Evaluation of post-mortem urine dipstick test and setting up of semi-quantification for benzodiazepines, tramadol, morphine and oxycodone on the LC-MS/MS

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Laymen's summary

This internship comprised two projects. The main project is about the performance of the urine dipstick test in death investigations. Death investigations are conducted by the police, a Public Health Service (GGD) and a forensic doctor to determine the cause of death. When an unnatural death is suspected, blood and urine samples of the decedent, if available, are send to the pharmacological laboratory of the Erasmus MC for toxicological tests. In addition, the urine dipstick test is used at the crime scene and gives a quick indication of the presence of 10 or 12 drugs in the urine. In this project, a digital database was made containing the results of the toxicological screenings on the blood samples, performed by a LC-MS/MS system in the Erasmus MC and the results of the urine dipstick test. This LC-MS/MS system can test blood samples for the presence of over 1200 drugs and other components. 164 post-mortem cases are used in this study to investigate whether there are differences between the results of the urine dipstick test strips of cocaine, MDMA and THC and the toxicological test results of the blood. For all these three drugs, cases were found for which both the urine dipstick test and the toxicological test on blood showed the presence of the drug. However, also cases were found for all three drugs in which the dipstick was false negative, meaning that the dipstick was negative, while the toxicological screening did pick up one of these drugs and/or the components to which the drug is turned into by the body, called metabolites. Furthermore, cases were found in which the dipstick for MDMA and THC were false positive, meaning that the dipstick was positive while the toxicological test did not show any presence of the drug or its metabolites in blood. Possible explanations for these false positive and false negative dipstick results are discussed in this report. For example, it could be that the drug was taken shortly before death in case of a false negative dipstick result. This could explain why the drug was present in blood but not yet in urine. In the future, the database will be filled with more cases, which will help recognizing patterns of factors that could be responsible for the false negative and false positive dipsticks, also for the other 9 dipstick test strips.

In a side-project, semi-quantification was set up for benzodiazepines, tramadol, morphine and oxycodone on two LC-MS/MS systems. After the blood samples are run on the LC-MS/MS, semi-quantification can be performed for certain compounds to make an estimation of the concentration. The hospital pharmacists in the Erasmus MC can then interpret the estimated concentration as either sub-therapeutic, therapeutic, supra-therapeutic or toxic. First, benzodiazepines, tramadol, morphine and oxycodone are added to separate samples of drug-free blood plasma in different concentrations. The samples are then prepared like they were blood samples from a patient to be measured on the LC-MS/MS. In one of these preparation steps, an internal standard is added. This is a specific component that is always added in the same concentration. This is done to see how well the system had worked, as the system sometimes picks up the presence of the internal standard better than other times. The samples containing the different concentrations of these drugs were then each measured 15 times on the LC-MS/MS systems. The responses are calculated for each measurement by comparing the outcome of the specific concentration of the drug to the outcome of the internal standard. The average responses for the different concentrations are used in a calibration curve, which will be used for the semi-quantification. An example of a calibration curve can be seen in **Figure 1**. Only for one benzodiazepine, called oxazepam, no calibration curve could be made because the measurements in both measured concentrations deviated too much from each other. Also for morphine, the calibration curve can only be made on one LC-MS/MS system, as the measured concentrations deviated too much from each other on the other LC-MS/MS system. In the future, samples of all the drugs for which semi-quantification has been set up must be measured periodically to check whether the responses would still be on the calibration curve and therefore, the calibration curves can still be used for semi-quantification.

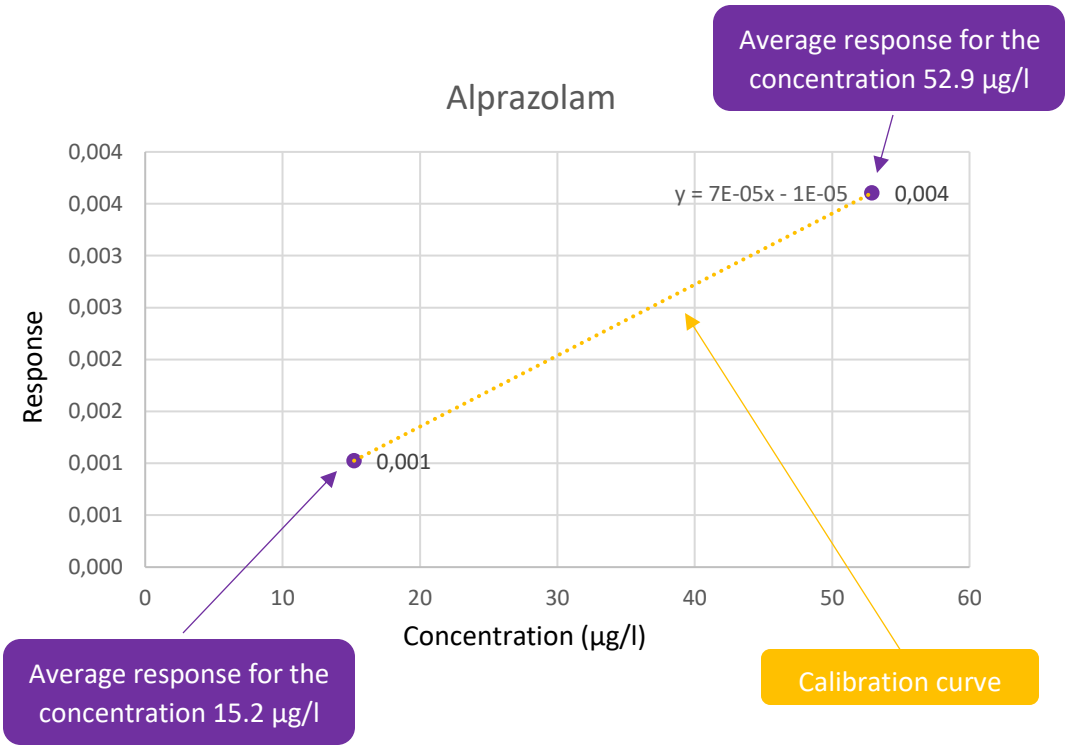

Figure 1 Example of a calibration curve. The responses are calculated for each measurement and the average responses for both measured concentrations (15.2 µg/l and 52.9 µg/l) of alprazolam are shown in this graph. The calibration curve is drawn between these two points and can be used for semi-quantification.

Introduction to two reports

In this internship, a digital database in Castor EDC was set up containing the results of the toxicological screenings of all the post-mortem cases that the pharmacy of the Erasmus MC has produced and analyzed. The main goal during my internship was to investigate the reliability of the urine dipstick test, while using the toxicological research on blood with the LC-MS/MS as a standard. Not only does this database contain the results necessary for my research like those of the LC-MS/MS on blood and the dipstick, it contains all the potentially relevant information that could be used for research in the future. This includes for example the results of the immunoassay on blood for ethanol, immunoassay for drugs of abuse (DOA) on urine, but also the semi-quantification results, additional tests and cause of death. In this way, the database will be used to its full potential and it will stay a useful pool of data for the future.

Because I wished to gain more practical experience in the laboratory, I also performed a smaller side-project to set up semi-quantification for benzodiazepines, tramadol, morphine and oxycodone on LC-MS/MS systems. These are drugs that are often seen in practice when performing toxicological screenings and of which the forensic doctor would like to know if the intake of these drugs has contributed to the death of the patient.

I enjoyed working on both projects, because together they gave me a deeper understanding of the process of the toxicological screening in death investigations. It was particularly interesting to see different causes of death pass by when filling in the database. In the end, I am most glad that my efforts have led to a database and semi-quantification data that the pharmacy is able to use in its daily work and research.

Acknowledgements

I would like to start by thanking Corine Bethlehem for supervising me during the start and larger part of my internship and teaching me all I had and wanted to know. I especially enjoyed working in her office as I learned a great deal from the discussions that occurred during a normal working day. Next, I would like to thank Dr. Brenda the Winter for taking over the supervision near the end of my internship. Also, I would like to thank Prof. Dr. Dylan de Lange and Prof. Dr. Birgit Koch for making time to read this report and attend my final presentation. Finally, I would like to thank the whole pharmacy laboratory department for having me as a part of their group and involving me in their meetings, in which I have learned a great deal. It is important to mention that this research is supported by the police units Rotterdam, Den Haag, Midden-Nederland and Oost-Brabant, by the Forensisch artsen Rotterdam Rijnmond (FARR) and the GGD regions Brabant-Zuidoost, Flevoland, Gooi en Vechtstreek, Haaglanden, Hart voor Brabant, Hollands Midden and Utrecht.

Report 1

Reliability of Post-mortem uRinE DIpstiCk Test (PREDICT)

Abstract

INTRODUCTION Death investigations are conducted by the police, a Public Health Service (GGD) and a forensic doctor to determine the cause of death. In case of a (suspected) unnatural death, post-mortem blood and urine samples, if available, are sent to the pharmacological laboratory of the Erasmus MC for toxicological research. Additionally, a urine dipstick test is used at the crime scene to give a quick indication of the presence of 10 or 12 drugs in the urine. The research question is: How often do discrepancies occur between the urine dipstick test strips of cocaine, MDMA and THC and the toxicological test on blood performed by LC-MS/MS? **MATERIALS & METHODS** In this retrospective study, a database was created in Castor EDC in which the results of all previously performed urine dipstick tests and toxicological tests performed on the LC-MS/MS could be gathered. **RESULTS** Due to time limitations, only 311 cases were put in this database, of which 164 cases were included for this study. Overall concordance of the cocaine dipstick results with the toxicological test results on blood by LC-MS/MS was 97%, with 3.0% false negative cocaine dipstick test strips (n=5). For MDMA, the overall concordance was 97.6%, with 1.2% false negative (n=2) and 1.2% false positive (n=2) MDMA dipstick test strips. Finally, the overall concordance for THC was 86.0%, with 0.6% false negative (n=1) and 13.4% false positive (n=22) THC dipstick test strips. **CONCLUSION & DISCUSSION** There was a discrepancy between the urine dipstick test and the toxicological test on the LC-MS/MS on blood in 3.0% of the cases for cocaine, 2.4% for MDMA and 14% for THC. Possible explanations for false negative results are a low drug concentration in urine, consumption of the drug shortly before death and dilution of urine by decomposition fluids. Possible explanations for false positive dipstick results of MDMA and THC are a lower sensitivity of the LC-MS/MS, post-mortem redistribution and formation of amino acids after a high PMI. In the future, the database will be filled with more cases, which will help recognizing patterns of factors that could be responsible for the false negative and false positive dipsticks, also for the other 9 dipstick test strips.

List of abbreviations

BE	benzoylecgonine
DOA	drugs of abuse
EME	ecgonine methyl ester
FARR	Forensisch artsen Rotterdam Rijnmond
GGD	Gemeentelijke Gezondheidsdienst or Public Health Service
GHB	Gamma-Hydroxybutyric acid
MDA	3,4-methyleendioxyamfetamine
MDMA	methylenedioxymethamphetamine
PMI	post mortem interval
Δ9-THC	Δ9-tetrahydrocannabinol
THC	tetrahydrocannabinol
WMO	Wet Medisch-Wetenschappelijk Onderzoek

Introduction

Death investigations are conducted by the police and a Public Health Service (GGD) or Forensisch artsen Rotterdam Rijnmond (FARR) in order to determine the cause of death and therefore find crimes (Bethlehem & Koch, 2020). Samples of blood and urine, if available, are send to a laboratory for toxicological research by a forensic doctor, who is in service of a GGD or the FARR and is responsible for the toxicological investigation. In the police regions Rotterdam, Den Haag, Midden-Nederland and Oost-Brabant, the samples are sent to the pharmacy laboratory of the Erasmus MC. If both urine and blood samples are sent to the Erasmus MC, toxicological screening of blood on the LC-MS/MS, immunoassay on blood for ethanol and the immunoassay for drugs of abuse (DOA) screening on urine are standardly conducted. In this DOA screening, the urine is checked for the presence of amphetamines, benzodiazepines, barbiturates, cocaine, cannabis, gamma-Hydroxybutyric acid (GHB), opiates, methadone and the level of creatinine is measured. This toxicological research can give more insight into the cause of death and to determine whether the death was natural or unnatural. It can also help in the decision to conduct a section or not (FARR, 2017). In 2016, a toxicological urine dipstick test was introduced as a standard screening within these death investigations. This urine dipstick can be used at the crime scene and gives a quick indication of the presence of 10 or 12 drugs in the urine. When the dipstick was first implemented it contained 10 test strips: amphetamine, barbiturates, benzodiazepines, cocaine, methadone, morphine, methamphetamine, methylenedioxymethamphetamine (MDMA), tetrahydrocannabinol (THC) and tricyclic antidepressants. Since February 2019, the test has been expanded with test strips for fentanyl and oxycodone. The results of the urine dipstick can lead to more elaborate investigations at the crime scene or the implementation of follow-up research (Bethlehem & Koch, 2020). In 53% of the death investigations, the urine dipstick test resulted in one or more positive test strips (Ceelen et al., 2010).

