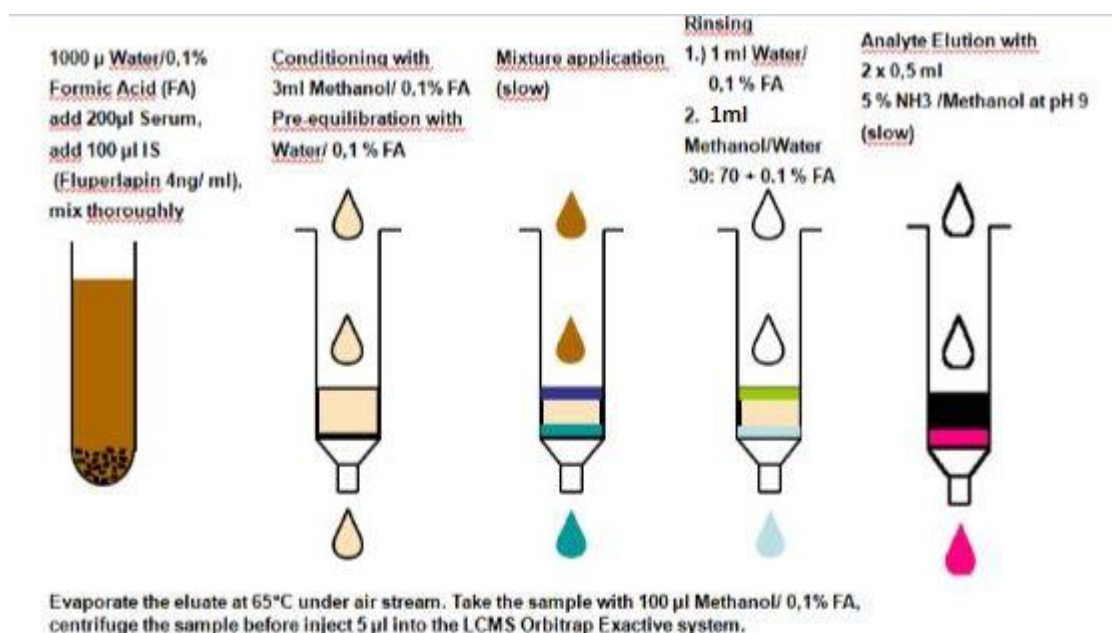


Figure 13 Sample preparation (five steps) method used for samples containing Flupentixol (Gerhards, Schaaf, Torzewska 2021)

## LCMS Method

To quantitate the Flupentixol samples, a method called “Flupentixol” has been created. An improvement of already existing LC method had to be done in order to separate two peaks occurring in chromatography for Z and E isomers.

“Flupentixol” measuring method lasts for 12 minutes, in order to give the sample time to react with the elution agent and also to enable peak separation but on the other hand not take too long for one sample run. The beginning condition is 64% of Elution agent A and 36% of Elution agent B to keep the gradient flat, over the course of time the percentage proportion changes. After 8 minutes there is 30% EA and 70% EB, to get to 10% and 90% respectively, in the end it goes back to the starting condition to 64% EA and 36% EB.

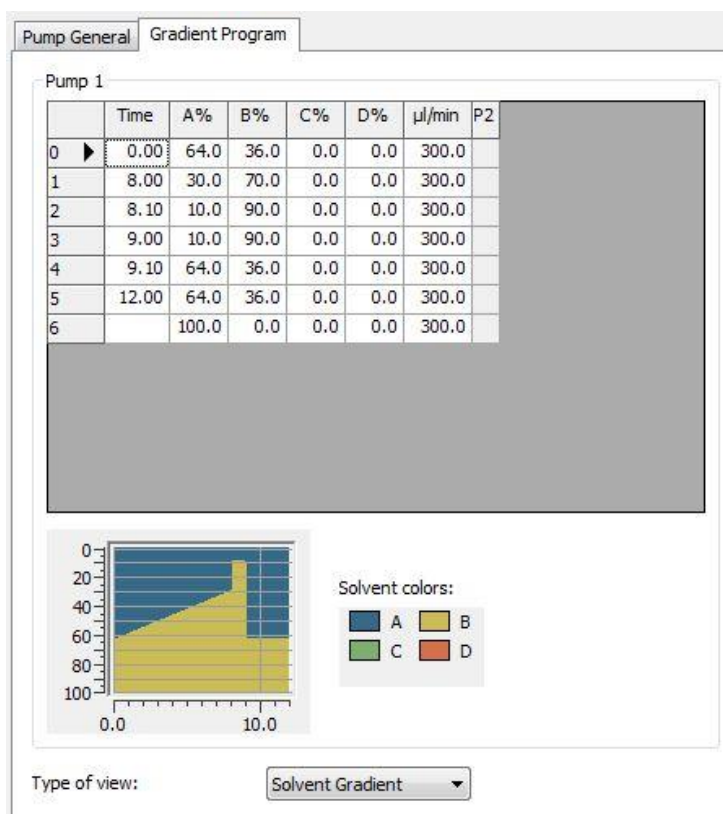


Figure 14 Final method called "Flupentixol" with starting condition 64% EA and 36% EB, the percentage difference between the Elution agents smaller than in the initial method

As already mentioned, Flupentixol is available in form of tablets or in depot form. Tablets contain both cis- and trans- isomers (which should be in 50-50% proportion, but further experiments prove that it is not an equal proportion), however depot contains only the active substance (100% of the Z-form). This phenomenon is really rare and makes the substance more attractive for analytics, since it brings some efforts with it. For method development, Flupentixol in form of tablets (and later on pure substance) has been used for the stock solution. As consequence, samples measured gave a double-

signal in chromatography, since the solution contains both isomers. Unfortunately, evaluation of this kind of results is not as precise as of one substance (one signal), also, prior to evaluation the pharmacist does not know if the patient becomes a pure Z-form of substance (depot) or a pill (containing both forms). The therapeutical range of the substance for a pill is 1ng/ml to 10ng/ml, however for a depot it is half of it (0,5ng/ml to 5ng/ml). For this reason, it is of big value for the pharmacist to be able to distinguish between which form of the substance patient is taking. Hence, trials of separating these two peaks were done at the very beginning.

In order to separate two signals, it is necessary to lengthen the time that the substances interact with the column material. This can be done by flattening the gradient between two elution agents, Elution agent „A“, which is polar (contains water) and Elution agent „B“ which is non-polar (contains Methanol). The difference between the percentages of elution agents should be lower, either at the beginning or at the end of the method.

In the following trials the method used for measurement of Flupentixol is changed to a longer one in order to flatten the curve, as well as the percentage difference in the beginning is set lower. As it can be seen in Figure 15, in the initial measuring method the gradient between both elution agents is relatively high, which makes the curve steep.

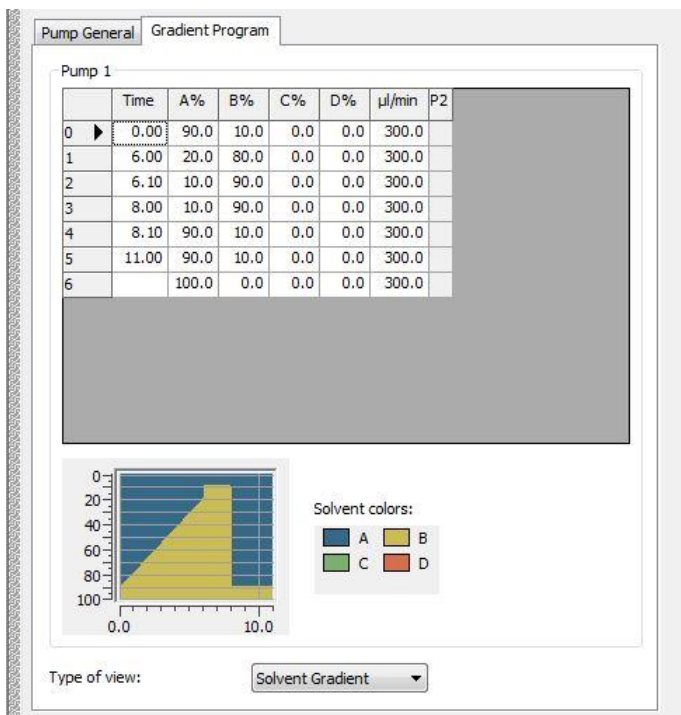


Figure 15 LC method initial - not changed. In the graph it is visible that the curve is pretty steep, since the beginning condition is 90% Elution agent A and 10% Elution agent B; also, the method is 11minutes long. (optimal time for most samples).

In the first method development trial, the gradient between the two Elution agents was changed. It was lowered (from 90% agent A and 10% agent B) to 70% agent A and 30% agent B, the flow time was not changed. These changes are depicted in Figure 16.

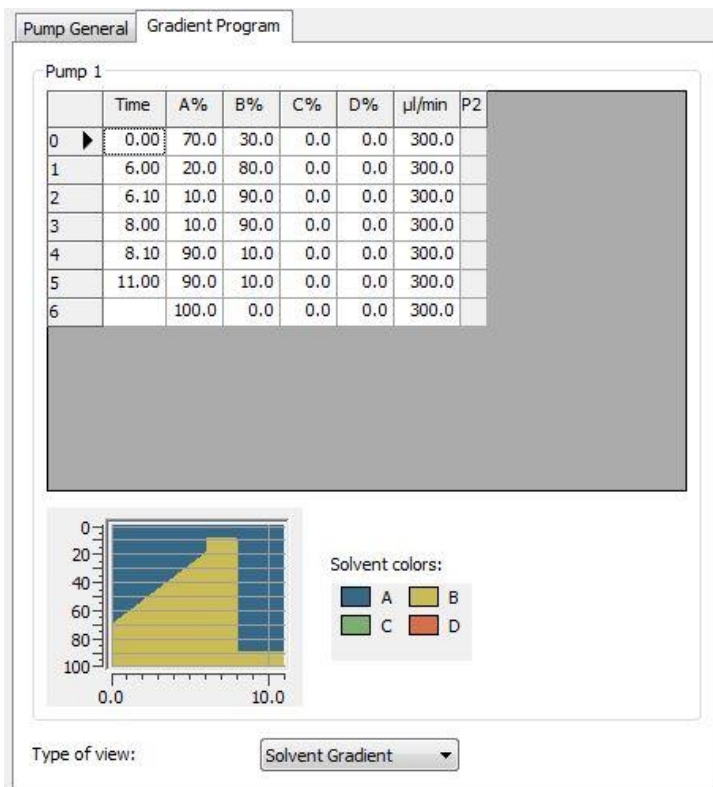


Figure 16 LC method - first change trial. The gradient is smaller because the percentage of Elution agent A is set to 70% and of Elution agent B to 30% in the beginning conditions (the percentage between them gets smaller).

In the second trial (which can be seen in Figure 17) the method was set to longer, one run takes now 14 minutes instead of 11 minutes. This step was done in order for substance to have more time to interact with the elution agents and to separate the two signals.