It is known that false positive and false negative test results can occur (Ceelen et al., 2010). However, it is not yet investigated thoroughly how many false dipstick results occur post-mortem. An evaluation study in 2018 in the region Rotterdam-Rijnmond showed that the result of the urine dipstick test did not match with the results of the toxicological research on blood and urine in 13% of the death investigations (Bethlehem & Koch, 2020). A false positive dipstick result could lead to unnecessary follow-up research and delay in upscaling or finishing of a death investigation, while the goal of the urine dipstick test was to give clues quickly. Moreover, a false negative result could cause a lead to be overlooked, which could result in follow-up research not to be conducted and possibly a crime to be overlooked. Therefore, it is important that the police investigators know how to perform the urine dipstick test and know its pros and cons.

The different test strips of the dipstick test should turn positive when certain concentrations of certain analytes are present. The incorrect results can have different causes. A false positive result can be caused by a reaction with another analyte for which it should not turn positive. This is called a cross reaction. The manufacturer only found compounds which did not lead to a false positive result. So, the research that has been done on these cross reactions is limited (UltiMed, 2018). Literature showed that the use of medication could cause false positive results (Lewandrowski et al., 2008). Practice showed that due to a high concentration, specificity of a test could get lost. For example, a high concentration of either amphetamine, MDMA or methamphetamine

could give a simultaneous positive result on all three of these test strips. False negative results could be caused by the presence of a certain analyte that blocked the performance of the dipstick test. Also, the concentration of the present analyte could be below the cut-off limit value, which would then also result in a negative test strip. Also, a possible cause for both false positive and false negative results could be the incorrect reading or interpretation of the dipstick result. Other possible explanations of a negative dipstick are given by Drummer. If the death followed shortly after the intake of the drug, the drug could be detectable in blood but not yet in urine. Further, the concentration of different drugs could decrease during the keeping of the samples after the samples are taken, but also during the post-mortem interval (PMI). The PMI is the time between the death and the taking of the samples (Drummer, 2004).

Gonzales *et al.* investigated the stability of multiple drugs and their metabolites in urine, including MDMA, cTHC and benzoylecgonine (BE), a metabolite of cocaine. This study shows that at 4°C and -20°C, the percent differences of MDMA and BE were within 10% of the original measurement (at time 0) when measured after 2,3 and 6 months. However, the average difference of cTHC appeared to be 40% or above when storing for 3 of 6 months at 4°C and -20°C (Gonzales et al., 2013). Another topic to consider is the post-mortem stability. Post-mortem changes occur for all the drugs of abuse, but the extent of these changes is significantly variable between drugs. Hydrolysis can even occur in the collection vessel if no special precautions are undertaken (Drummer, 2004). Caution should be taken when attempting to interpret the significance of any results. Key elements for this interpretation are the stability of the drug in the case generally and in the specimen particularly, quality and state of the specimen, and the effects of drug diffusion away from or to other tissues. Decomposition and eventually, the liquefaction of tissues, occurs during post-mortem periods and is heavily depending on the PMI, the temperature and other environmental factors. Weeks at freezing temperatures often showed little detectable changes while one day in a hot environment could result in significant putrefaction (Drummer, 2004).

Cocaine, MDMA and THC were chosen for this study because they are three of the most used drugs in the Netherlands. Cannabis is the most used drug in the Netherlands but also in other western countries. In 2020, 22.9% of the Dutch population of 18 years and older had once used cannabis. In the year 2020 itself, 7.8% of this population had used cannabis. Most of these users were between 18 and 24 years old. 1.7% of this population was a daily or almost daily user of cannabis in 2020 (Trimbos, 2021). The main active cannabinoid is Δ9-tetrahydrocannabinol (Δ9-THC) and the other cannabinoids that show significant activity are Δ8-tetrahydrocannabinol, Δ9-tetrahydrocannabivarol and cannabinol. Most forms of cannabis have THC yields of 2-8%, but can also be over 20%. Plasma concentrations of THC in blood exceed 50 ng/ml within 15 min of smoking and can reach 200 ng/ml with cigarettes with a higher content of THC. THC is quickly distributing to muscle and fat because of its low water solubility. This results in a reduction of THC concentration in blood plasma. The half-life in this phase of distribution is less than 1 hour. After this 1 hour, even when moderate to high doses of cannabis are consumed, THC concentrations in plasma greater dan 10 ng/ml are rare (Drummer, 2004). The terminal elimination half-life of THC is 3 to 13 days, but blood concentrations are normally below 2 ng/ml a few hours after last use. Only highly sensitive analytical methods are able to detect the terminal stages of drug elimination. This is demonstrated by Johansson *et al.* who use an improved GC/MS assay. As an explanation for the long terminal elimination half-live they give the possible explanation that this is due to the redistribution from the adipose tissues into the blood before excretion (Johansson, Halldin, Agurell, Hollister, & Gillespie, 1989).

MDMA is the purest form of ecstasy. Of the Dutch population of 18 years and older in 2020, 9.7% had once used ecstasy. The users were mostly between 20 and 29 years old. In 2020 itself, 3.1% of that population had used ecstasy. The percentage of the population that has used ecstasy (in their lives but also in the last year) in the Netherlands is much higher than in other European countries (Trimbos, 2021). Consumption of 100 mg MDMA results in peak levels of 0.4 mg/l after 2 hours. A single dose of 75 or 125 mg MDMA significantly increases the systolic blood pressure up to 10 mmHg, the heart rate to 30 beats/min, and the pupillary diameter (mydriasis), but not the body temperature. The maximum plasma concentration of MDMA after a single dose is about 0.13 mg/l after 2.4 hours and 0.24 mg/l after 1.8 hours. The terminal elimination half-life is about 8 hours for both low and high MDMA doses (Drummer, 2004).

Of cocaine, 1.6 % of the Dutch population of 18 years and older had used it in the year 2020. Cocaine is mostly used as a powder to snort (Trimbos, 2021). Cocaine is a potent stimulant of the nerve function. It inhibits the reuptake of dopamine, norepinephrine and serotonin in the nerve terminals in the CNS. The terminal elimination half-life of cocaine is between 40 min to 4 hours, depending on the dose. Cocaine is rapidly metabolized and BE and ecgonine methyl ester (EME) are the most significant metabolites. If ethanol is co-consumed, cocaethylene is found in significant amounts in the tissue. The formation of cocaethylene does not occur post-mortem, which suggests the active involvement of enzymes. Anhydroecgonine methyl ester or methylecgonidine is only formed when smoking cocaine. The profile of activity is different to that of cocaine, as it acts as a muscarinic agonist to lower the blood pressure. BE and EME can be used to identify past use of cocaine if the parent drug is no longer present in the blood. In urine, BE and EME are predominately present, although about 1 to 9% is cocaine. Detection time is about 1 to 4 days when using a 300 ng/ml cut-off, but can be a week in long term users. Greater amounts of EME and cocaethylene arise when used orally, because of the first-pass metabolism. A common doses of cocaine ranges between 10 to 100 mg. However, tolerance can occur quickly resulting in a rapid escalation of daily doses up to over 1 g. There is no defined safe or therapeutic blood concentration. Next to this, post-mortem hydrolysis continues after death. So, measurements of cocaine concentrations are unlikely to result in useful interpretive information. Excessive use of cocaine can result in life threatening conditions like pathological core temperatures leading to rhabdomyolysis, convulsions, ischemic heart disease typified by contracting bands and sudden arrhythmic death, intravascular coagulation and renal failure. Using cocaine with heroin, alcohol and other narcotics will significantly increase the toxicity (Drummer, 2004).

In this retrospective study, the reliability of the test strips of cocaine, MDMA and THC of the urine dipstick test were investigated. The research question is: How often do discrepancies occur between the urine dipstick test strips of cocaine, MDMA and THC and the toxicological test on blood performed by LC-MS/MS? An in-house database was created in which all previously performed urine dipstick and toxicological test results were gathered from cases that were send to the Erasmus MC. These are now around 1500 cases, but not all of these were entered in the database during this project due to time limitations. With this data, it was investigated how many similarities and discrepancies there are between the results of the urine dipstick test and the toxicological test on blood. Control variables were then investigated that could explain the discrepancies, for example when the body was found in water, a certain average temperature, PMI or a new cross reaction.

Materials & methods

Dipstick

The DrugControl MultiDip (UltiMed) test, in this paper referred to as the dipstick, was conducted

on the urine specimens collected by a forensic doctor at the crime scenes. The dipstick was conducted according to the protocol of Multi DrugControl MultiDip 008SLM12 (UltiMed, 2018) by a forensic doctor at the crime scene. Before February 2019, the dipstick consisted of 10 test strips: Amphetamine, barbiturates, benzodiazepines, cocaine, methadone, methamphetamine, MDMA, morphine, THC and tricyclic antidepressants. Since then, either the dipstick with 10 or the dipstick with 12 test strips, to which fentanyl and oxycodone were added, was used. Normally, the forensic doctor informed the hospital pharmacist of the Erasmus MC of the dipstick results.

Toxicological analysis

Blood samples were collected by a forensic doctor in service of the GGD or FARR and sent to the Erasmus MC. The blood samples were prepared to be measured in the LC-MS/MS (Waters) according to the protocol of toxicological screening of the Erasmus MC. In short, the blood samples were centrifuged so that 125 µl of plasma could be derived. 125 ul ice cold sample-prep was added to this plasma sample and this was vortexed for about 30 seconds and then centrifuged for 5 minutes at 14680 rpm. 100 ul of the supernatant was pipetted in a clean autosampler vial to which 400 ul MilliQ was added. The sample was subsequently vortexed for at least 10 seconds and the sample was then ready to be measured in either the micro 1 or the micro 2, which were both LC-MS/MS systems. A sample with blanco plasma was always taken along. The results of the toxicological screening were checked in MassLynx and the results were discussed with an authorized analyst and a hospital pharmacist.

Building of database

The database was created in the program Castor EDC, which is an online validated data management system, designed for the collection of clinical research data. Therefore, the system has an audittrail. The following data was collected for every deceased person: demographic data (gender, age, place of deceasing), results of dipstick on urine, results of toxicological tests on blood and control variables (PMI, average temperature if the body had been outside, finding place of the body and known home medication). Identifiable data are pseudonymised and recorded via a research number in the database.

Ethical evaluation

Because this was a retrospective study that involves departed persons, no approval was needed of an ethics committee. Nonetheless, the study plan of the project is submitted for a statement that the study is not encompassed by the law of medical-scientific research (in dutch: Wet Medisch-Wetenschappelijk Onderzoek or WMO, in this case non-WMO) at the Medisch Ethische Toetsingscommissie (METC) of the Erasmus MC. In this application it is also guaranteed that the study will be conducted according to the Dutch behavioural code for scientific integrity (KNAW; NFU; NWO; TO2-federatie; Vereniging Hogescholen; VSNU, 2018).

Results

Data refinement

Available data and the results of the toxicological analysis of 311 post-mortem cases from the period 2019 until present were entered into the database. From these cases, 1 case had to be excluded because the patient was still alive when samples were taken. Another 143 cases had to be excluded because either no dipstick was performed at the crime scene or it was unknown to the Erasmus MC whether a dipstick was performed. From the 167 cases left, again 3 cases were excluded because no blood sample was sent to the Erasmus MC or the blood sample was unusable to perform toxicological screening with the LC-MS/MS. This resulted in 164 cases

suitable to analyse the similarities and differences between urine dipstick and blood LC-MS/MS results. In 54.9% (n = 90) of these cases, the urine dipstick test resulted in one or more positive test strips.

Cocaine

The dipstick was positive for cocaine in 23 cases, in these cases no discrepancies could be found, meaning that either cocaine, BE and/or EME was found in the blood. These are analytes which are known to result in a positive cocaine dipstick according to the protocol of Multi DrugControl MultiDip 008SLM12 (UltiMed, 2018). In five cases, the dipstick was negative, yet cocaine, BE and/or EME were found in the blood. The chart in **Figure 1** depicts these cases in which cocaine was found. For these five false negative dipstick cases, there was no resemblance in the PMI, place the body was found or temperature (**Supplementary Table 1**). Interestingly, for four of these cases, urine was also sent to the Erasmus MC for a DOA screening. No cocaine was found in the DOA screenings of these four urine samples. In two cases, EME was found, in two others cocaine and BE were found and in the last case only BE was found in the blood.

Overall concordance (total n=164) of the cocaine dipstick results with the toxicological test results on blood by LC-MS/MS was 97%, with 3.0% false negative cocaine dipstick test strips (n=5)

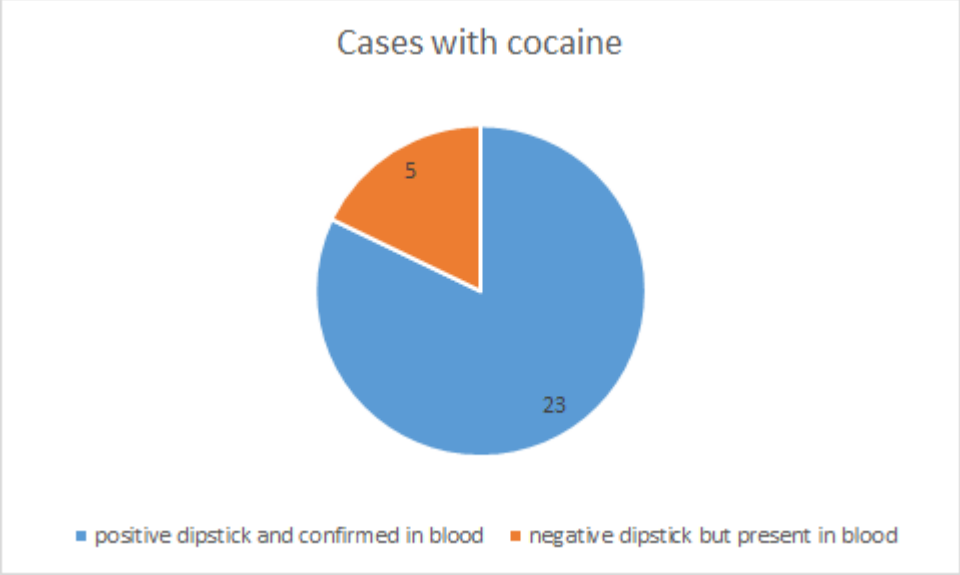

Figure 1 Overview of the number of cases in which cocaine was present. In all these 28 cases, cocaine, BE and/or EME were present in the blood. In 23 cases, the dipstick also tested positive for cocaine (blue), but in 5 cases the dipstick was negative for cocaine (orange).

MDMA

For MDMA, the dipstick was positive in six cases, of which two had discrepancies. This means that in two cases, no MDMA, (±) 3,4-Methylenedioxy-amphetamine HCl and/or 3,4-Methylenedioxyethyl-amphetamine were found in the blood which are known to result in a positive dipstick (protocol of Multi DrugControl MultiDip 008SLM12 (UltiMed, 2018). In these two false positive cases other factors that could have influenced the result of the dipstick were explored. No analyte was found that was present in both cases, neither was there amphetamine or methamphetamine present of which it is known that a high concentration can result in a positive MDMA dipstick. For one of these two cases, it was suspected that the decedent had consumed MDMA. Both decedents used omeprazole as home medication, but it was not found in the blood of both decedents. One of the decedents

had a PMI of 4.5 hours, was found in the water outside while the average day temperature was 11.1°C. For the other, the PMI was 8.25 hours, there was no information about where the body was found and the average day temperature was 14.6°C. Additionally, there were two cases in which the dipstick was false negative. An overview of the number of cases in which MDMA was found in blood or urine can be found in **Figure 2**. In one of these two false negative cases 3,4-methyleendioxyamfetamine (MDA) and MDMA were found in blood and in the other case MDMA was found in blood. No similarity was found in the home medication between these two cases. In one case, the time of death was an estimation of more than 24 hours. The PMI in this case was 60 hours and 20 minutes, there was no information on where the body was found and the average temperature in the middle of that period was 18.3°C. For the other case, the PMI was 3.93 hours, the body was found on land (not in water) outside and the average temperature was 13.9°C. One similarity was that cocaine and benzoylecgonine were found in the blood in both cases. There are no cases in which the dipstick was positive for MDMA but also cocaine, BE and/or EME were found in blood.

Overall concordance (total n=164) of the MDMA dipstick results with the toxicological test results on blood by LC-MS/MS was 97.6%, with 1.2% false negative (n=2) and 1.2% false positive (n=2) MDMA dipstick test strips.

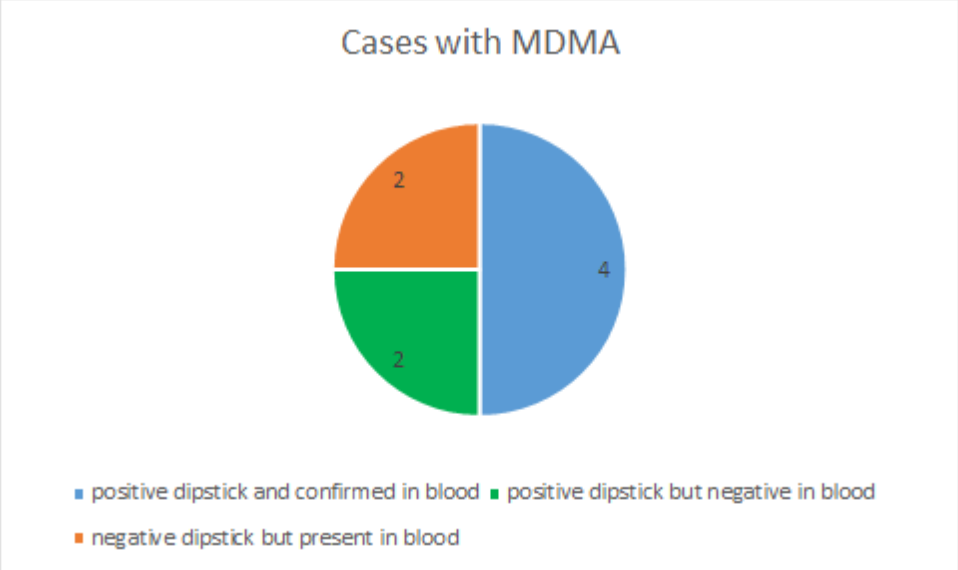

Figure 2 Overview of the number of cases in which the LC-MS/MS found MDMA and/or the dipstick was positive for MDMA. In six cases, MDMA was present in the blood (blue and orange). However, in two of these cases, the dipstick was negative for MDMA (orange). Next to this, there were two cases in which the dipstick was false positive, as no MDMA was found in the blood (green).

THC

The dipstick was positive for THC in 30 cases, of which 22 cases had discrepancies. This means that in those 22 cases, no 11-nor-Δ9-THC-9 COOH, 11-nor-Δ8-THC-9 COOH, cannabinol, Δ8-THC and/or Δ9-THC were found in the blood which are known to result in a positive THC test strip on the dipstick (UltiMed, 2018). Therefore, these 22 dipstick results are called false positive. Compared with cocaine and MDMA, THC has the highest percentage of false positive dipstick results. Of these 22 discrepancies, 9 times the THC metabolite called THC-COOH glucuronide was found in blood when the dipstick should not be sensitive for this. There is only one case in which THC-COOH glucuronide is present in the

blood, but the dipstick was negative, but in this case the dipstick was false negative as also 11-nor-Δ9-THC-9 COOH was found (this case is described later in this paragraph).

That leaves 13 cases for which the dipstick was false positive. Factors that could have influenced the dipstick result are examined. There was not one analyte that was present in the blood of all these cases, nor was there a drug that was prescribed as home medication to all of these decedents. Interestingly, two of these cases were also the cases in which MDMA was found in the blood but the dipstick was false negative for MDMA. On top of that, among these false positive THC cases, there was also one case of which the MDMA dipstick was false positive. For all these 13 cases, also urine was sent to the Erasmus MC and a DOA screening could be performed on this material. Notably, in only 5 cases, THC was also found in the urine. For these 8 cases in which no THC was found in the urine in the DOA screening, there was no resemblance in the PMI, place the body was found or temperature (**Supplementary Table 2**). Hopefully when the database has been filled with more cases, a relation can be found between a certain factor and the positive dipstick results for THC. For the 5 cases in which THC was found in the urine by the DOA screening, again it was checked whether a certain analyte was present in the blood or was prescribed as home medication for all these decedents. However, no such analyte was found. In three of these 5 cases, the PMI was within 9 hours. In none of these cases the body was found in water. A more elaborate overview of the control variables can be found in **Supplementary Table 3**. Again, there was not one control variable that could be pointed out as the cause of these false positive dipstick results.

Furthermore, in one case the THC dipstick was false negative, as 11-nor-Δ9-THC-9 COOH, a metabolite of THC, was found in the blood. Also, THC-COOH glucuronide was present in this blood sample. An overview of the number of cases in which THC was found in blood or urine can be found in **Figure 3**. In this false negative dipstick case, the body was found on land (not in water) and inside. The PMI was unknown and the average temperature would not have been an influence on the state of the body.

Overall concordance (total n=164) of the THC dipstick results with the toxicological test results on blood by LC-MS/MS was 86.0%, with 0.6% false negative (n=1) and 13.4% false positive (n=22) THC dipstick test strips.

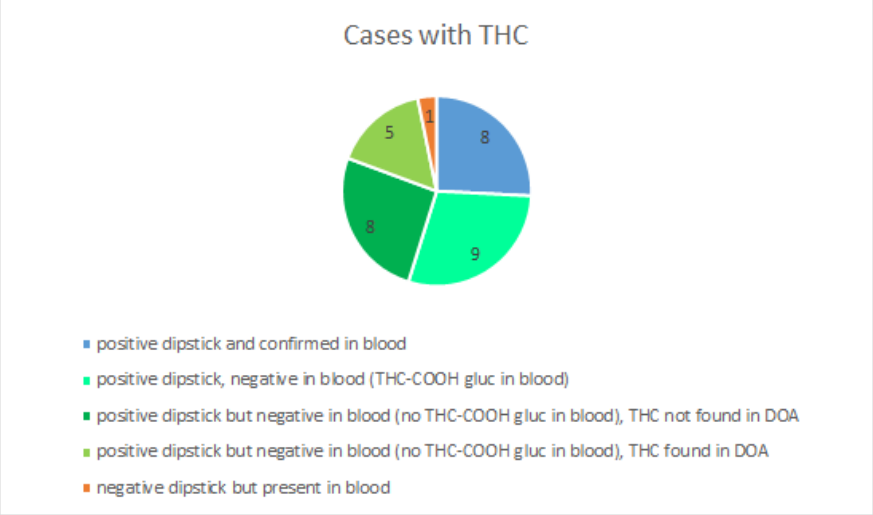

Figure 3 Overview of the number of cases in which the LC-MS/MS found THC and/or the dipstick was positive for THC. In nine cases, THC was present in the blood (blue and orange). However, in one of these cases, the dipstick was negative for THC (orange). Next to this, there were 22 cases in which the dipstick was false positive, as no THC or its metabolites which are known to make the dipstick positive for THC were found in the blood (three green colors). In nine of these false positive dipstick cases, THC-COOH glucuronide was found in the blood, for which the THC test strip of the dipstick apparently also turns positive (bright green). Of the 13 other unexplainable false positive dipstick cases, 8 cases had THC present in the urine (dark green) and the other 5 cases had no presence of THC in the urine (light green), as was shown by DOA screenings.

Conclusion and discussion

In conclusion, using toxicological testing with LC-MS/MS as a standard, discrepancies with dipstick results were found for THC, cocaine and MDMA. The dipstick was positive for cocaine in 23 cases in which the presence of cocaine or one of its metabolites was confirmed in blood. However, 5 cases were found in which the dipstick was negative for cocaine, yet cocaine, BE and/or EME were found in the blood. So, 3% of the cocaine dipstick test strips were false negative. Possibly the concentrations of cocaine and its metabolites were too low for the dipstick to pick up. This makes especially sense for those two cases in which only EME was found and for that last case in which only BE was found in the blood, since there was no more parent drug present in these samples. Because the metabolism of cocaine is so rapid and this can also continue after death, it is possible that cocaine and a metabolite are present in the blood but not yet in the urine, especially if cocaine was consumed shortly before death. For MDMA, the dipstick was positive in 4 cases in which the presence of MDMA was confirmed in blood as well. In 2.4% there was a discrepancy between the urine dipstick test for MDMA and the toxicological test on the LC-MS/MS on blood. In 1.2% (n=2), the dipstick was false positive as there was no presence of MDMA detected in the blood. An explanation could be that the dipstick is more sensitive to MDMA than the LC-MS/MS. Another possible explanation could be that the decedents had consumed MDMA some time before their death, causing MDMA to be no longer present in the blood but still present in the urine. Both decedents used omeprazole as home medication, but it is unlikely that has influenced the dipstick result. Omeprazole is one of the drugs with the most users in the Netherlands (Stichting Farmaceutische Kengetallen, 2021), therefore one would expect omeprazole to be responsible for many more false positive dipstick results which is not the case. The sensitivity of the LC-MS/MS for omeprazole is presumably very low, as the drug is not often found in blood, though it has many users. In another 1.2% (n=2), the dipstick was false negative, as MDMA was found in blood. Maybe the concentrations of MDMA were below the cut-off limit value of the MDMA dipstick, but just high enough in the blood to be picked up. Or maybe MDMA was consumed shortly before death and it was not yet present in the urine. Interestingly, cocaine and BE were found in the blood of both cases. Therefore, it could be possible that cocaine and/or BE block the function of the MDMA dipstick test strip. To confirm this, more cases should be filled in the database, because this is only based on two cases and could therefore also be a coincidence. However, this could also be tested by spiking urine with both MDMA and cocaine and/or BE and testing whether the dipstick turns positive for MDMA or not. If not, cocaine and/or BE could indeed be responsible for the false negative MDMA dipsticks and this could be valuable knowledge in death investigations. Lastly, the dipstick test strip for THC was positive in 30 cases, but only in 8 cases was the presence of THC and/or its metabolites which are known to result in a positive dipstick confirmed in blood. In 14.0% there was a discrepancy between the urine dipstick test for THC and the toxicological test on the LC-MS/MS on blood. In 9 cases, the THC metabolite THC-COOH glucuronide was present in the blood, but the dipstick should not be sensitive for this metabolite according to the user guide (UltiMed, 2018). Since the dipstick was positive for THC in these 9 cases, it could be possible that the dipstick is sensitive for THC-COOH glucuronide. To be sure, drug-free urine could be spiked with THC-COOH glucuronide and the THC dipstick test strip could be used on this sample. Still, in another 13 cases the dipstick test