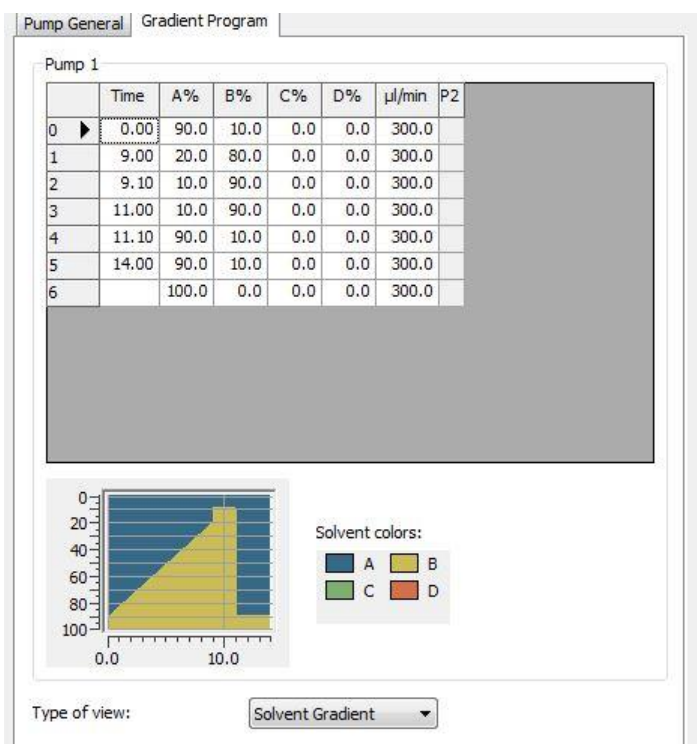


Figure 17 Second change trial. In the second trial the method is set longer. A single sample run takes now 14minutes instead of initial 11minutes.

In the third trial (see Figure 18) both previous outcomes were put together to achieve a better result. The slope was flattened, so the gradient between solvents was set lower (70% EA to 30% EB) and the duration of the method is 3 minutes longer for one sample (lasts for 14mins). This method gave the best results compared to previous trials, and a baseline separation of the peaks was reached. However, 14minutes for a single run is unfortunately too long for daily basis in the laboratory, so one more adjustment was done to get the final method (see Figure 14).

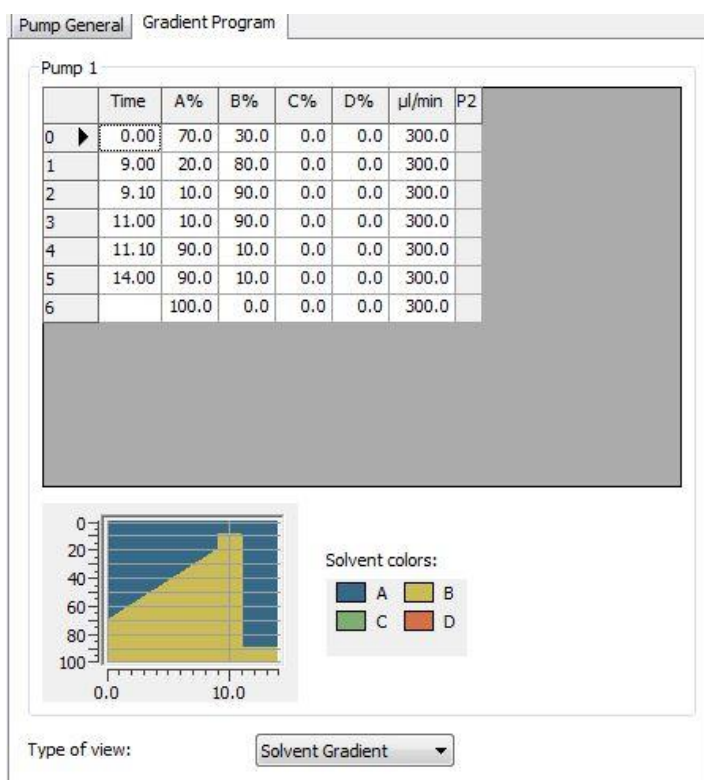


Figure 18 Third trial, the method is set to longer with the gradient 70% EA and 30% EB.

Different measurement settings were put together in a table for a clear overview.

Table 3 Method adjustment for Flupentixol samples - different settings with flow rates

Variation	Flow rate in ml/min	Gradient (EA=Elution Agent)
initial	0,3	0-6min: 90% EA A, 10% EA B 6-6,10min: 20% EA A, 80% EA B 6,10-8,10min: 10% EA A, 90% EA B 8,10-11min: 90% EA A, 10% EA B
1	0,3	0-6min: 70% EA A, 30% EA B 6-6,10min: 20% EA A, 80% EA B 6,10-8,10min: 10% EA A, 90% EA B 8,10-11min: 90% EA A, 10% EA B
2	0,3	0-9min: 90% EA A, 10% EA B 9-9,10min: 20% EA A, 80% EA B 9,10-11,10min: 10% EA A, 90% EA B 11,10-14min: 90% EA A, 10% EA B
3	0,3	0-9min: 70% EA A, 30% EA B 9-9,10min: 20% EA A, 80% EA B 9,10-11,10min: 10% EA A, 90% EA B 11,10-14min: 90% EA A, 10% EA B
4 the final	0,3	0-8min: 64% EA, 36% EB 8-8,10min: 30% EA, 70% EB 8,10-9,10: 10% EA, 90% EB 9,10-12: 64% EA, 36% EB

### **3 Results**

In order to validate analytical procedures, among others the following quality features are used: limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, selectivity, specificity, linearity, sensitivity, ruggedness. (Rücker, Neugebauer and Willems 2001)

#### **3.1 Selection of the solvent**

In order to develop a new method for quantitation of patient samples, different properties and characteristics, like for instance retention time of the given analyte have to be examined. This can only be done by analysis of the solution of this substance.

Since water as solvent brings in the problem of easily growing microorganisms, and thus stability of watery solutions is relatively low, it is wiser to choose another solvent if possible. Both of studied drugs are soluble in methanol and for this reason it is taken as solvent for Fluphenazine and Flupentixol.

Later quantifications showed that for Flupentixol, there had to be also Formic acid added to the analyte in solvent in order to protonate the substance before it is prepared for entering an analytical device.

#### **3.2 Retention time determination**

Retention time is the time that the solute needs to pass through the chromatographic column, so the duration between the sample injection and a peak of the substance at the detector.

##### **3.2.1 Measurement of methanolic solutions in defined concentrations using different methods**

For retention time determination, and all other measurements, a solution of the analyte in methanol had to be done. Since it is optimal for the laboratory to have as many



substances as possible that can be quantised using one method, and since both analysed substances have given therapeutic reference range from 1ng/ml to 10ng/ml (for Flupentixol in form of a tablet), first trial was to produce one solution containing both analytes.

It is an advantage for the laboratory if more substances can be measured in one solution, because then it is only one instead of several solutions that have to be prepared for quantitation. This saves a lot of time as well as resources and money.

For this purpose, a solution of Fluphenazine from a pure substance in powder form (which is available in the pharmacy in the clinic, because it is needed for the patients) was done by putting 51,52mg to 100ml MeOH to get the concentration of 515µg/ml. Then, it had to be diluted once 1:10 (so 1ml substance into 10ml methanol). For Flupentixol a 5mg pill was taken for the stock solution and put into 100ml methanol to get the concentration of 50µg/ml. After both stock solutions were prepared, 20µl of both substances was taken and put into one flask with 10ml methanol, in order to get concentration of 100ng/ml. This solution was diluted four times, in order to get solutions of 10ng/ml, 1ng/ml, 0,1ng/ml and 0,01ng/ml and then these were prepared for quantitation. An already existing and used in the laboratory method called "Biphenyl03\_new" was used.

These concentrations are always checked in the beginning of a method development to see if under selected conditions (solvent, method etc.) the assumed concentrations of analyte can be detected and quantised. For Fluphenazine this worked perfectly, unfortunately for Flupentixol the concentrations after the sample precipitation were too low, and so the limit of quantitation could not be reached using this method. Also, (as it is written in the package leaflet for Flupentixol) the content of both isomers in the tablet should be 1:1, so 50% of Z-isomer and 50% of E-isomer. However, this is unfortunately not true for Flupentixol. Already after a few measurements it became obvious, that the percentage ratio of two forms is different.

These discoveries led to separation of these two substances. From this point, separate measurements for Fluphenazine and Flupentixol were done. In order to make the quantitation of Flupentixol more accurate and easier, a pure substance in powder form was ordered for a new stock solution (Flupentixol dihydrochloride 50mg in 50ml water

which corresponds to 42,815mg Flupentixol). Nevertheless, the pure substance also contained both isomers, but this solution was taken for further examinations. The content of calibration standards produced can also be verified by the external control materials.

### **Retention time Fluphenazine**

Since both analytes are quite nonpolar, they need some time for interaction with the separation column, for this reason a peak for Fluphenazine can be detected first at 6,30 minutes.

### **Retention time Flupentixol**

For Flupentixol, very first trials were done with precipitation method and buffer solution added, this led to retention time at 6,92 minutes for Z-isomer. However, after the precipitation was changed to sample preparation, the retention time also has changed slightly to 6,80 minutes.

### **3.2.2 Selection of suitable Internal Standard Solution**

An Internal Standard solution (IS) is a substance which is used in order to balance the possible measurement inaccuracies that can be caused for instance by fluctuations in the injector or in the temperature in the analytical instrument or inaccuracies in the sample preparation. The Internal Standard solution cannot contain the analyte of interest. However, it should be a substance (or mix of few) which is chemically and physically similar to the analyte. Also, it must not cause any interference in the chromatography. IS is added in a defined amount to all samples, as well as to the calibration standards and external control materials. Thus, it serves as a reference and can enable calculation of deviations between the samples.

### **Internal Standard for Fluphenazine**

This selection was also based on previous experience. Fluphenazine was prepared with

the precipitation method and the Internal Standard Solution used in the laboratory for other analytes prepared via precipitation method occurred to be suitable for Fluphenazine as well. IS consisting of 40ng/ml Fluperlapin and 40ng/ml Bromperidol solved in Acetonitrile showed no interference in the chromatography and proved to be optimal. Fluperlapin is taken as the IS. Moreover, it got chosen because it is already used for other similar substances that are quantified in the laboratory.

### **Internal Standard for Flupentixol**

In case of Flupentixol, a few substances were checked for suitability as Internal standard solution. For instance, IS used for Fluphenazine (containing Fluperlapin and Bromperidol in concentration of 40ng/ml) was analysed first. Bromperidol as standard resulted in much better linearity as Fluperlapin, however it could not be chosen as IS because the external control solutions contain it as analyte. Also, Haloperidol in concentration 4ng/ml was examined, but can also not be taken since the proficiency test contains it too. After some trials, Remoxiprid 6ng/ml (in a solution with Fluperlapin 4ng/ml) got chosen as an IS for Flupentixol.

### **3.3 Determination of measurement sensitivity**

The measurement sensitivity is defined as “Quotient of the change in the display of a measuring system and the corresponding change in the value of measured variable”. (Brinkmann 2012)

The measurement sensitivity for a prescription drug, required to reliably determine the active substance level, is min. 50% of the lowest concentration value for therapeutical area of that drug. For Fluphenazine the measurement sensitivity lies at 0,5ng/ml and for Flupentixol it is 0,25ng/ml.

#### **3.3.1 Limit of quantitation (LOQ)**

Limit of quantitation is applied for qualitative analyses and provides the lowest mass or the lowest content (limiting concentration) of a substance that still can be reliably

detected using provided method. (Rücker, Neugebauer and Willems 2001) It is the lowest level at which recognizable signal and a satisfactory calibration of assured precision and accuracy are provided.