strip for THC was false positive. In three of these false positive cases, the PMI was multiple days, as can be seen in **Supplementary table 2**. It is known that amino acids can be produced due to the breakdown of proteins in a decaying body (Boumba, Ziavrou, & Vougiouklakis, 2008)(Butzbach, 2009). It could be possible that these amino acids are responsible for the false positive THC dipstick. Another possible explanation could be that THC was consumed a long time before death. THC would then not be present in the blood or the concentration would be too low in the blood to be picked up, but the THC would still be present in the urine. Two explanations for all these false positive THC dipstick test strips that are more likely, are as follows. It is known in the laboratory of the pharmacy in the Erasmus MC that the LC-MS/MS is possibly not very sensitive to THC and its metabolites. Next, it is known that a reduction of THC concentration in blood plasma can happen rapidly due to distribution to muscle and fat tissue (Drummer, 2004). Therefore, it could be possible that the presence of THC and its metabolites are missed in the blood. Normally, this would not have an impact on the cause of death, since consumption of THC presumably does not result in acute death. In these false positive cases, the DOA results could best be used as a standard. So, 13.4% (n=22) of the THC dipstick test strips were false positive, but it seems that for most of these cases this can be explained by the presumably low sensitivity of the LC-MS/MS for THC and by the post-mortem redistribution of THC. Additionally, in 0.6% (n=1), the THC dipstick test strip was false negative, because the THC metabolite called 11-nor-Δ9-THC-9-COOH was found in the blood. In this case also THC-COOH glucuronide was present in the blood. A possible explanation for this case could be that THC was consumed shortly before death. This would result in the presence of THC in the blood but not yet in the urine. Another factor that may have contributed to this phenomenon is that the concentration in the urine might have been just too low for the dipstick and the DOA to pick up, but in the blood it was just high enough for the LC-MS/MS to pick up. In general, in case of a false negative result, decomposition fluids could play a role in diluting the urine, causing the concentration of the drugs and their metabolites to drop.

Ceelen *et al.* found that in 53% of the death investigations, the urine dipstick had one or more positive test strips. Their study population also consisted of decedents that have suffered a (potentially) unnatural death (Ceelen et al., 2010). Their percentage is comparable to our finding, namely that in 54.9% of the cases of which blood samples were send to the Erasmus MC and a urine dipstick was performed, the urine dipstick was positive for one or more test strips. Only in our study population, sometimes a dipstick containing test strips for 12 drugs instead of 10 drugs was used, but this was not very frequently used.

Prevalence in usage of THC, MDMA and cocaine in the Dutch population is different from the presence of these drugs in the decedents of this study. THC use is most prevalent in both populations. However, MDMA is used by more people in the Dutch population than cocaine (Trimbos, 2021), whereas in the post-mortem population, cocaine was present in more cases (n=28) than MDMA (n=8, but 2 of these cases had a false positive MDMA dipstick). This may be because cocaine is more addictive than MDMA (Trimbos, 2020). This is also evident when considering the number of people that have used cocaine in the year 2020 and want to stop using cocaine (34.2%) and the number of people that have used MDMA in the year 2020 and want to stop using it (13.0%). This explains the difference in frequency of use. Of the users of the year 2020, cocaine was used by 32% in the last month, while MDMA was used by 19% in the last month (Monshouwer et al., 2021). So, even though there are less people consuming cocaine than MDMA, cocaine is consumed more often by these people and this can explain why cocaine

was found in more post-mortem cases than MDMA.

There were some impediments when filling in the database. If it was unclear whether a dipstick with 10 or with 12 test strips was used, the database had to be filled in as if a dipstick with 10 test strips was used, since we did not want to draw false conclusions about the test strips for fentanyl and oxycodone. However, it was hardly ever clear which dipstick was used, making the data available on fentanyl and oxycodone very little. Luckily, it is now specifically asked to the forensic doctor which dipstick is used so that more data can be collected for these two drugs. Additionally, another difficulty was interpreting the dipstick result when only boxes were ticked by the forensic doctor which were meant for indicating which drug they wanted to test for. In general, these boxes were used to indicate the dipstick test result. However, sometimes it was not explicitly written next to the boxes that it showed the dipstick test results and it could also mean that the forensic doctor was interested in those drugs and wanted the Erasmus MC to test for that. Fortunately, this was only dubious in some cases and the hospital pharmacists explicitly ask the forensic doctor for the dipstick test result. Next, the PMI is an estimation that could deviate, even in hours. True, it is indicated whether the estimation of the time of death is more than 24 hours. Nonetheless, sometimes when the estimation was within 24 hours, it was still an estimation of a few hours. This will make it very difficult to say with great certainty wat the average PMI was. Yet, it can be used to give an indication about approximately how long the body had been laying before the samples were taken and this information could be very useful and insightful. Finally, the average temperature does not always give a good view of the temperatures the body has been exposed to. On some days the temperature could variate to a great extent and especially in the afternoon the body could have been exposed to higher temperatures. The maximum temperature of that day would be valuable additional information in those cases. Still, the average temperature does give an indication whether the body could have been exposed to potentially higher temperatures and this knowledge can be valuable when looking for explanations of false positive and false negative dipstick results.

False negative and false positive dipstick test results occur for THC, cocaine and MDMA. Unfortunately, a reason for these false positive and false negative results cannot be found in all these cases. Adding more cases to the database can be helpful in recognizing patterns of factors that could be responsible for false positive and false negative dipsticks. Also, basing these possible reasons for false negative results on more cases will provide stronger evidence for these possible explanations. When the database is filled with all the cases for which toxicological tests have been performed at the Erasmus MC, the database will be used to check for discrepancies in all the 12 dipstick test strips. Even though it was not the scope of this research, it might be interesting to explore the discrepancies between the DOA screening on urine and the toxicological tests on blood and the urine dipstick. In the results, the DOA results were mentioned in some cases to see whether the Erasmus MC had found certain analytes in the urine and to compare this with the dipstick results. This might give an indication of whether the dipstick was performing as expected or whether the analyte just was not present in urine. Next, the dipstick results of cases in which a discrepancy has been found between the dipstick and the toxicological tests on blood and potentially also the cases for which the dipstick result was unknown or unclear will be checked again. Someone will examine the photos of the dipstick test results to make sure that no mistake has been made in reading of the result from the dipstick test. In order to save time, this will not be examined for cases in which no discrepancy has occurred. So, false negative and false positive results which are possibly unknown to us will not be found. In the end, this study will hopefully result in recommendations for the improvement of the work instructions of forensic doctors, for education material that can be used to update the knowledge of forensic

doctors, police officers from the regions involved in this research and police academy students and in recommendations for the manufacturer of the urine dipstick test. In this way, the professionals that are involved in the death investigations know the potential flaws of the urine dipstick and this will hopefully improve the effectiveness of the dipstick in death investigations.

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Supplementary material

Supplementary Table 1 Overview of control variables for the cases in which the dipstick was false negative for cocaine. For these five cases, the following control variables are described: whether the estimation of the time of death was within 24 hours or more than 24 hours, the PMI, whether the body was found on land/in the water and outside/inside and the average temperature on the day the body was found (when the body was found on the same day or the day after the death had occurred) or on the day in the middle of the PMI. The average temperature was not notated in some cases of which it was known that the body had been found inside, since the average temperature outside would not be of influence to the state of the body. No factor can be pointed out as the cause of the false negative dipstick, as the control variables are different in all these cases.

Estimation of time of death	PMI	Circumstances in which the body was discovered	Average temperature (°C) on the day in the middle of the PMI
≤ 24 hours	22 hours	On land (not in water) inside	7.6
unknown	unknown	unknown	
≤ 24 hours	5 hours and 35 minutes	On land (not in water) outside	19.1
≤ 24 hours	1 day and 6 hours	Inside	
unknown	7 hours and 15 minutes	In water	16.8

Supplementary Table 2 Overview of control variables for the cases in which the dipstick was false positive for THC but no THC could be found in the urine when doing the DOA screening. For these 8 cases, the following control variables are described: whether the estimation of the time of death was within 24 hours or more than 24 hours, the PMI, whether the body was found on land/in the water and outside/inside and the average temperature on the day the body was found (when the body was found on the same day or the day after the death had occurred) or on the day in the middle of the PMI. The control variables are quite different in all these cases. Therefore, it cannot be said for certain if they have contributed to the false positive result of the THC dipstick.

Estimation of time of death	PMI	Circumstances in which the body was discovered	Average temperature (°C) on the day in the middle of the PMI
≤ 24 hours	9 hours and 15 minutes	unknown	19
unknown	unknown	unknown	unknown
unknown	4 hours and 30 minutes	In the water outside	11.1
>24 hours	2 days and 7 hours	unknown	21.8
>24 hours	3 days and 12 hours	unknown	14.7
≤ 24 hours	5 hours and 15 minutes	unknown	13.5
>24 hours	2 days, 12 hours and 20 minutes	unknown	18.3
≤ 24 hours	3 hours and 56 minutes	On land (not in water) outside	13.9

Supplementary Table 3 Overview of control variables for the cases in which the dipstick was false positive for THC and THC could also be found in the urine when doing the DOA screening. For these 5 cases, the following control variables are described: whether the estimation of the time of death was within 24 hours or more than 24 hours, the PMI, whether the body was found on land/in the water and outside/inside and the average temperature on the day the body was found (when the body was found on the same day or the day after the death had occurred) or on the day in the middle of the PMI. The average temperature was not notated in some cases of which it was known that the body had been found inside, since the average temperature outside would not be of influence to the state of the body. No factor can be pointed out as the cause of the false positive dipstick, as the control variables are different in all these cases.

Estimation of time of death	PMI	Circumstances in which the body was discovered	Average temperature (°C) on the day in the middle of the PMI
≤ 24 hours	2 hours and 45 minutes	On land (not in water) inside	16.4
unknown	unknown	unknown	
≤ 24 hours	4 hours	unknown	17.0
≤ 24 hours	2 hours and 30 minutes	On land (not in water) inside	
> 24 hours	3 days and 30 minutes	On land (not in water)	20.6

Report 2

Setting up of semi-quantification for benzodiazepines, tramadol, morphine and oxycodone on the LC-MS/MS

Abstract

INTRODUCTION Blood can be sent to the Erasmus MC for a toxicological screening, which can be conducted on two LC-MS/MS systems, called micro 1 and micro 2. Semi-quantification of different analytes has been set up, so that estimations can be made about these concentrations that can be interpreted as either sub-therapeutic, therapeutic, supra-therapeutic or toxic. In this project, semi-quantification for benzodiazepines was set up on the micro 1. Subsequently, semi-quantification for tramadol, morphine and oxycodone were set up on both the micro 1 and micro 2. **MATERIALS & METHODS** Plasma was spiked with different concentrations of the benzodiazepines, tramadol, morphine and oxycodone. These samples were then prepared to be measured on the LC-MS/MS. All these concentrations were measured 15 times on the applicable LC-MS/MS system. Responses were calculated for each measurement and relative standard deviations (RSDs) were calculated for each chosen concentration. Calibration curves were made to obtain the equations necessary for semi-quantification. **RESULTS** The calibration lines of all the benzodiazepines can be used for semi-quantification, except for oxazepam, of which the RSDs of both the low and high concentration were above 25%. For tramadol, the concentrations of 0.01, 0.1 and 5 mg/l can be used for the calibration line. The concentrations of 0.1 and 0.5 mg/l morphine can be used to set up semi-quantification on the micro 1. Unfortunately, the measurements on the micro 2 cannot be used for semi-quantification. Lastly, only the concentration of 0.01 mg/l oxycodone on the micro 2 had to be left out because the RSD is above 25%. Without this condition, the calibration lines of both the micro 1 and the micro 2 can be used to set up semi-quantification. **CONCLUSION & DISCUSSION** Semi-quantification can be set up for benzodiazepines, tramadol, morphine and oxycodone based on these data, except for oxazepam on the micro 1 and morphine on the micro 2 LC-MS/MS system. In the future, validation must take place periodically for all the different analytes for which semi-quantification has been set up.

List of abbreviations

IS	internal standard
LLOQ	lower limit of quantification
QC	quality control
RSD	relative standard deviation
SSRIs	selective serotonin reuptake inhibitors
TCAs	tricyclic antidepressants

Introduction

Toxicological screenings are conducted in the laboratory of the pharmacy in the Erasmus MC. For this, blood and urine can be sent in. The blood samples were prepared to measure the substances it contains on the LC-MS/MS. In the laboratory of the pharmacy, there are two of these LC-MS/MS systems, one called the micro 1 and the other called the micro 2. Depending on which one the measurements were conducted, an estimation could be made about the concentration present of different substances. Semi-quantification has been set up in the past for benzodiazepines, anti-psychotics, antiarrhythmics, antihypertensives, tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). Semi-quantifications of all these drugs have been validated on both the micro 1 as the micro 2, except for the benzodiazepines, who have only been validated on the micro 2. In this project, the benzodiazepines were also validated on the micro 1, so that semi-quantification could take place for these drugs if the blood sample was run on the micro 1. Additionally, semi-quantification of tramadol, morphine and oxycodone was validated on both the micro 1 and the micro 2. These are three substances of which it was not yet possible to semi-quantificate, but were often seen in practice with the question if an estimation could be made of the quantity present in the blood.

Semi-quantification can be useful for determining the cause of death in post-mortem cases, but can also be useful to determine the need for treatment of living patients that have poisoned themselves for example. Real quantification is difficult, because only a few concentrations of every substance were measured and especially below and above these concentrations it cannot be said with complete certainty what the quantity of the substance was. This is also because of the uncertainty in which part of the S-curve the semi-quantification has been set up. Therefore, the hospital pharmacists in the Erasmus MC interpret the results of the semi-quantification and label it with either sub-therapeutic, therapeutic, supra-therapeutic or toxic. Interpreting in post-mortem cases is done with the help of multiple sources, one of them is an article written by Ketola and Ojanperä. This is done, because the post-mortem drug concentrations are mostly not the same as concentrations in living persons, for instance because of post-mortem drug redistribution. Drug levels could vary according to the sampling site and the interval between death and the collection of specimens. The most optimal procedure is to take femoral venous blood as soon as possible after death, as this gives results that are least susceptible to post-mortem changes. The paper from Ketola and Ojanperä followed an all-causes-of-death approach to calculate concentrations for 183 drugs and metabolites, based on 122 234 autopsy cases (Ketola & Ojanperä, 2019). Because of this large overview of concentrations of drugs and their metabolites, this article is particularly valuable and useful when interpreting the results of toxicological research in daily post-mortem cases.

The goal of this project was to set up semi-quantification for benzodiazepines on the micro 1 and for tramadol, morphine and oxycodone on both the micro 1 and 2. This was done by creating solutions of different known concentrations of each drug. These were run 15 times in the LC-MS/MS and the responses resulting from these measurements were used to create the graphs shown in the results section and to draw conclusions whether these drugs can be semi-quantified or not.

Materials & methods

Diluting tramadol

A vial of 100 mg Tramadol hydrochloride in 2 ml injection fluid (50mg/ml) of Tramadol®100 (Grunenthal) was used to make dilutions of 2000 mg/l (stock solution), 20 mg/l, 12.5 mg/l, 5 mg/l 0.1 mg/l and 0.01 mg/l. The stock solution with the concentration of 2000 mg/l was made by using 1 ml of the Tramadol®100 (Grunenthal) and 24 ml MilliQ. The 20 mg/l solution was made by adding 9.90 ml Omniplasma (Octapharma®) to 100 ul stock solution. The solution of 12.5 mg/l was made by adding 7.95 ml Omniplasma (Octapharma®) to 50 ul of the stock solution. The 5 mg/l solution was made by mixing 2 ml of the 12.5 mg/l solution with 3 ml Omniplasma (Octapharma®). The 0.1 mg/l solution was made by adding 4.975 ml plasma to 25 ul of the 20 mg/l solution. Finally, the 0.01 mg/l solution was made by mixing 500 ul of the 0.1 mg/l solution with 4.5 ml Omniplasma (Octapharma®). Of all the dilutions, except for the stock solution, 125 ul was filled out per eppendorf. These eppendorfs were stored in the -80°C freezer. The stock solution was stored in the 4°C refrigerator.

Diluting morphine

A vial of 1 mg morphine HCl 3-water in 1 ml was used to make dilutions of 100 mg/l (stock solution), 1.0 mg/l, 0.5 mg/l, 0.1 mg/l and 0.01 mg/l. The stock solution with the concentration of 100 mg/l was made by using 1 ml of the morphine HCl 3-water and 9 ml MilliQ. The 1.0 mg/l solution was made by adding 4.95 ml Omniplasma (Octapharma®) to 50 ul stock solution. The solution of 0.5 mg/l was made by adding 9.95 ml Omniplasma (Octapharma®) to 50 ul of the stock solution. The 0.1 mg/l solution was made by mixing 500 ul of the 1.0 mg/l solution with 4.5 ml Omniplasma (Octapharma®). Lastly, the 0.01 mg/l solution was made by mixing 500 ul of the 0.1 mg/l solution with 4.5 ml Omniplasma (Octapharma®). Of all the dilutions, except for the stock solution, 125 ul was filled out per eppendorf. These eppendorfs were stored in the -80°C freezer. The stock solution was stored in the 4°C refrigerator.

Diluting oxycodone

A capsule of 10 mg of OxyNorm® containing oxycodone was used to make dilutions of 100 mg/l (stock solution), 1.5 mg/l, 0.5 mg/l and 0.01 mg/l. The stock solution with the concentration of 100 mg/l was made dissolving the entire capsule of 10 mg in 100 ml MilliQ. The 1.5 mg/l solution was made by adding 9.85 ml Omniplasma (Octapharma®) to 150 ul stock solution. The solution of 0.5 mg/l was made by adding 4 ml Omniplasma (Octapharma®) to 2 ml of the 1.5 mg/l solution. At last, the 0.01 mg/l solution was made by mixing 100 ul of the 0.5 mg/l solution with 4.9 ml Omniplasma (Octapharma®). Of all the dilutions, except for the stock solution, 125 ul was filled out per eppendorf. These eppendorfs were stored in the -80°C freezer. The stock solution was stored in the 4°C refrigerator.

Diluting benzodiazepines

The tablets in the flasks of level I (low concentration) and level II (high concentration) of ClinChek® Serum control, lyophil., for Benzodiazepines (Recipe) were dissolved according to protocol. To each flask 1 ml MilliQ was added and after at least 15 minutes of waiting, the flasks were put on the rollerbank for at least another 15 minutes. Of both solutions, 125 ul was filled out per eppendorf. These eppendorfs were stored in the -80°C freezer.

Preparing samples for LC-MS/MS measurement

The samples were prepared to be measured in the LC-MS/MS (Waters) according to the protocol of toxicological screening of the Erasmus MC. In short, 125 ul ice cold sample-prep was added to the samples of 125 ul and this was vortexed for about 30 seconds and then

centrifuged for 10 minutes at 14680 rpm. 100 ul of the supernatant was pipetted in a clean autosampler vial to which 400 ul MilliQ was added. The sample was subsequently vortexed for at least 10 seconds and the sample was then ready to be measured in a LC-MS/MS system (Waters). During multiple days 2 or 3 samples per condition were prepared and measured on the LC-MS/MS (Waters). At least 15 samples of every condition had to be measured on the micro 1 and at least 15 samples of every condition had to be measured on the micro 2. One exception were the benzodiazepines, both its concentrations only had to be measured in the micro 1 since it already had been measured 15 times for both concentrations on the micro 2 in the past. The results of the toxicological screening on these LC-MS/MS systems were checked in MassLynx and the results were assembled in Excel files, in which these results were also analyzed.

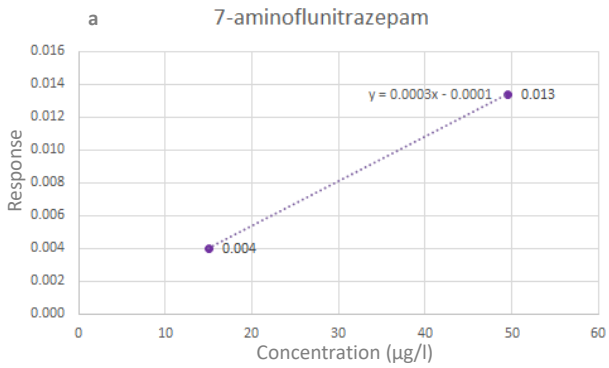
For a semi-quantification to be set up, the relative standard deviation (RSD) of the response has to be below 25%. The response is the area of the drug divided by the area of the internal standard (IS), which is Verapamil-d6.

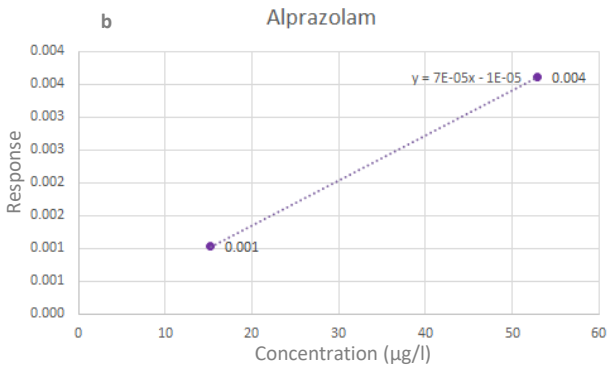
Results

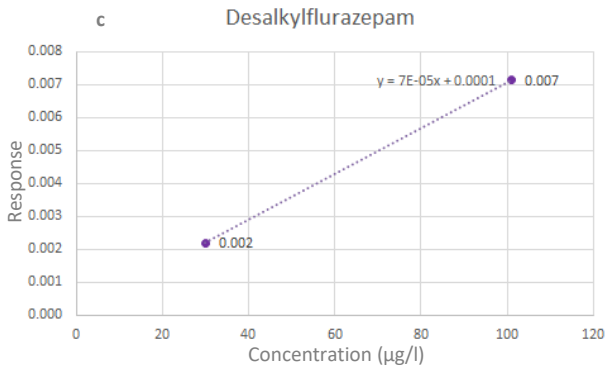
The areas of the internal standard (Verapamil-d6) and the measured drugs in the different concentrations were assembled in excel files. The results of every drug on each machine is discussed separately in this section.

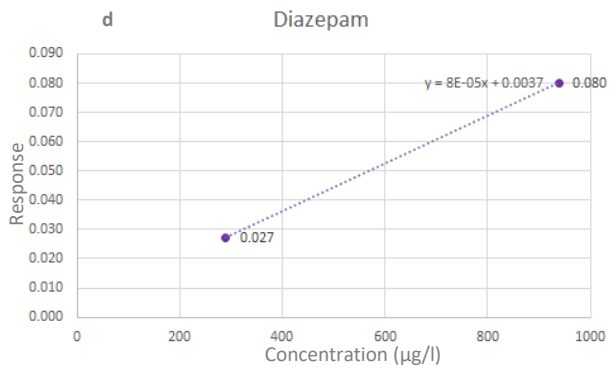
Benzodiazepines on the micro 1

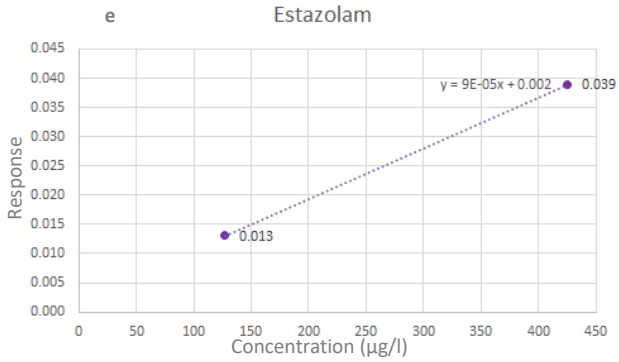
The course of the average response of the two concentrations are plotted for each benzodiazepine in **Figure 1**. Only oxazepam is not shown, since the RSD of the responses of both the low and high concentration were above 25%. Also, the R^2 is not shown, since for each benzodiazepine only 2 concentrations were measured and the R^2 is therefore always 1, giving no relevant information. In **Supplementary Figure 1** you can see the scattering of the responses of the low and high concentrations of oxazepam. Some samples of the low and high concentration seem to give the same response. Due to this and to the high RSD, oxazepam cannot be semi-quantified based on these measurements. The scattering of all the other benzodiazepines can be seen in **Supplementary Figure 2**. All these benzodiazepines had a RSD for both concentrations below the 25% and the scattering of both concentrations did not overlap.