In this laboratory, it is important to quantify values which are 50% lower than the lower therapeutical reference range in order to provide the patients and clients with accurate results and check the compliance of the patients.

### LOQ Fluphenazine

The desired limit of quantitation for Fluphenazine was set to 0,5ng/ml since the lower therapeutical reference range lies by 1ng/ml. The external laboratory, to which the samples were sent before a quantification method was established, has set the LOQ to 0,7ng/ml. However, the laboratory in LVR Clinic wants to develop a method which is more sensitive. For this reason, it is required that the LOQ value is minimum 50% of the lower reference range. As it can be seen in the Figure 19, the desired results were obtained.

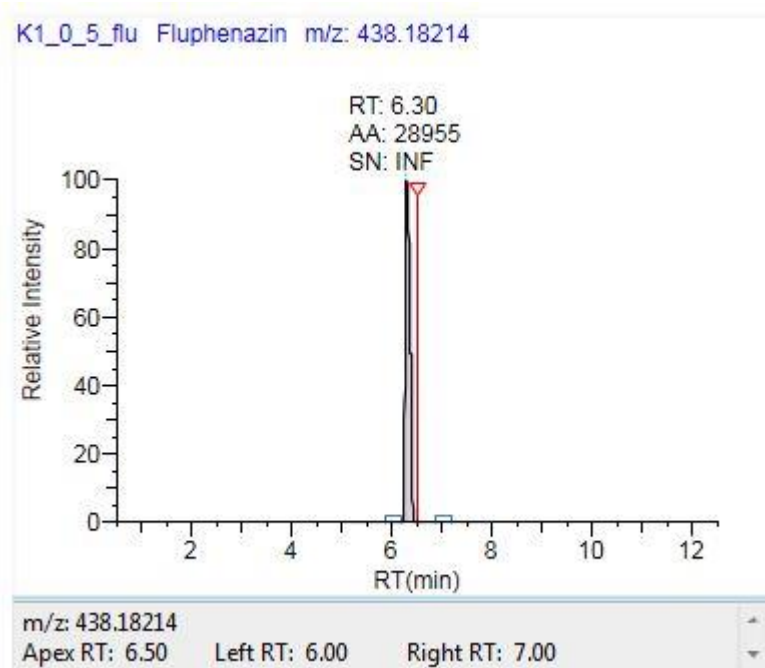


Figure 19 Limit of quantitation for Fluphenazine 0,5ng/ml

## LOQ Flupentixol

The desired limit of quantitation for Flupentixol should be the concentration 0,25ng/ml, which was actually quantified in different solvents and altering the sample preparation method in order to get the most of the substance detected. This figure shows a detected peak after the final sample preparation method.

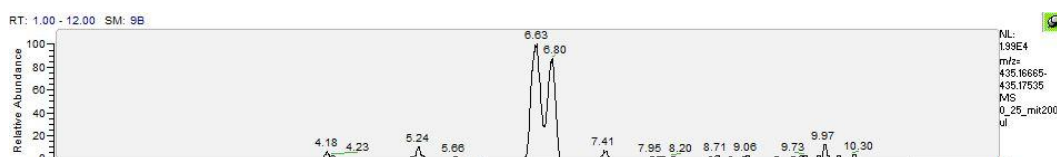


Figure 20 Limit of quantitation for Flupentixol - 0,25ng/ml after adjustments of the method

### 3.3.2 Limit of detection (LOD)

Limit of detection is also applied for quantitative analyses and means the lowest mass or content that can be still determined with acceptable accuracy and precision under specified analysis conditions and available material. (Rücker, Neugebauer and Willems 2001)

#### Limit of detection Fluphenazine

In order to define the LOD of an analyte, further dilutions are prepared. Thus, concentrations of 0,05ng/ml and 0,025ng/ml were prepared. The results showed that 0,025ng/ml is already too low concentration to get any reliable results, so 0,05ng/ml was taken as the limit of detection for Fluphenazine.

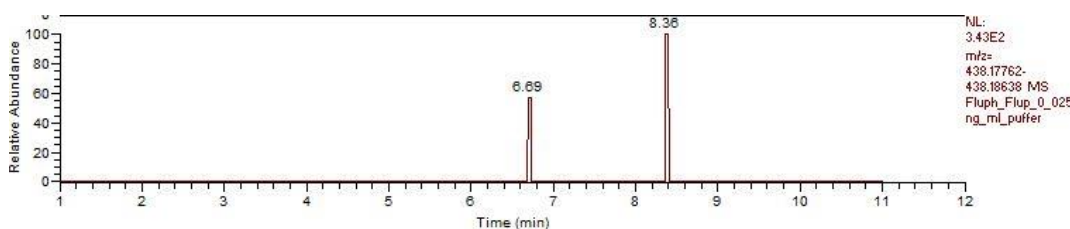


Figure 21 Fluphenazine 0,025ng/ml in buffer solution, concentration too low

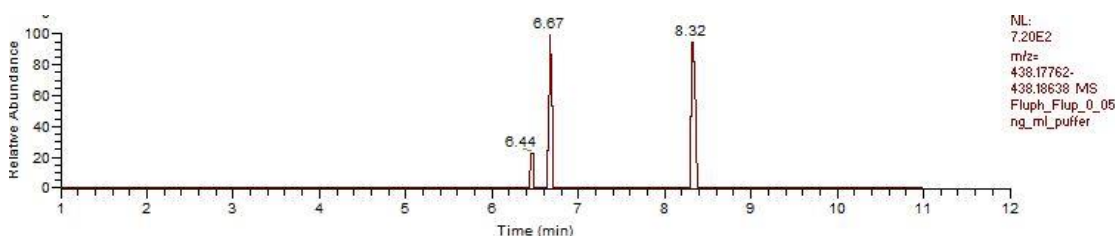


Figure 22 Fluphenazine 0,05ng/ml in buffer solution - limit of detection

### Limit of detection Flupentixol

The same procedure was done for Flupentixol. For this substance, the limit of detection is also at the concentration 0,05ng/ml.

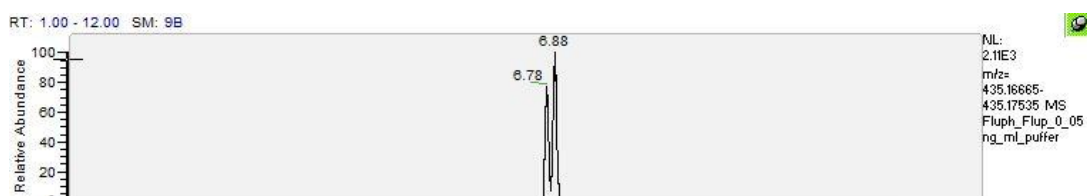


Figure 23 Flupentixol 0,05ng/ml in buffer solution, limit of detection

### 3.4 Linearity of the method

In order to validate the quantification method and introduce it to the daily routine of the laboratory, there has to be a linear relationship between the amount of the analyte in the analysed sample and the peak height. The linearity of a quantitative analysis method describes the proportionality between the measurement results and the actual concentration of the substance. It can be either direct, or it can be made visible through a mathematical transformation of given values. (Rücker, Neugebauer and Willems 2001)

Additionally, for each calibration curve, an R-value is obtained. This value is calculated by dividing the “distance travelled by substance” by “distance travelled by solvent”. R-values can vary from 0 (meaning that the substance is not attached to the mobile phase) to 1 (the substance is not attached to the stationary phase). A value which is equal

almost 1 is proving a precise measurement.

Table 4 Concentrations of the calibration standards for both analytes Fluphenazine and Flupentixol

Analyte	K1	K2	K3	K4	K5
Fluphenazine	0,52ng/ml	2,06ng/ml	5,15ng/ml	7,7ng/ml	13,0ng/ml
Flupentixol	0,51ng/ml	2,14ng/ml	5,14ng/ml	8,56ng/ml	12,84ng/ml

### 3.4.1 Five-point calibration Fluphenazine

The 5-point calibration of Fluphenazine can be seen in the following figure. The measurement is sensitive and accurate, what is visible in the graph. The R value is 0,9926 which means that almost no substance is attached to the stationary phase.

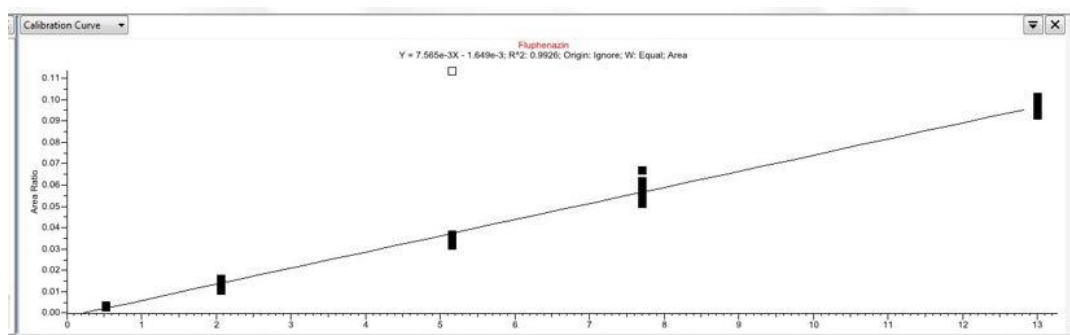


Figure 24 Five-point calibration of Fluphenazine with R-value on top of the figure, indicating a very good linearity

### 3.4.2 Five-point calibration Flupentixol

For the 5-point calibration of Flupentixol, the chromatogram containing only 5 samples instead of 10 was chosen. This was done because of an important observation which was made during these measurements.

In a laboratory of a psychiatric clinic, it is most important to take care about patient's health. For this reason, patient samples have priority over the method development. Samples for method development can be run after the others were quantified, so in the

afternoon or at night. However, they are prepared and put into the analytical device already during the daily routine. Flupentixol samples were also treated in the same way, which led to a very important observation.

As already mentioned, Flupentixol exists as an isomer, with an unequal ratio of Z- and E-form. This ratio in the produced stock solutions was always around 60% E-isomer to 40% Z-isomer, when the samples were quantified directly after sample preparation; and the external control solution contained only the Z-form. Nonetheless, what could be observed is a really interesting and surprising finding. With passing time, a rearrangement takes place, so that the E-form is more favourable as a result. When the samples were left in the analytical instrument and the measurement began more than two hours after the sample preparation, the process of rearrangement began. In the following figure the assumed rearrangement taking place in Methanol and Formic acid can be seen.