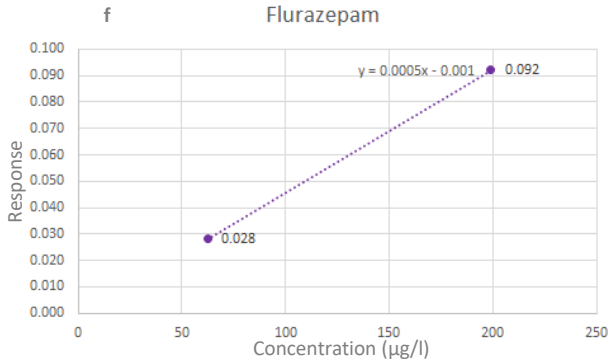


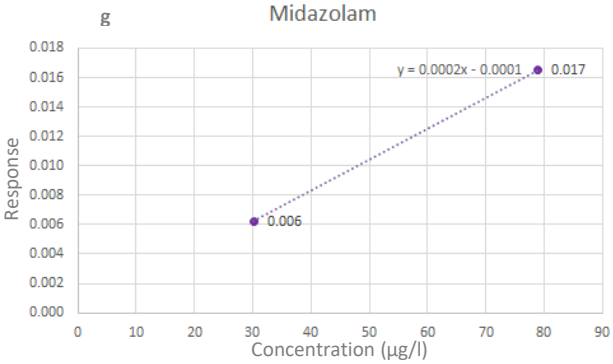


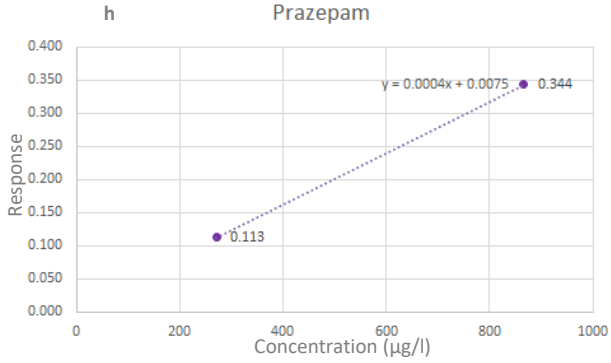

c
Desalkylflurazepam
y = 7E-05x + 0.0001
0.007
0.002
Response
Concentration (µg/l)

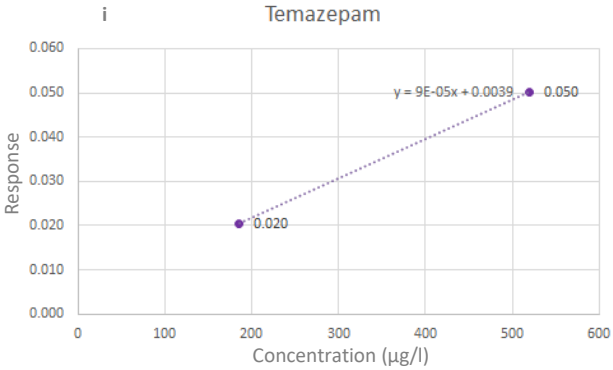

d
Diazepam
y = 8E-05x + 0.0037
0.080
0.027
Response
Concentration (µg/l)

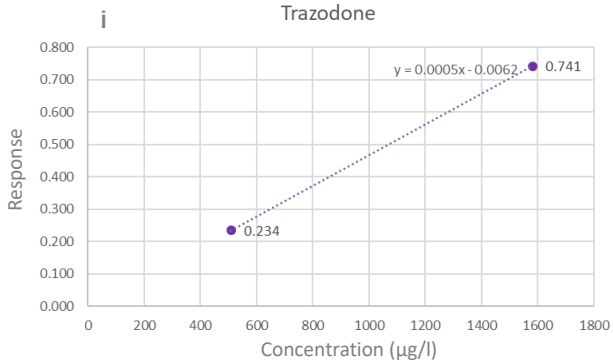

e
Estazolam
y = 9E-05x + 0.002
0.039
0.013
Response
Concentration (µg/l)


f
Flurazepam
y = 0.0005x - 0.001
0.092
0.028
Response
Concentration (µg/l)


g
Midazolam
y = 0.0002x - 0.0001
0.017
0.006
Response
Concentration (µg/l)


h
Prazepam
y = 0.0004x + 0.0075
0.344
0.113
Response
Concentration (µg/l)


i
Temazepam
y = 9E-05x + 0.0039
0.050
0.020
Response
Concentration (µg/l)


j
Trazodone
y = 0.0005x - 0.0062
0.741
0.234
Response
Concentration (µg/l)

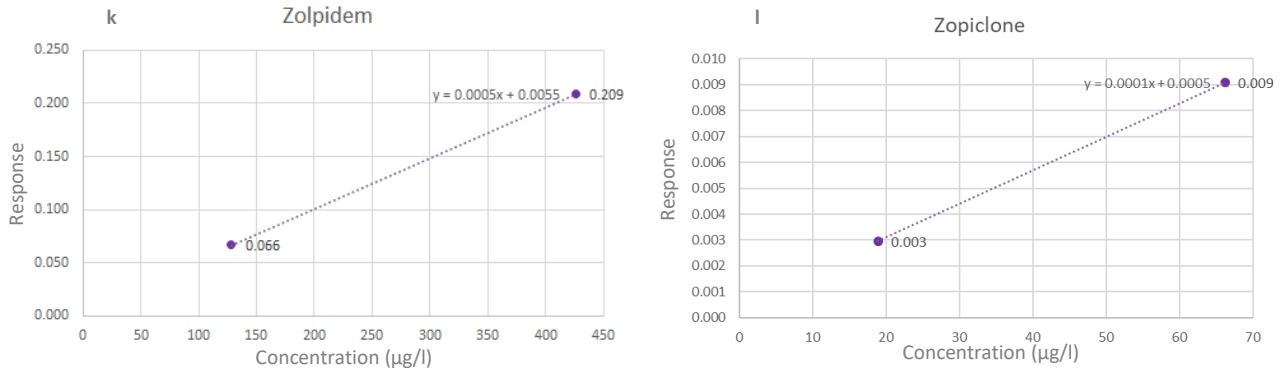
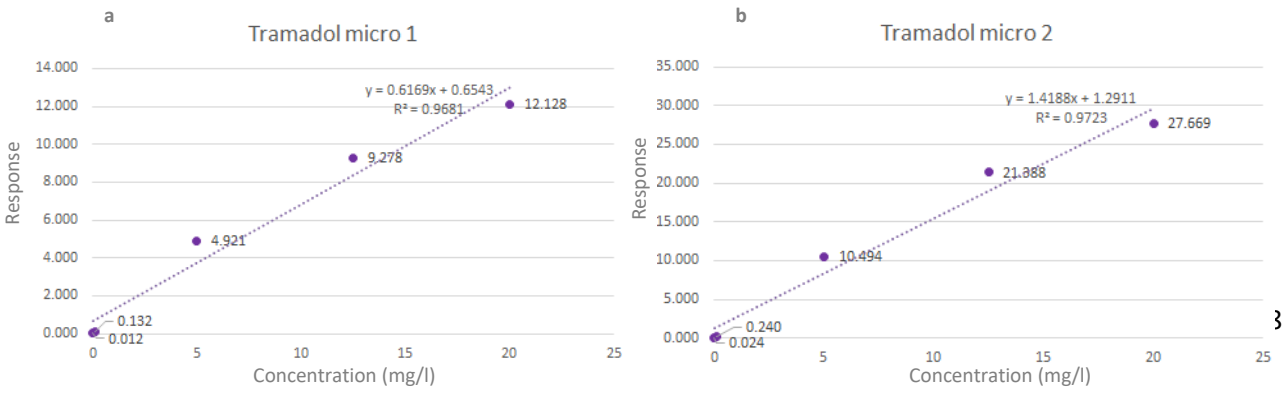


Figure 1 The average responses of the two concentrations of the benzodiazepines. For every benzodiazepine, two concentrations were measured on the LC-MS/MS, each 15 times. The area of the benzodiazepine was divided by the area of the IS, resulting in the response. A calibration line was drawn between these two average responses and the equation is also shown. All these benzodiazepines can be semi-quantified in the future based on these graphs. The benzodiazepines and the measured concentrations are (**a**) 7-aminoflunitrazepam, 15µg/l, 49.6 µg/l; (**b**) alprazolam, 15.2 µg/l, 52.9 µg/l; (**c**) desalkylflurazepam, 29.9 µg/l, 101 µg/l; (**d**) diazepam, 290 µg/l, 939 µg/l, (**e**) estazolam, 127 µg/l, 425 µg/l; (**f**) flurazepam, 62.3 µg/l, 199 µg/l; (**g**) midazolam, 30.2 µg/l, 78.9 µg/l; (**h**) prazepam, 271 µg/l, 866 µg/l; (**i**) temazepam, 186 µg/l, 520 µg/l; (**j**) trazodone, 509 µg/l, 1581 µg/l; (**k**) zolpidem, 128 µg/l, 426 µg/l; (**l**) zopiclone, 18.9 µg/l, 66.2 µg/l.

Tramadol on the micro 1 and 2

The course of the average response of the chosen concentrations for tramadol can be seen in **Figure 2a** for the micro 1 and **Figure 2b** for the micro 2. At the concentration of 12.5 mg/l tramadol, the curve is already flattening. In order to calculate concentrations from given responses of patient samples, the semi-quantification has to be set up in the linear part of the curve. If the concentrations at which the curve flattens are left out, **Figure 2c** is created for micro 1 and **Figure 2d** is created for micro 2. Both graphs show that R^2=1, which means that the graphs are completely linear. In the future, the concentration of 10 mg/l tramadol could be tested, to see if that would still result in linear graphs or if the graphs already flatten at that particular concentration. The scattering of the responses of the individual measurements can be seen in **Supplementary Figure 3a** for micro 1 and **Supplementary Figure 3b** for micro 2. This shows that even though the RSDs of the concentrations 12.5 and 20 mg/l tramadol were below the 25%, there is an overlap in responses of some samples. This overlap happens partly because the graph flattens at these concentrations, but also because the responses were scattered quite broadly. In conclusion, the concentrations of 0.01, 0.1 and 5 mg/l tramadol could be used to create a calibration line as a basis for the semi-quantification for both the micro 1 and 2. In the future, the concentration of 10 mg/l tramadol can be measured to track down where the graphs flatten and to see whether this concentration can also be implemented in the calibration lines.



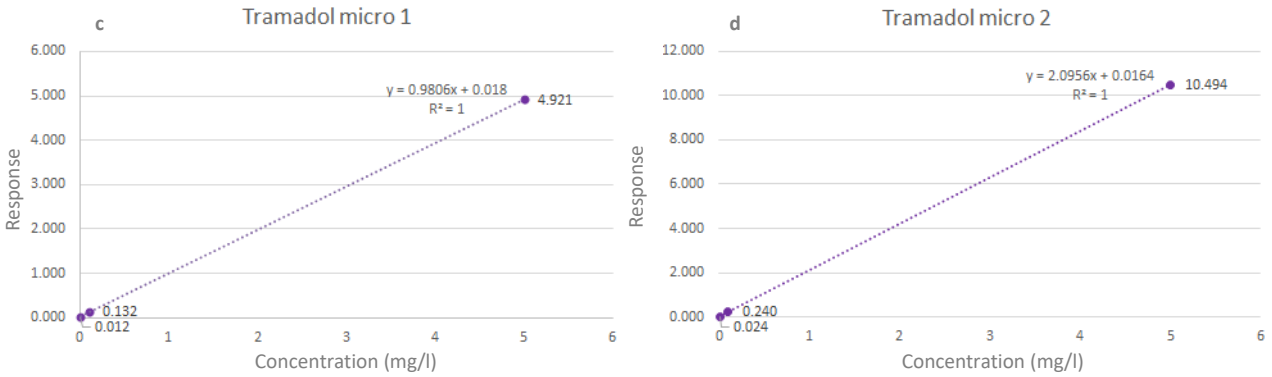


Figure 2 The average responses of the different measured concentrations of tramadol. The measured concentrations are 0.01 mg/l, 0.1 mg/l, 5 mg/l 12.5 mg/l and 20 mg/l. Both the measurements on (**a**) the micro 1 and (**b**) the micro 2 LC-MS/MS systems are shown. Both curves seem to flatten from 12.5 mg/l, that is why the concentrations 12.5 and 20 mg/l are left out to show the linear part of the curve (**c**) for the micro 1 and (**d**) for the micro 2. These last two figures can be used as a basis for semi-quantification.