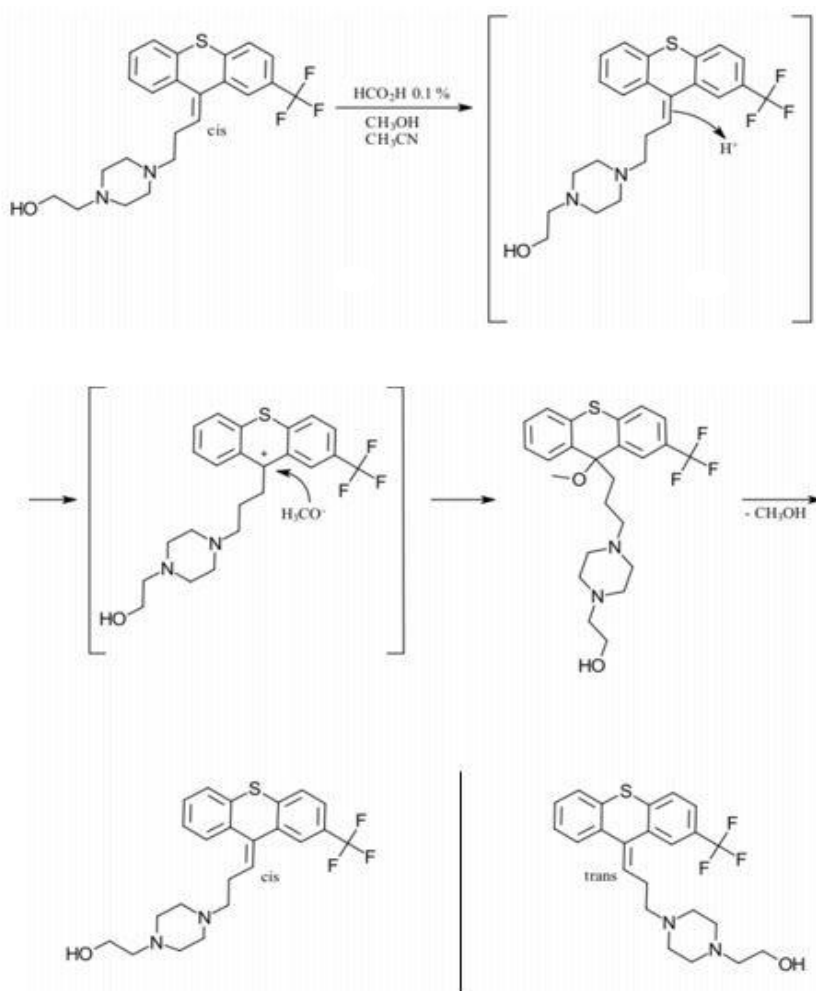


Figure 25 Assumed rearrangement of Flupentixol taking place in Methanol and Formic acid. On the left-hand side, the *Trans*-Flupentixol can be seen, whereas on the right-hand side the *Cis*-Flupentixol (Gerhards, Schaaf and Torzewska 2021)



Even in the external control solutions, where initially no E-isomer was present, with time, the percentage of E-isomer arose. This can be clearly seen in the chromatograms below (see Figure 26 and 27). This rearrangement is most probably caused by the small percentage of formic acid in MeOH which is added after the sample preparation to the dry sample to fill it and take up with a syringe.

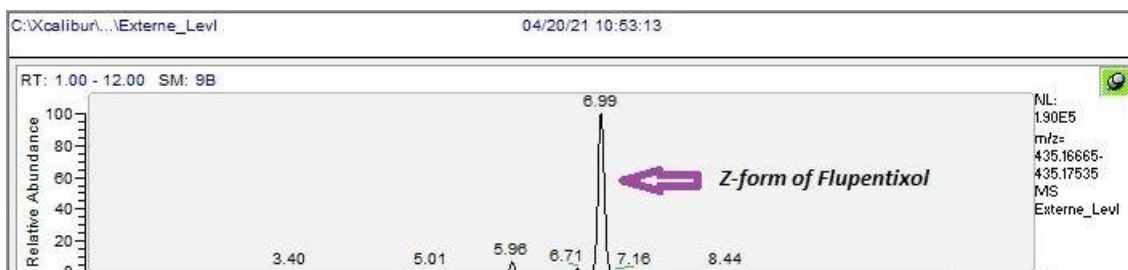


Figure 26 Chromatogram of an external control material measured directly after sample preparation, only one signal of the active form of the substance (Z-Flupentixol) is visible in the chromatogram

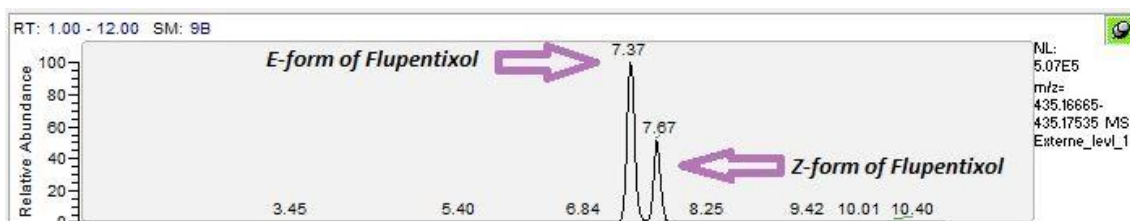


Figure 27 Chromatogram of the same external control material (containing initially only Z-form) measured 48 hours after sample preparation, two signals showing both Flupentixol isomers are visible

Below, the calibration curves are shown. The 5-point calibration of five samples (see Figure 28) is linear (with an R-value of 0.9904) and the given results are satisfying, because no rearrangement takes place. There are 5 samples that are being measured in a row, so the overall process lasts 1 hour (5 samples  $\times$  12 minutes each = 60 min). However, the 5-point calibration for ten samples per level, shows that a rearrangement takes place already 4 hours after sample preparation and with passing time the standard deviation is getting bigger. This observation led to a next crucial conclusion, namely that the samples have to be quantised directly after the sample preparation, maximal interval of 2 hours is allowed between preparation and measurement; otherwise, the obtained results are not reliable.

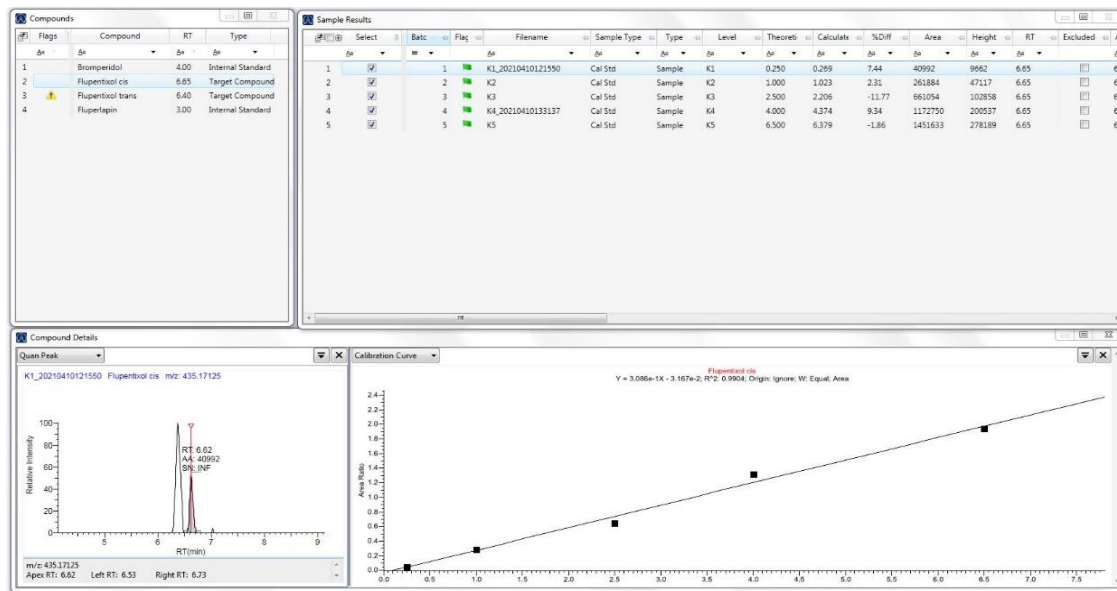


Figure 28 Flupentixol 5-point calibration of five samples: K1 to K5, one run each, the results are stable and precise

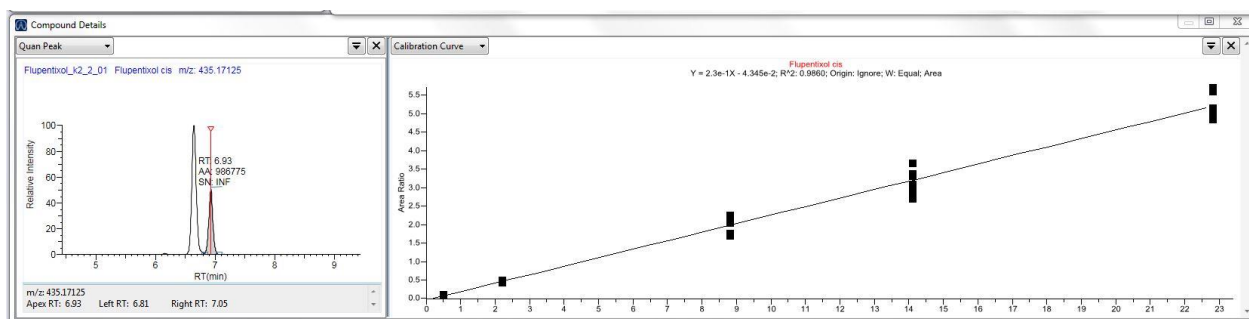


Figure 29 Flupentixol 5-point calibration for ten samples: K1 to K5, run 10 times each, standard deviation between the results increasing with increasing time

### 3.5 In-day-variation

To check for any fluctuations and possible measuring errors occurring during a day, the sample containing the analyte is prepared 10 times during a day.

#### 3.5.1 In-day-variation Fluphenazine

The results of In-day-variation for Fluphenazine are shown in the Table below.

Table 5 Values for In-day-variation Fluphenazine, see Attachment for more detailed example of the results

Theoretical value in ng/ml	Measured value in ng/ml	Standard deviation in %
13,00	13,63	4,87
13,00	12,95	0,39
13,00	12,49	3,96
13,00	13,04	0,32
13,00	12,94	0,43
13,00	12,74	1,99
13,00	13,39	3,04
13,00	13,55	4,25
13,00	12,99	0,05
13,00	12,86	1,08
Mean value	13,058	2,038

In a precise measurement, the standard deviation (STD) between the given results should be lower than 5%. These values show small STD percentage.

### 3.5.2 In -day-variation Flupentixol

Values of ten measurements of Flupentixol done during a day are put hereunder in a table together with the standard deviation percentage and mean value. The in-day measurement was challenging, because of the fluctuations occurring in the results which are due to the rearrangement which is taking place if the samples are quantified more than 2 hours after the sample preparation. Nevertheless, all results are in the acceptable norm (below 25% fluctuation).

Table 6 In-day-variation-Flupentixol, see Attachment for more detailed example of the results

Theoretical value in ng/ml	Measured value in ng/ml	Standard deviation in %
2,200	2,220	0,91
2,200	2,092	4,91
2,200	2,111	4,06
2,200	2,239	1,79
2,200	2,238	1,72
2,200	2,300	4,56
2,200	2,469	12,21
2,200	2,202	0,09
2,200	2,475	12,51
2,200	2,115	3,85
Mean value	2,246	4,66

### 3.6 Day-to-day-variation

Also, there are variations that can occur between different work days. These can be caused by changes in temperature in the analytical device, or because of the elution agents that are freshly made every day, the syringe which is being exchanged every few days and other small changes. Even a negligible change can cause a difference in the results.

#### 3.6.1 Day-to-day-variation Fluphenazine

The values shown below show slight variations occurring between daily measurements of serum samples containing Fluphenazine.

Table 7 Day-to-day-measurements Fluphenazine, see Attachment for more detailed example of the results

Date	Theoretical value in ng/ml	Measured value in ng/ml	Standard deviation in %
22.03	13,00	13,074	0,57
25.03	13,00	13,005	0,04
26.03	13,00	13,019	0,15
27.03	13,00	13,071	0,55
28.03	13,00	13,080	0,62
29.03	13,00	13,125	0,96
02.04	13,00	13,084	0,65
06.04	13,00	13,000	0,00
09.04	13,00	13,043	0,33
10.04	13,00	13,018	0,13
	Mean value	13,0519	0,4

These values show a very small percentage difference from the theoretical value (setpoint), meaning a very precise and accurate procedure.

### 3.6.2 Day-to-day-variation Flupentixol

As well as the previous results, the day-to-day-variation of serum samples containing Flupentixol is linear and the standard deviation percentage is smaller than 5%. The table below provides an overview.

Table 8 Day-to-day values - quantification of Flupentixol samples, see Attachment for more detailed example of the results

Date	Theoretical value in ng/ml	Measured value in ng/ml	Standard deviation in %
05.05	6,900	6,915	0,21
06.05	6,900	6,966	0,95
08.05	6,900	7,058	2,29
11.05	6,900	6,837	0,91
12.05	6,900	6,900	0,00
14.05	6,900	6,916	0,23
27.05	6,900	6,900	0,00
01.06	6,900	6,990	1,31
04.06	6,900	6,981	1,16
08.06	6,900	6,900	0,00
	Mean value	6,9363	0,706

### 3.7 Participation in Proficiency Test (Round Robin Test)

Accuracy of done measurements can also be proven by participation in a test provided by an external institution. In Germany it is for example the GTFCH – Society of Toxicological and Forensic Chemistry. All laboratories take part in a Proficiency Test (also called Round Robin Test) which is offered three times in a year. The Proficiency Test is a quality control and is also used to validate measurement methods. Laboratories which want to take part have to register themselves. All participating laboratories get the same sample material with an unknown content of different antipsychotic drugs. The samples are then treated like regular patient material, and quantified using various methods for diverse substances. The obtained results are sent back to the GTFCH. In the Proficiency Test, 48 laboratories took part in case of Flupentixol, and 40 in case of Fluphenazine. (Society of Toxicological and Forensic Chemistry).

As it can be seen in the following figures, the quantification of Fluphenazine and Flupentixol with the developed methods is accurate. The LVR laboratory got a certificate which proves that the values determined in the laboratory and passed on to

the doctors and patients are correct.

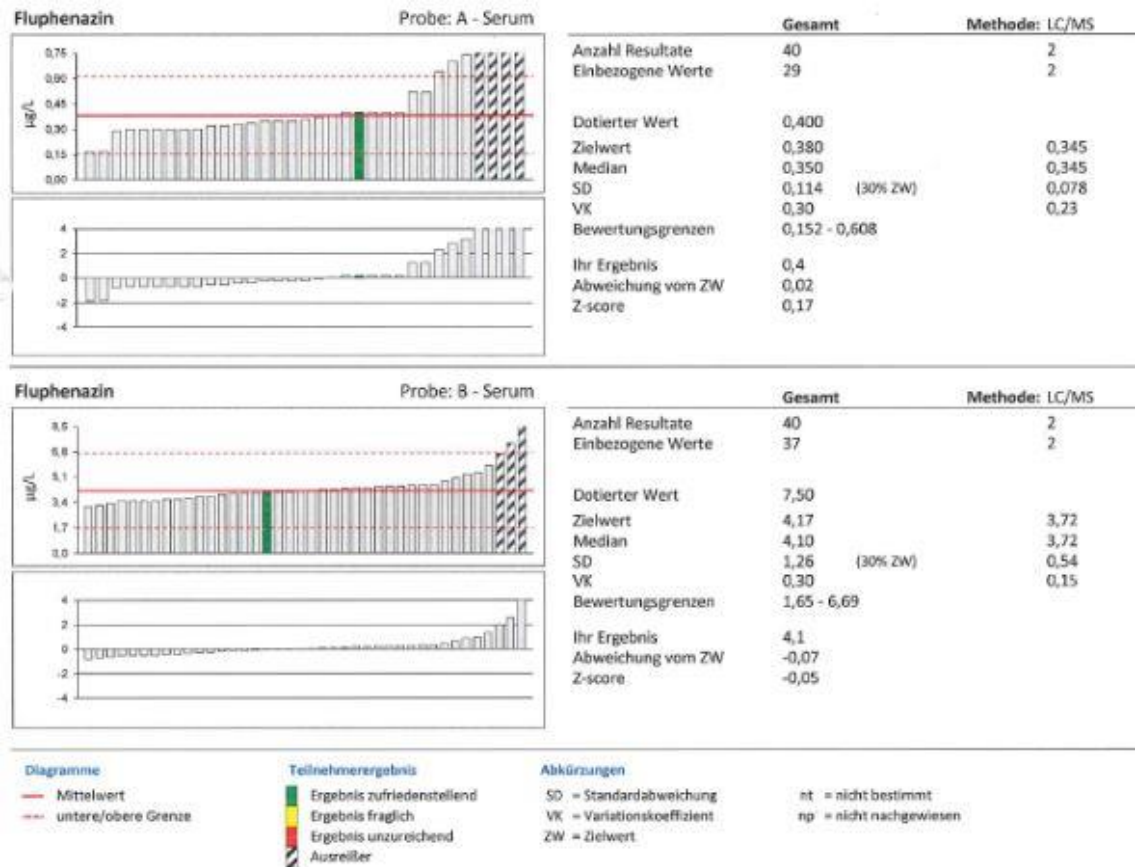


Figure 30 Round Robin Test evaluation of two samples (A and B) containing Fluphenazine showing the result the laboratory got and a comparison among other laboratories, for the sample A, the result obtained was exactly the same as the target value, in case of sample B the result obtained was only slightly different from the target value (Society of toxicological and forensic chemistry)

There were two samples, A and B with unknown content. The sample A containing Fluphenazine had a target value of 0,380µg/L, the outcome of the quantification was 0,4µg/L so there was a deviation from the target value of 0,02. The valuation limit for this sample was 0,152-0,608µg/L. The second sample, B contained 4,17µg/L of the substance, measured in the laboratory was 4,1µg/L so the inaccuracy in this case is 0,07. The range of valuation was 1,65-6,69µg/L. The summary and combination of the results, mean values etc. can be seen in the Figure 30.

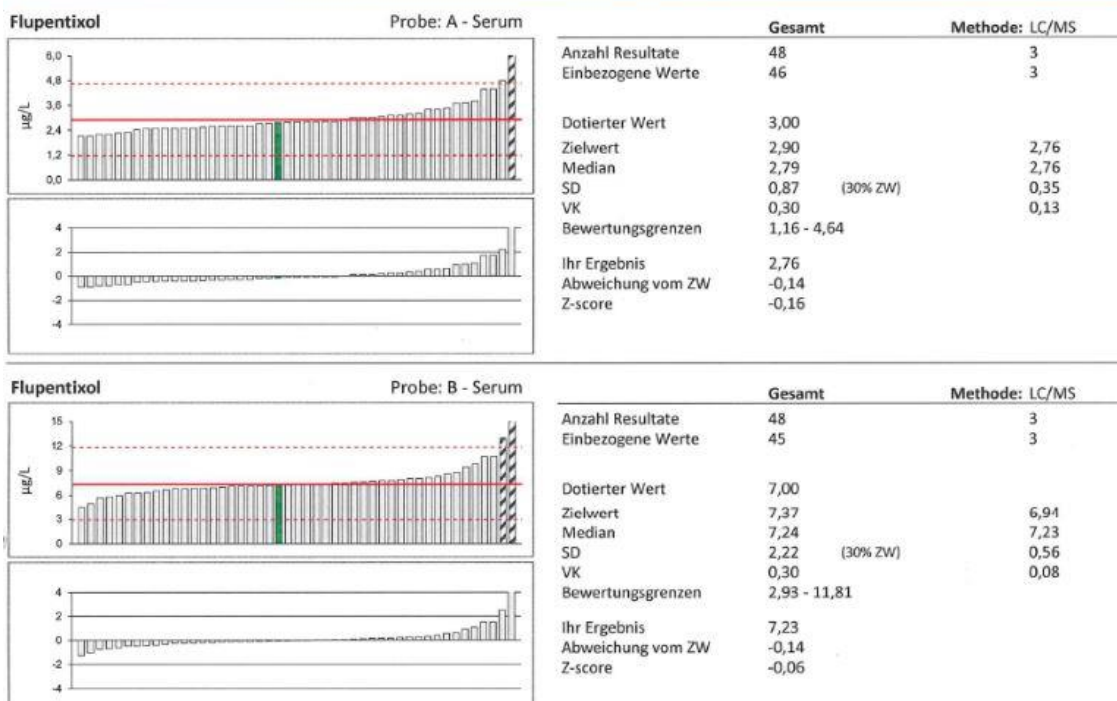


Figure 31 Round Robin Test evaluation of two serum samples containing Flupentixol, both obtained by the laboratory results were very precise, with a slight difference from the target value, namely 0,14 (Society of toxicological and forensic chemistry)

For Flupentixol, the theoretical (setpoint) value of sample A was 2,90µg/L, and the result obtained by the laboratory was 2,76µg/L, so the standard deviation from the setpoint equals to 0,14. The range in which the results were allowed was 1,16-4,64µg/L. The content of sample B was 7,37µg/L, whereas the measured value: 7,23 µg/L, so the standard deviation from setpoint equals again 0,14. For this sample the deviation allowed was from 2,93µg/L to 11,81µg/L. A resume of the values is depicted in the Figure 31.



## 4 Discussion

The aim of this project was the development and validation of a method for quantification of two antipsychotic drugs which are similar to other drugs already being quantitated in the laboratory. Due to the fact that a method development has already been done at this pharmacy laboratory more often, it was initially planned to follow the existing regulations and conduct similar measurements. Because of an increasing number of patients in the clinic who get Fluphenazine or Flupentixol prescribed to fight their mental disorders, also the number of samples containing these two substances kept increasing in the past years. Before, the laboratory in LVR Clinic used to send these samples to an external laboratory for quantification. Nevertheless, quantification in the own laboratory is an advantage – it saves time and costs, as well as the new method can be more precise than the one used by the others. These possible benefits induced this development, because the LVR Clinic puts a high value on the precision of their analysis. Therapeutic drug monitoring is an important factor in a psychotherapy. It provides the information about the drug concentration in patient's material (for instance in serum). TDM can improve the therapy by obtaining a dosage which is special for a particular patient. (Hiemke 2011)

Before the methods for the quantitation of analytes could be chosen and adjusted, a solvent solution and internal standard solution for each substance had to be determined. Solvent selection was done with help of the *List of Pharmaceutical Substances*, where the solubility of the substance in various liquids is given. According to this information, methanol should be a good solvent for both substances, so it got chosen. However, later measurements showed that Flupentixol needs also an acid in order to get protonated before being prepared for further quantitation. Therefore, 0,1% formic acid was mixed with methanol as the solvent for this substance. The initial idea was the establishment of one mixture containing these two analytes, in order to add these to the daily routine of the laboratory. However, because of the chemical features of Flupentixol, which also make the method development more complex, the substances have to be quantitated separately using different methods. The internal standard solution (IS) is used as a reference in the sample preparation to balance any possible inaccuracies occurring. These can be for example due to fluctuations in the injector, changes in temperature in the analytical device and some errors during the sample preparation itself. This substance must not contain the analyte of interest, but should be similar to it. Thus, in

case of Fluphenazine, an IS already applied in the laboratory for other similar substances (Fluperlapin 40ng/ml with Bromperidol 40ng/ml in Acetonitrile) was chosen for the first experiments and revealed satisfying results. Nevertheless, for Flupentixol, various mixtures have been examined. This standard solution could not serve as IS for Flupentixol because the external control material contains Bromperidol as an analyte. Similarly, other substance, namely Haloperidol, is present in the proficiency test as an analyte too. After some trials, Remoxiprid 6ng/ml in a solution with Fluperlapin 4ng/ml was chosen as an IS for Flupentixol. Other possibilities of the quantitation of Fluphenazine and cis-(Z)-Flupentixol are described in some publications. First of them is a work titled "*Sensitive gas-liquid chromatographic method for the assay of the neuroleptic drug cis-(Z)-flupentixol in human serum or plasma.*" (Ulrich, 1995). In this writing, as the title says, a gas-liquid chromatographic (GLC) assay was developed for the analysis of Flupentixol. The Internal Standard solution used by them for Flupentixol samples is also different than used in this method, they choose Perphenazine. This substance is also used as IS for Fluphenazine in publication titled "*Quantitation of Fluphenazine in Equine Serum Following Fluphenazine Decanoate Administration*" (Costello et al, 2013), in which equine serum is analysed with help of a LC-MS-MS device.