Morphine on the micro 1 and 2

For morphine, the graph depicting the average responses for differences concentrations of morphine can be seen in **Figure 3a** for micro 1 and **Figure 3b** for micro 2. A concentration of 0.01 mg/l morphine was measured in the micro 1 but not shown in the figure, because the RSD was above 25%. The same accounts for the concentrations 0.01 and 0.1 mg/l morphine measured in the micro 2. For both the micro 1 and 2, it seems as if the graph is flattening at the concentration of 1.0 mg/l. To be sure that the curve is really flattening from this point, a higher concentration could be measured in the future. Considering that both graphs seem to be flattening, I think it is likely that the curve is indeed flattening. When looking at the results of the measurements of the micro 1, that would mean that only the concentrations of 0.1 and 0.5 mg/l morphine were suited to draw a calibration line, resulting in **Figure 3c**. The scattering of the responses of the individual measurements of the different concentrations of morphine can be seen in **Supplementary Figure 4a**. This shows no overlap in the responses of the concentrations of 0.1 and 0.5 mg/l. The measurements of the micro 2 resulted in a RSD for the concentrations of 0.5 and 1.0 mg/l of 24.0 and 24.5 respectively. Even though the RSDs were just below 25%, the responses of the individual measurements overlap **(Supplementary Figure 4b).** If these two concentrations would be used for a calibration line, the outcome of the translation of a patient sample response would not be accurate. Therefore, I would not use this calibration line. All in all, the calibration line and equation arising from the measurements of 0.1 and 0.5 mg/l morphine on the micro 1 can be used for semi-quantification. Unfortunately, the measurements on the micro 2 cannot result in a reliable calibration curve. Therefore, no semi-quantification can be done for morphine on the micro 2.

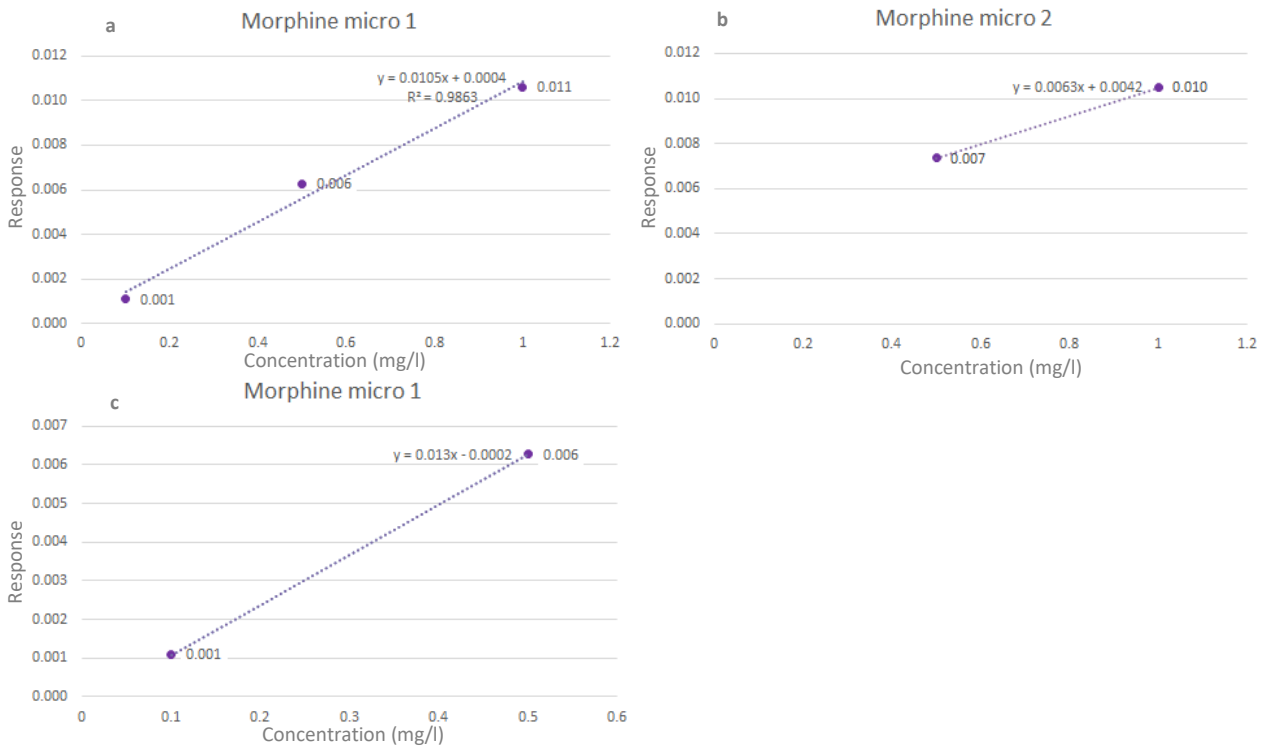


Figure 3 The average responses of the different measured concentrations of morphine. Both the measurements on (**a**) the micro 1 and (**b**) the micro 2 LC-MS/MS systems are shown. The concentrations for which the RSD was above 25% are not shown. The measured concentrations are 0.01 mg/l (RSD above 25% for both micro 1 and micro 2), 0.1 mg/l (RSD was above 25% for the micro 2), 0.5 mg/l and 1.0 mg/l. Since the curves seem to flatten, the linear part of the graph containing the measurements on the micro 1 is shown in (**c**).

Oxycodone on the micro 1

The graph showing the average responses of different concentrations oxycodone can be seen in **Figure 4a and 4b** for the micro 1 and micro 2 respectively. For micro 1, the RSDs for all three concentrations of oxycodone were below 25%. Also, the scattering of the responses of the individual measurements (**Supplementary Figure 5a)** does not overlap. So, the calibration line is suitable for semi-quantification on the micro 1. For micro 2, the lowest concentration of 0.01 mg/l oxycodone had a RSD above 25%. The calibration line that would be the result if the lowest concentration would be implemented can be seen in **Supplementary Figure 6**. This shows that the R^2 would have been 1 and it is still 1 if the average response of the lowest concentration is left out. The scattering of the responses of the individual measurements (**Supplementary Figure 5b**) do not overlap, making this calibration line suitable for semi-quantification on the micro 2 as well. In conclusion, both calibration lines can be used for semi-quantification, if the lowest concentration of 0.01 mg/l oxycodone is left out on the micro 2.

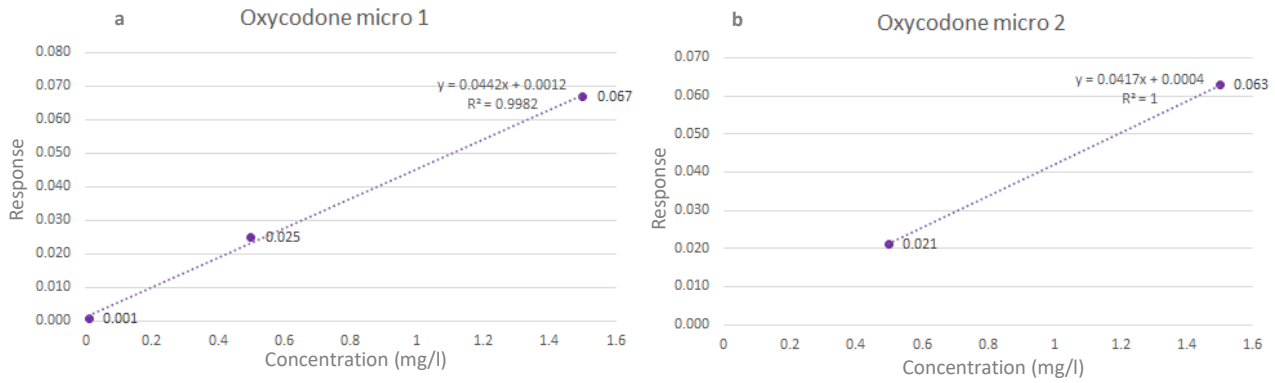


Figure 4 The average responses of the different measured concentrations of oxycodone. The measurements on both (**a**) the micro 1 and (**b**) the micro 2 LC-MS/MS systems are shown. The concentration for which the RSD was above 25% are not shown. The measured concentrations are 0.01 mg/l (RSD above 25% for the micro 2), 0.5 mg/l and 1.5 mg/l

Conclusion & discussion

In conclusion, all benzodiazepines, except for oxazepam, can be semi-quantified on the micro 1 based on the created calibration lines. Also, the calibration lines of tramadol and oxycodone on both the micro 1 and micro 2 are suited for semi-quantification. The LC-MS/MS systems appeared to be very sensitive for tramadol, which can be seen by the relative high responses for even the lowest concentrations. The graph shows that at the concentrations of 12.5 and 20 mg/l tramadol, the curve is already flattening. So, the concentration of 10 mg/l tramadol can be measured in the future, to see where the curve starts to flatten. For oxycodone, a tablet was dissolved to make the dilutions. This is not ideal, because the different concentrations were probably not very accurate. Oxycodone was ordered in a liquid, but it could not be delivered before the end of this internship, therefore the tablet was used. If the oxycodone liquid arrives in the future, it could be used to check whether the created calibration curve indeed gives a reliable concentration. Otherwise, semi-quantification has to be set up again from this oxycodone liquid. For morphine, only the results of the micro 1 can be used for semi-quantification in the future. Unfortunately, the results of morphine on the micro 2 were not suited to base semi-quantification on. The LC-MS/MS systems are not very sensitive for morphine, since the low concentrations result in a very low area. However, it can be relevant to be able to measure lower concentrations, since the concentration of 0.10 mg/l morphine is considered therapeutic (Ketola & Ojanperä, 2019). So, it might be important to be able to distinguish a therapeutic from a sub-therapeutic concentration of morphine.

With this method, benzodiazepines, tramadol, morphine and oxycodone present in patient plasma can be semi-quantified, but not completely quantified. As said before, real quantification is not possible, because below and above the measured concentrations it cannot be said with complete certainty what the quantity of the substance is. This is partly because you cannot be sure in which part of the S-curve graph these measured concentrations are. This is especially the case for the benzodiazepines, as only two concentrations were measured for these drugs. The non-linear behavior of the curve when the response of the drug exceeds a certain response threshold can be explained by the detector saturation of the MS detector (Yuan, Zhang, Jemal, & Aubry, 2012). Next to this non-linear behavior of the curve above a certain response, real quantification cannot be done because the RSD has to be below 25%. If a response is allowed to deviate up to 25%

percent, it makes sense that no statement can be made about the exact concentration. This 25% is chosen for the semi-quantification methods in the pharmacy laboratory in the Erasmus MC, but is not a standard requirement for other laboratories or institutes. For example, in a study by Svedberg *et al.* the measured points had to be within 15% of the nominal value, except for the lower limit of quantification (LLOQ) which had to be within 20% of the nominal value (Svedberg, Green, Vikström, Lundeberg, & Vikingsson, 2015).

The spiked plasma samples in this project have undergone one freeze-thaw cycle, but the patient samples that will be used for semi-quantification will be measured mostly without freezing and thawing. Therefore, it would be wise to evaluate freeze-thaw stability. This could be determined by measuring freshly spiked plasma samples and compare the results with the results of this project. In the future, periodic validations will be performed for the benzodiazepines, tramadol, morphine and oxycodone but also for all the other drugs for which semi-quantification has previously been set up. This will be done by measuring a quality control (QC) sample, which will consist of a plasma sample spiked with a known concentration, to see whether the response is indeed on the calibration line. Another option is to compare the outcome of a patient sample on the LC-MS/MS to the results of the oncology department, to which some samples are send for quantification (most often of morphine). Also, a dedicated method could be used periodically to perform an extra validation. The pharmacy laboratory of the Erasmus MC has plans to use a dedicated method for morphine in the future, which could be useful for this purpose. There is also a dedicated method for the benzodiazepine midazolam, which could be used for the same validation.

References

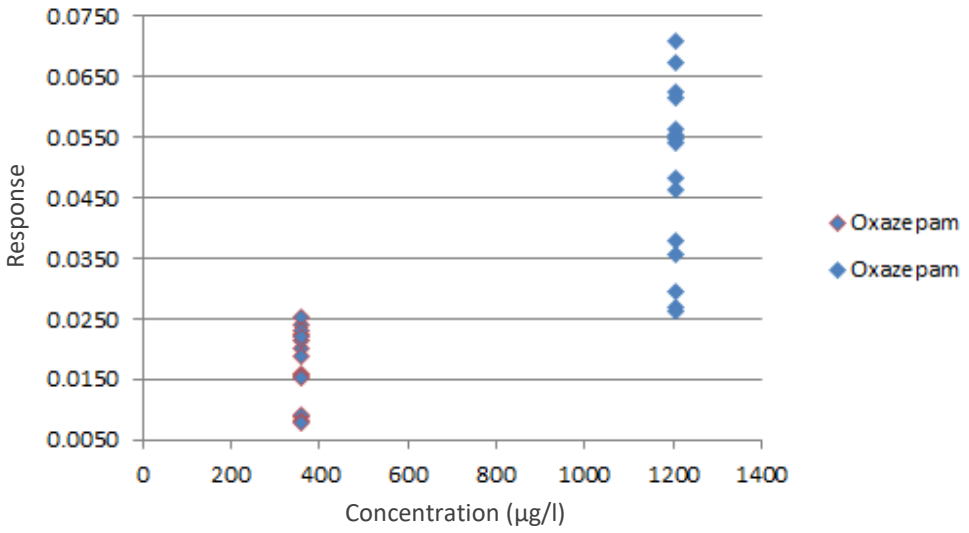
Ketola, R. A., & Ojanperä, I. (2019). Summary statistics for drug concentrations in post-mortem femoral blood representing all causes of death. *Drug Testing and Analysis*, *11*(9), 1326–1337.