In case of Fluphenazine, the standard procedures for a method development were followed. The sample precipitation method used for other similar substances, as well as the LCMS method were applied to Fluphenazine samples in the first trial. The sample precipitation is an elimination of proteins from serum, it is done by taking 100µl of the sample and adding 400µl of Internal Standard (40ng/ml Fluperlapin and Bromperidol in Acetonitrile), after centrifugation this mixture is filled into a vial with 400µl of Buffer solution. The LCMS method used is called "Biphenyl03\_new", lasting for 11 minutes. In fact, both resulted in satisfying results for this analyte, so no improvements needed to be done. Therefore, without a need for a completely different method than already applied, a better result than in the external laboratory can be obtained, and the samples can be introduced into the daily routine. The Limit of Quantitation for Fluphenazine in this developed method is 0,5ng/ml, and the Limit of Detection is 0,25ng/ml. In the external institution, the LOQ is 0,7ng/ml, whereas the optimal and desired by the laboratory LOQ was 0,5ng/ml (50% of the lowest therapeutic reference range). The ability of a quantitation of the analyte 50% under and above the therapeutic range provides an exact result for the customers. Even if the amount of Fluphenazine is

smaller than 1ng/ml, a result between 0,5ng/ml and 0,9ng/ml tells the doctor if the patient takes the prescribed drug at all. Furthermore, as it occurred at the end of the method development, so precise method is required for a correct result in the Proficiency Test. The samples of unknown concentrations which the laboratory got, contained 0,38µg/L and 4,17µg/L of Fluphenazine. If the method would not be sensitive enough (the LOQ and LOD values would be set higher), then the sample A could not be measured and the laboratory would not get the certificate. This problem can be seen in the Round Robin Test evaluation (see Attachment, Figure 33), which shows that only 65% of the laboratories taking part in the Round Robin Test, got a successful result for the sample A (sample with smaller concentration), whereas 95% for sample B (sample with higher analyte concentration). Also, in the next comparison of the results of the Proficiency Test (see Attachment, Figure 32), it can be noticed, that for sample A, a lot of results were significantly higher than the desired value. This was most likely caused by the fact, that a lot of laboratories have set their LOD values higher than 0,4µg/L.

Similarly, for Flupentixol a more professional approach to the method development is required. The laboratory, where the samples were sent before, quantified only the sum of both isomers of Flupentixol and did not distinguish between Z- and E-form, so the therapeutic reference range was 1ng/ml to 10ng/ml for both, injection and a pill. This actually led to incorrect results for all patients getting the substance in form of a depot, since the range for pure Z-isomer content in a depot should be 0,5ng/ml to 5ng/ml. This mistake can possibly expose patients to a risk of getting a wrong dosage of the medication and following side effects. The first attempt of modification of the method was separation of one signal into two (separate signal for each isomer). For this purpose, the measuring method on the LCMS analytical device had to be adjusted. In order to separate two signals, to provide enough time for each signal to occur separately, the time was set longer as well as the gradient between the elution agents A and B was set smaller (gradient in the beginning and end: Agent A 64%, Agent B 36%). To get to the final method, four trials and corrections were done for an optimal result. Because this laboratory gets a lot of patient samples every day, it is important to save time and other resources. For this reason, the final method (12 minutes for a single sample) was adjusted for a few times in order to achieve a shorter method than in the beginning (14 minutes one run), but so that the desired signal-separation was possible. After readjustments and fitting, a baseline separation of two isomers was achieved for every

sample. This is an enormous advantage, because the exact content of the sample and each of the components: active and non-active form can be determined. Furthermore, also the sample preparation method had to be altered in order to get the desired limit of quantitation. In comparison to work of S. Ulrich, "*Sensitive gas-liquid chromatographic method for the assay of the neuroleptic drug cis-(Z)-flupentixol in human serum or plasma.*", where the minimal quantifiable concentration for Flupentixol is 0,5ng/ml (whereas using the developed here method, a concentration of 0,25ng/ml was detectable), and the day-to-day coefficient of variation was 11,2% at 1,0ng/ml and 8,7% at 10,0ng/ml (measurements done for this work led to 0,653% deviation).

Sample precipitation method used for Fluphenazine, leads to a dilution of the sample, which unfortunately resulted in very small signals, when applied to serum containing Flupentixol. Therefore, another sample preparation method, called solid phase extraction (SPE) was chosen. Normally, seven steps, including also two washing steps, are done. For this analyte, an additional procedure was needed. As already mentioned, Flupentixol needs to be protonated before entering the analytical device, whereas for other neuroleptics, the acidity of the elution agent on the column is sufficient.

Moreover, it needs to get protonated before the sample preparation begins, otherwise the linearity of the results is not satisfying, it cannot be quantified and the outcome cannot be forwarded to the doctors. It was also tried to do some improvements in the protonation step: mix the sample with IS and 0,1%FA in H<sub>2</sub>O in the cartridge, and not as initially in an extra vial, this way time and some laboratory resources could be saved, but this experiment led to poor and invalid results. For the second washing step, which should remove all other substances than the analyte of interest, 1ml mixture of 30% MeOH + 0,1% FA and 70% H<sub>2</sub>O + 0,1% FA was applied. Two other mixtures (proportion 50% to 50% of the same liquids, and 70% to 30%) did not result in as pure substance as with the one which got chosen, and a high percentage of the analyte was lost using these mixtures. After the sample was extracted and put into an evaporator to get rid of the liquid and get only pure, concentrated analyte, it had to be filled with a medium for quantitation in the LCMS device.

In contrast, S. Ulrich (1995) extracts the Flupentixol sample with a solvent containing *n*-hexane-isoamyl alcohol (in proportion 98,5 to 1,5 units). The author also mentions the light and temperature protection as an important aspect, however in this method development these were applied from the beginning. At the laboratory in LVR Clinic, the samples are stored at -18°C, in brown glass bottles. For the sample preparation, S.

Ulrich uses a liquid-liquid extraction method and one washing step in order to obtain a highly concentrated and very pure extract. The analytical device used is a Hewlett-Packard 5890 Series II Plus gas chromatograph with a nitrogen-phosphorus detector (NPD). However, in another work titled "*Quantification of antipsychotics flupentixol and haloperidol in human serum by high performance liquid chromatography with ultraviolet detection*" (Walter et al. 1998) a HPLC method is used, but with a different separation column than the one taken for this assay. They used a Hypersil column, which is a C-18 type, so it is built of 18-Carbons chain, whereas the Biphenyl column is built up of phenol-rings. According to the ThermoFisher company, which produces the columns and other laboratory equipment: "Generally, C18 columns offer the ability to resolve a wide variety of analytes without issue. However, there are times when alternative chemistry is required for the separation of aromatic and moderately polar analytes. A biphenyl chemistry offers complementary selectivity to a C18 column, and the ability to resolve isomeric compounds, such as drugs of abuse and steroids by taking advantage of  $\pi$ - $\pi$  interactions between the analyte and the stationary phase. This can help pull critical peaks out of matrix interferences." (*When to use Biphenyl selectivity?*, ThermoFischer Scientific). The Biphenyl column provides significant selectivity for separation of aromatics, and thus separation of cis- and trans-flupentixol was easier with this column. Nevertheless, it can be conducted from the other publication (Walter et al. 1998), that it is also possible with a Hypersil column when some improvements are done. Separation of the racemates is also possible using a chiral separation column, which is a very efficient method for separation of racemic drugs. In some chiral drugs, the desired pharmacological effect is induced only by one enantiomer, whereas the other one can possibly cause side effects (*Chiral stationary phases, Always the proper column for enantiomer analysis*, 2021). Using a chiral HPLC column, this issue can be overcome; however, it is an advantage for the laboratory to use one column for analysis of all substances, instead of changing the column for different analytes. Also, using a derivatization method could be applied to separate the isomers, however this costs a lot of time and money. (Zhu et al. 2019)

Also, the package leaflet information of Flupentixol says, that the ratio between cis- and trans-form is 1 to 1 (an equimolar mixture). The measurements and experiments done, prove this wrong. These showed, that the Trans-(E)-Flupentixol is energetically and spatially favourable form of the substance, and in a favourable solvent, a rearrangement takes place with passing time. These facts strengthened the idea of a method

development, but it was much more challenging than the one for Fluphenazine. In the very beginning, Flupentixol was also quantified using the same LCMS method and the sample precipitation method. However, the obtained results, using these methods, were surprising and disappointing at the same time. First trials led to bad linearity of the quantitation, and the first reason one could think of, was an inaccuracy occurring during the sample precipitation or any other man-made errors. Possible mistakes that can happen are for example: inaccurate pipetting, swapping the vials with different samples, problems in the LCMS device. For these reasons, the experiments were repeated, resulting again in incorrect values. The next reason which could possibly lead to that outcome, was the wrong preparation of the stock solution or any of the dilutions. However, this can be easily checked in the documentation which is done for every prepared solution (see attachment). After all these errors were eliminated, an adjustment in the method for this analyte was concerned. It was noticed in all the failed trials, that there was a build-up of an E-form of the substance in the external control solution, where initially only the Z-isomer was present. This focused the attention and induced consideration of the structure of the analyte and the potential influence it could have on the measurements.

Because a rearrangement from Z- to E-isomer takes place after the preparation, which makes this analyte more challenging than other neuroleptics, different solutions with which the substance can be filled for the measurement, were examined. This experiment was done in order to search for an optimal mixture, which would not lead to a rearrangement, or slow down this process and at the same time would protonate the analyte enough to get a good linearity and the LOQ. For this reason, four other combinations of liquids were tested: 1) 0,1% Acetic acid in MeOH, 2) 0,1% Formic acid in H<sub>2</sub>O, 3) Drops of Phosphoric acid in 10ml MeOH, 4) 0,05% Formic acid in MeOH. Surprisingly, all four resulted in a smaller rearrangement percentage; Phosphoric acid led to increase in the area of the signals (due to the decomposition of substance), however the results of the calibration were not linear. These trials led to a conclusion that the initial solution provided the best results.

After all these adjustments and improvements, the developed sample preparation and LCMS methods had to be validated. For this purpose, a five-point linearity of the method, in-day variation as well as day-to-day variation were done. In case of Fluphenazine, also these measurements resulted in excellent outcomes, the values of the five-point calibration were linear with the R-value equal to 0,99. Also the

quantifications done during a day and between different days showed an insignificantly small percentage of standard deviation (STD in % for in-day measurement: 2,038, for day-to-day measurement: 0,4; whereas the allowed deviation is 25%). These small percentages difference ensure, that the newly developed method is accurate, exact and precise. Flupentixol occurred again a challenging substance. Once the method was adjusted to get the best results, a five-point calibration was done also for Flupentixol samples. However, this measurement, like several others also failed. Nevertheless, it appeared to be one of the most important quantifications done. In the graph showing the linearity of the samples (each calibration solution prepared ten times) (Figure 30), it can be easily seen, that the rearrangement of Z-Flupentixol to E-Flupentixol takes place already around 4 hours after the samples were prepared. This phenomenon was also the cause of failure of the few initial quantifications. In the beginning of this method development the samples were treated exactly like other trial samples, so they were prepared, put into the analytical device and then had to wait until the patient samples, which are more important, were run. Normally, the samples which are not containing the patient material are run at night, after the ones containing the patient serum are done. The outcomes of these trials were not linear at all, only when run directly after the sample preparation, the results were satisfying. This 5-point calibration also proved the theory of a rearrangement happening and showed for how long the samples are evaluable. From the figure number 29, it is clear, that after 4 hours the standard deviation between the given values for the same sample is getting bigger. This is the reason for analysing Flupentixol directly after the sample preparation, which was concluded after these experiments. This analyte is stable when the sample is dry, but instable in 0,1% FA in MeOH. The calibration was then created out of five samples in order to avoid the uncertainty of results. Also, the in-day and day-to-day variations were successful in case of Fluphenazine, where the measurements led low STD percentages (2,038% STD for in-day measurement, and 0,4% for day-to-day). The in-day experiment for Flupentixol showed some fluctuations between the results, however the overall percentage was within the allowed norm (4,66% mean deviation between the results, with one result being 12,21% bigger than desired). Measurements of Flupentixol samples done between different days led to very precise outcomes, with only 0,706% STD.

Participation in the Proficiency Test (also called Round Robin Test) was a significant chance of validation by an external institution of the developed methods. The certificate

which the laboratory receives if the measured values lie in the range of allowance, shows a high quality and accuracy of the provided by the laboratory data. The deviation range which is allowed in the test is rather high, however provided by this method results were very close to the set-point

Another important aspect to mention is the theory that is made after the developed method was applied to patient samples. During the daily routine, a lot of patient blood samples containing Flupentixol are quantified; between some of these, a significant difference in the signals can be observed. Two chromatograms of blood samples from two different patients, both getting Flupentixol in form of tablets prescribed were compared, and an important difference between these can be noticed. (see Attachment, Figure 34 and 35)

Although both patients take orally the same medication, in the material from one of them, the found amount of the active substance is noteworthy higher than in the other one. This observation proves again, how important it is for a laboratory, pharmacists and the doctors to be able to quantify both isomers separately. Assuming that the laboratory quantified only the sum of both forms, the outcome of serum analysis, so the area of signals found, would lie in the therapeutical reference range, even though the substance would not have a sufficient effect on the patient's health. The assumption, which can be made is the following: a pill taken orally gets into the stomach, and because of the acidic environment in the stomach, a rearrangement of the active form of the substance into the inactive takes place already before it reaches the blood. Also, there are a lot of patients with gastrointestinal and stomach disorders. These can be for instance, overproduction or underproduction of gastric acid. Moreover, patients with such illnesses become often medication to either stop the overproduction or prompt the production of the acid. These could have essential impact on the absorbance of Z-Flupentixol in the patient's blood. This hypothesis would explain why there are different proportions of isomers among various patients taking the same medicine.



## **5 Conclusion**

The methods were successfully established with use of materials and analytical devices available in the laboratory. This is a financial advantage, because without a need for a new equipment, the laboratory can quantify the samples which it used to send to an external institution before. The LCMS analytical device proved over years of practical application in this laboratory, that it is very robust, sensitive and reliable. The quantitation of pharmacologically active substance, namely the cis-Flupentixol, has led to an improvement and optimization of therapy for patients.

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## 7. Attachment



Figure 32 Results of the Round Robin Test for Fluphenazine, values higher than allowed circled in red, number of results included for sample B much higher than for sample A (Society of toxicological and forensic chemistry)

### ZUSAMMENFASSUNG DER ERGEBNISSE

Substanz	Probe	Teilnehmer			Dotierter Wert	Einheit	Zielwert	SD <sub>T</sub>	SD <sub>RV</sub>		VK	Bewertungsgrenzen
		Anzahl	Ausreißer	Erfolg					Teilnehmer	Ringversuch		
Aripiprazol	A	61	1	59 (97 %)	175,0	µg/L	179,0	24,0	38,0	Horwitz	0,21	103,0 - 255,0
Aripiprazol	B	61	1	59 (97 %)	315,0	µg/L	316,0	43,0	61,0	Horwitz	0,19	194,0 - 438,0
Dehydroaripiprazol	A	43	2	41 (95 %)	100,0	µg/L	102,0	12,0	24,0	Horwitz	0,24	54,0 - 150,0
Dehydroaripiprazol	B	43	0	43 (100 %)	160,0	µg/L	151,0	20,0	33,0	Horwitz	0,22	85,0 - 217,0
Flupentixol	A	48	1	45 (94 %)	3,00	µg/L	2,9	0,6	0,87	30% ZW	0,30	1,16 - 4,64
Flupentixol	B	48	2	45 (94 %)	7,00	µg/L	7,37	1,22	2,22	30% ZW	0,30	2,93 - 11,81
Fluphenazin	A	40	4	26 (65 %)	0,40	µg/L	0,38	0,133	0,114	30% ZW	0,30	0,152 - 0,608
Fluphenazin	B	40	3	38 (95 %)	7,50	µg/L	4,17	0,61	1,26	30% ZW	0,30	1,65 - 6,69
Haloperidol	A	59	1	58 (98 %)	4,50	µg/L	4,68	1,03	1,41	30% ZW	0,30	1,86 - 7,50
Haloperidol	B	59	2	58 (98 %)	13,0	µg/L	13,9	2,2	4,2	30% ZW	0,30	5,5 - 22,3
Sertindol	A	41	0	40 (98 %)	65,0	µg/L	65,5	8,4	15,8	Horwitz	0,24	33,9 - 97,1
Sertindol	B	41	0	40 (98 %)	85,0	µg/L	87,2	10,4	20,2	Horwitz	0,23	46,8 - 127,6
Sulpirid	A	40	0	40 (100 %)	225,0	µg/L	236,0	26,0	47,0	Horwitz	0,20	142,0 - 330,0
Sulpirid	B	40	0	40 (100 %)	480,0	µg/L	527,0	56,0	93,0	Horwitz	0,18	341,0 - 713,0
Zotepin	A	29	0	29 (100 %)	40,0	µg/L	42,9	8,9	11,1	Horwitz	0,26	20,7 - 65,1
Zotepin	B	29	1	27 (93 %)	105,0	µg/L	110,0	23,0	25,0	Horwitz	0,23	60,0 - 160,0
Melperon	A	37	1	36 (97 %)	100,0	µg/L	101,0	13,0	23,0	Horwitz	0,23	55,0 - 147,0
Melperon	B	37	0	37 (100 %)	270,0	µg/L	282,0	30,0	55,0	Horwitz	0,20	172,0 - 392,0
Pipamperon	A	44	2	42 (95 %)	200,0	µg/L	205,0	26,0	42,0	Horwitz	0,20	121,0 - 289,0
Pipamperon	B	44	1	41 (93 %)	330,0	µg/L	371,0	47,0	69,0	Horwitz	0,19	233,0 - 509,0

Figure 33 Summary of the results of Round Robin Test, only 65% success rate for Fluphenazine, sample A, whereas 95% success rate for sample B, Fluphenazine (Society of toxicological and forensic chemistry)

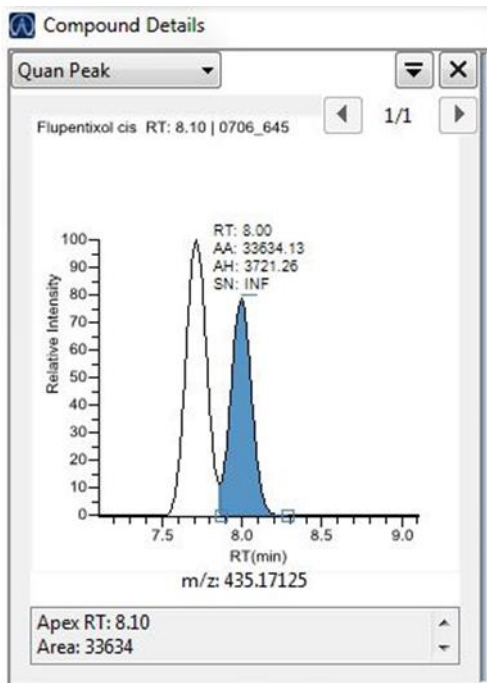


Figure 34 Flupentixol - patient sample with a ratio almost like in a pill, around 60% to 40% trans- to cis.

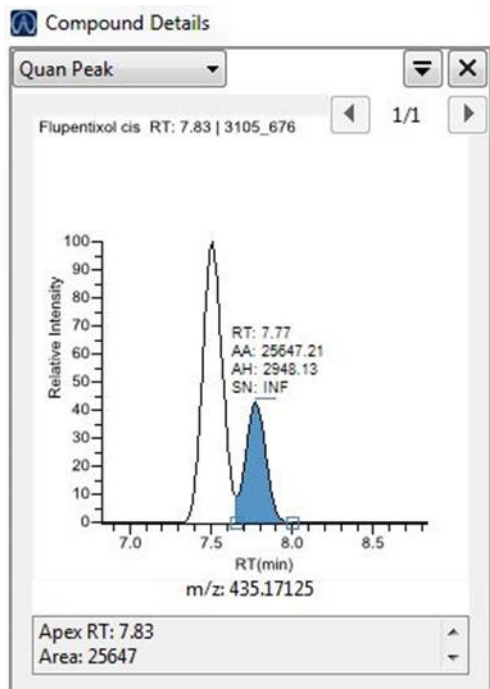


Figure 35 Flupentixol- patient sample with a high difference in ratio between two isomers, proportion of trans-form much higher than cis-form.

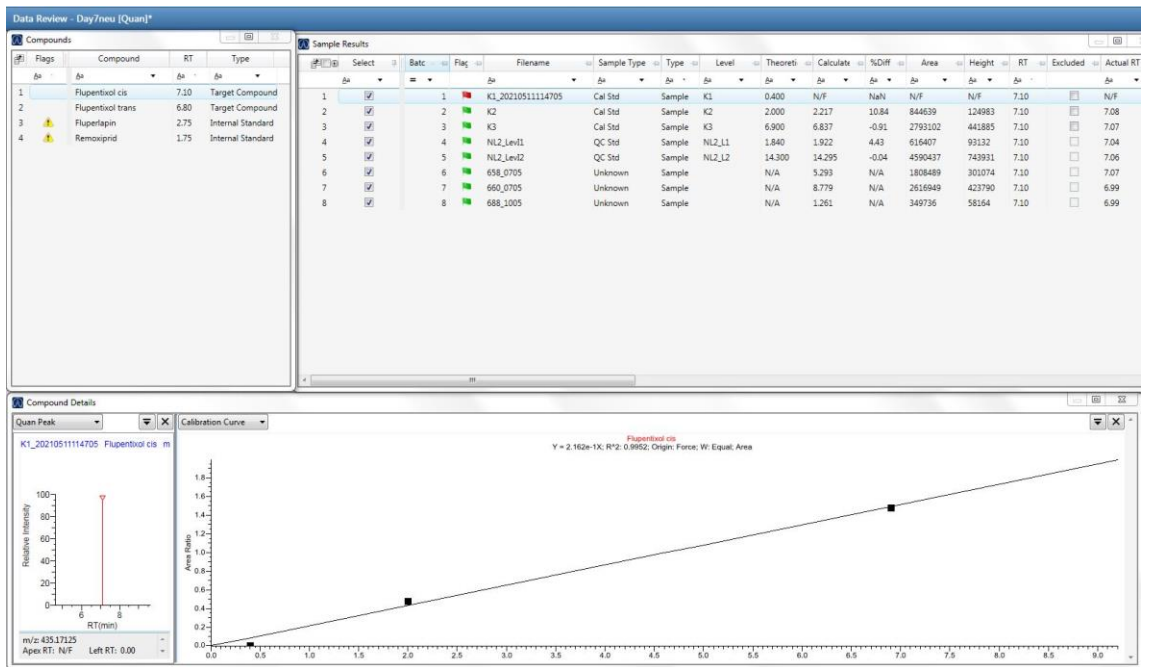


Figure 36 Example - Flupentixol one of the day-to-day measurements

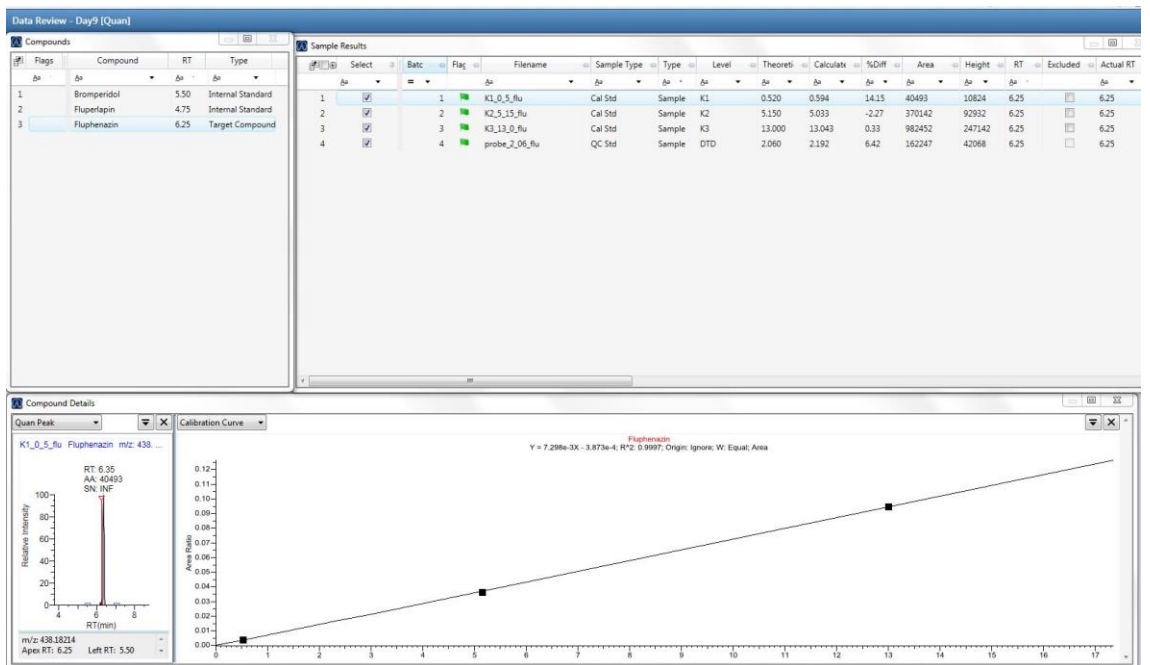


Figure 37 Example - one of the day-to-day measurements for Fluphenazine

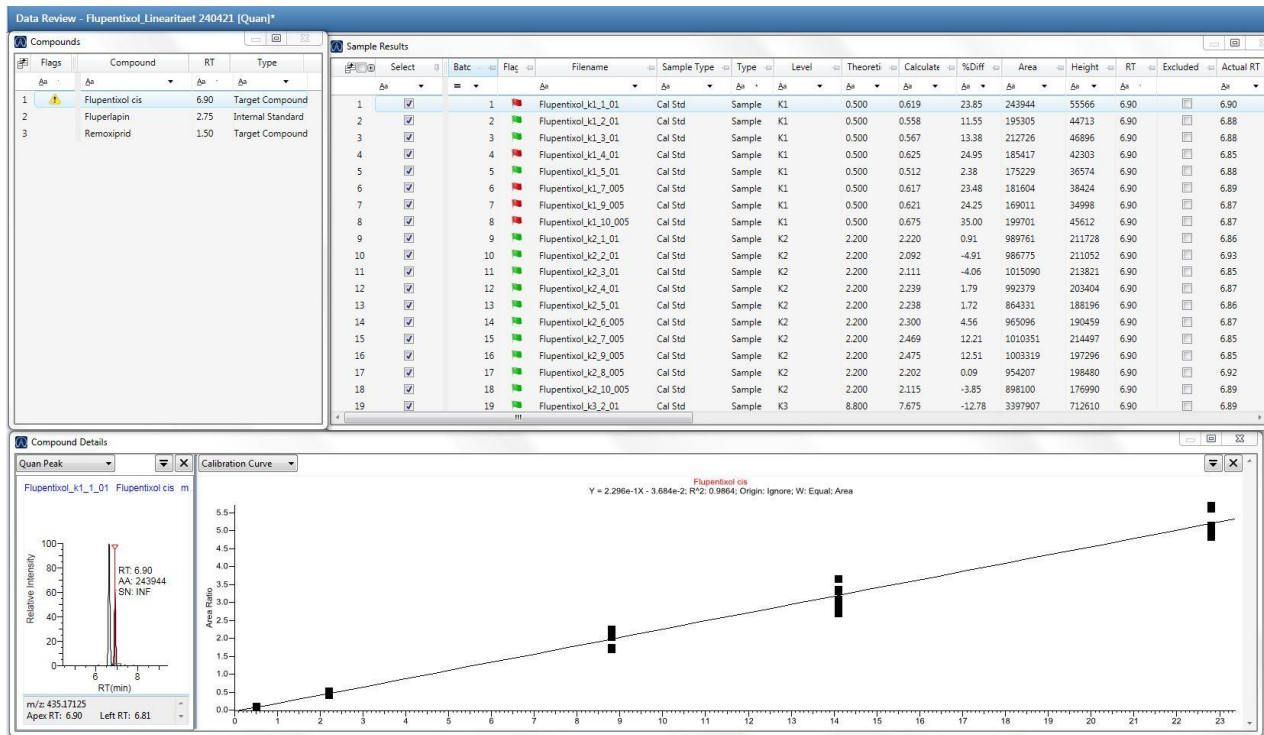


Figure 38 Example - the in-day measurement of Flupentixol

**Concentrations/Konzentrationen**  
**MassCheck® Neuroleptics 2/EXTENDED 2**  
**Plasma Control Level I**

Order no: 0228/XT2  
 Lot no: 1820  
 Exp. date: 2023-04-01

**Additionally Extended  
 Now 28 Parameters**

Substance Substanz	Method of analysis Analysemmethode	Unit Einheit	Target value* Zielwert*	Range Bereich
Amisulpride	LC-MS/MS	µg/l	53,8	43,0 - 64,5
Benperidol	LC-MS/MS	µg/l	1,93	1,54 - 2,32
Brexiprazole	LC-MS/MS	µg/l	34,8	27,9 - 41,8
Bromperidol	LC-MS/MS	µg/l	4,68	3,74 - 5,62
Cariprazine	LC-MS/MS	µg/l	9,30	7,44 - 11,2
Chlorpromazine	LC-MS/MS	µg/l	50,3	40,3 - 60,4
Chlorprothixene	LC-MS/MS	µg/l	46,2	36,9 - 55,4
Flupentixol	LC-MS/MS	µg/l	1,84	1,47 - 2,21
Fluphenazine	LC-MS/MS	µg/l	1,85	1,48 - 2,22
Guanfacine	LC-MS/MS	µg/l	1,67	1,34 - 2,00
Iloperidone	LC-MS/MS	µg/l	4,28	3,42 - 5,14
Levomepromazine	LC-MS/MS	µg/l	20,1	16,1 - 24,1
Loxapine	LC-MS/MS	µg/l	7,10	5,68 - 8,52

Figure 39 Package leaflet information - External Control Material - Neuroleptics 2, Level I



**Concentrations/Konzentrationen**  
**MassCheck® Neuroleptics 2/EXTENDED 2**  
**Plasma Control Level II**

Order no: 0229/XT2  
 Lot no: 1820  
 Exp. date: 2023-04-01

**Additionally Extended  
 Now 28 Parameters**

Substance Substanz	Method of analysis Analysemmethode	Unit Einheit	Target value* Zielwert*	Range Bereich
Amisulpride	LC-MS/MS	µg/l	438	350 - 525
Benperidol	LC-MS/MS	µg/l	8,36	6,69 - 10,0
Brexpiprazole	LC-MS/MS	µg/l	106	85,1 - 128
Bromperidol	LC-MS/MS	µg/l	20,4	16,4 - 24,5
Cariprazine	LC-MS/MS	µg/l	17,9	14,3 - 21,5
Chlorpromazine	LC-MS/MS	µg/l	428	343 - 514
Chlorprothixene	LC-MS/MS	µg/l	269	215 - 323
Flupentixol	LC-MS/MS	µg/l	14,3	11,4 - 17,1
Fluphenazine	LC-MS/MS	µg/l	14,4	11,5 - 17,2
Guanfacine	LC-MS/MS	µg/l	10,5	8,43 - 12,6
Iloperidone	LC-MS/MS	µg/l	8,87	7,10 - 10,6

Figure 40 Package leaflet information - External Control Material - Neuroleptics 2, Level II

## 8. Declaration of Authenticity

I, Agata Torzewska, hereby declare that the work presented herein is my own work completed without the use of any aids other than those listed. Any material from other sources or works done by others has been given due acknowledgement and listed in the reference section. Sentences or parts of sentences quoted literally are marked as quotations; identification of other references with regard to the statement and scope of the work is quoted. The work presented herein has not been published or submitted elsewhere for assessment in the same or a similar form. I will retain a copy of this assignment until after the Board of Examiners has published the results, which I will make available on request.

*Agata Torzewska*  
Kleve, 13.11.2021