Svedberg, A., Green, H., Vikström, A., Lundeberg, J., & Vikingsson, S. (2015). A validated liquid chromatography tandem mass spectrometry method for quantification of erlotinib, OSI-420 and didesmethyl erlotinib and semi-quantification of erlotinib metabolites in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, (107), 186–195. https://doi.org/10.1016/j.jpba.2014.12.022

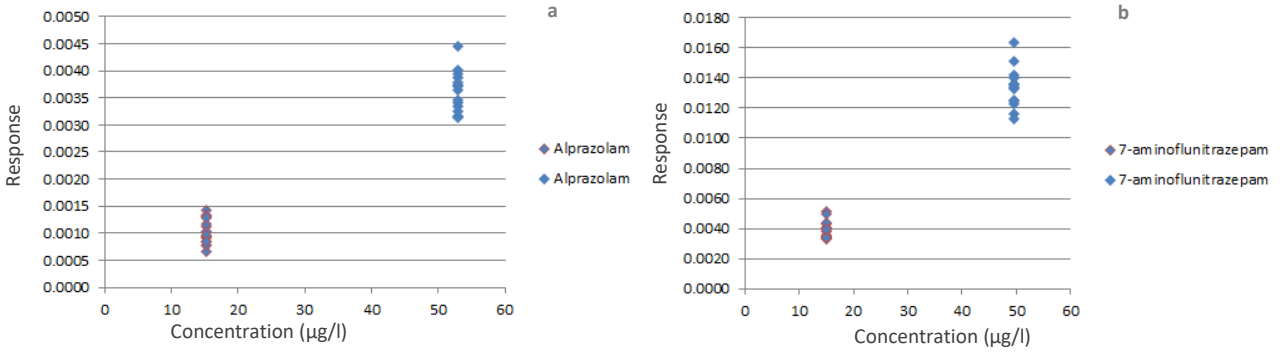
Yuan, L., Zhang, D., Jemal, M., & Aubry, A.-F. (2012). Systematic evaluation of the root cause of non-linearity in liquid chromatography/tandem mass spectrometry bioanalytical assays and strategy to predict and extend the linear standard curve range. *Rapid Communications in Mass Spectrometry*, *26*(12), 1465–1474. https://doi.org/10.1002/RCM.6252

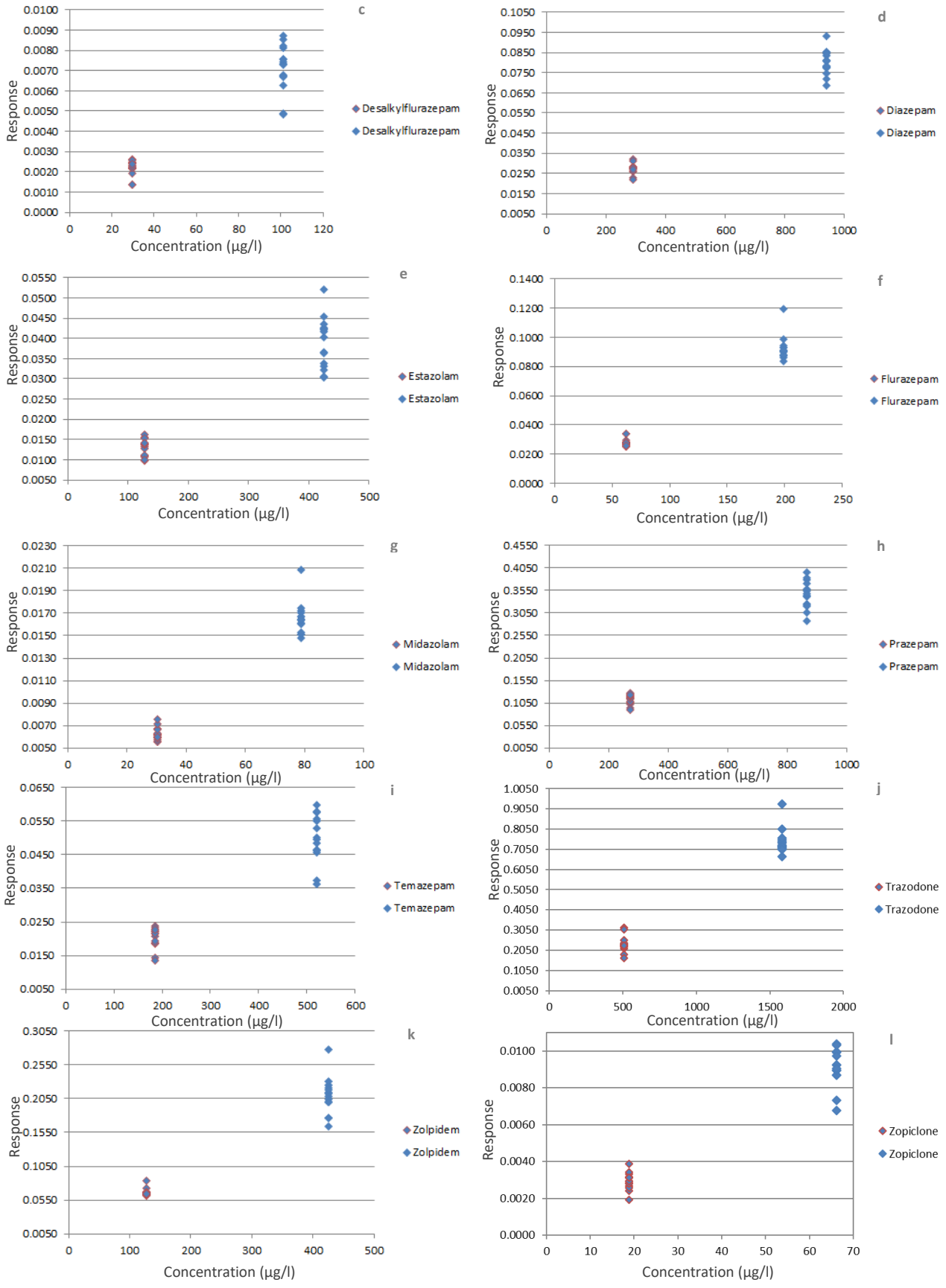
Supplementary material

Supplementary Figure 1 Scattering of the responses of the individual measurements of the two concentrations of oxazepam. The individual responses are scattered quite broadly and the highest response of the 360 ug/l oxazepam is close to the lowest response of the 1205 µg/l oxazepam.

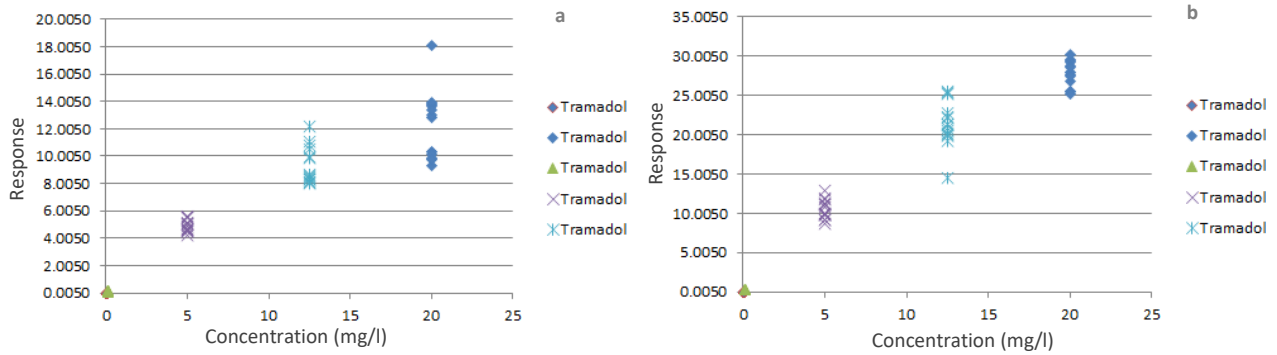


Supplementary Figure 2 The scattering of the responses of the individual measurements of the benzodiazepines that are approved for setting up semi-quantification. None of the responses of the lower concentration overlap with the higher concentration. The benzodiazepines and the measured concentrations are (**a**) 7-aminoflunitrazepam, 15µg/l, 49.6 µg/l; (**b**) alprazolam, 15.2 µg/l, 52.9 µg/l; (**c**) desalkylflurazepam, 29.9 µg/l, 101 µg/l; (**d**) diazepam, 290 µg/l, 939 µg/l, (**e**) estazolam, 127 µg/l, 425 µg/l; (**f**) flurazepam, 62.3 µg/l, 199 µg/l; (**g**) midazolam, 30.2 µg/l, 78.9 µg/l; (**h**) prazepam, 271 µg/l, 866 µg/l; (**i**) temazepam, 186 µg/l, 520 µg/l; (**j**) trazodone, 509 µg/l, 1581 µg/l; (**k**) zolpidem, 128 µg/l, 426 µg/l; (**l**) zopiclone, 18.9 µg/l, 66.2 µg/l.

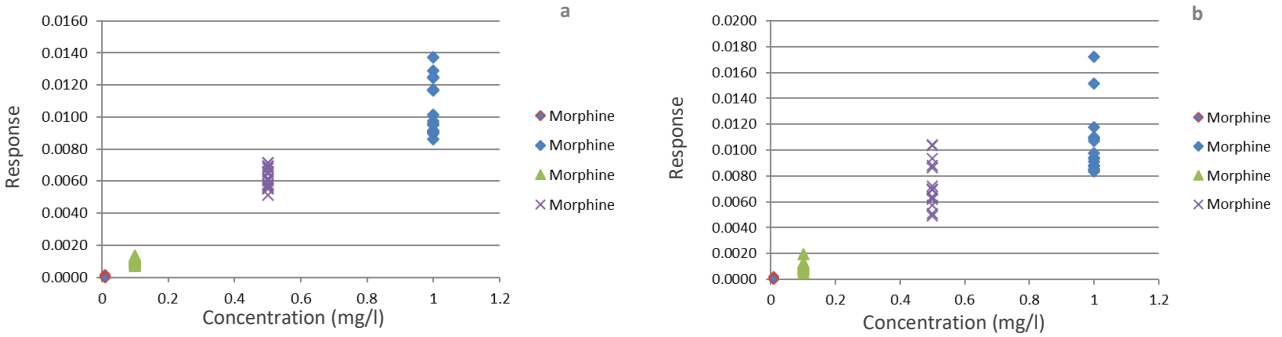



c
Response
Concentration (µg/l)
Desalkylflurazepam
Desalkylflurazepam
d
Diazepam
Diazepam
e
Estazolam
Estazolam
f
Flurazepam
Flurazepam
g
Midazolam
Midazolam
h
Prazepam
Prazepam
i
Temazepam
Temazepam
j
Trazodone
Trazodone
k
Zolpidem
Zolpidem
l
Zopiclone
Zopiclone

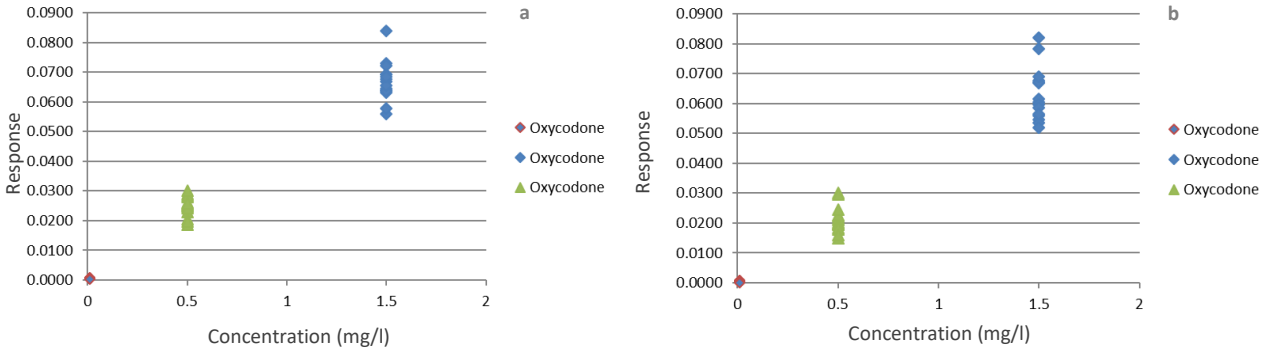
Supplementary Figure 3 Scattering of responses of individual measurements of tramadol. The measurements (**a**) on the micro 1 and (**b**) on the micro 2 are shown. The responses of 12.5 and 20 mg/l overlap on both systems, because the curve seems to flatten from this point. The measured concentrations are 0.01 mg/l, 0.1 mg/l, 5 mg/l, 12.5 mg/l and 20 mg/l.



Supplementary Figure 4 Scattering of responses of individual measurements of morphine. The measurements are performed (**a**) on the micro 1 and (**b**) on the micro 2. The measured concentrations are 0.01 mg/l, 0.1 mg/l, 0.5 mg/l and 1.0 mg/l. The responses of 0.5 and 1 mg/l overlap on the micro 2, making it not ideal to use these concentrations for the calibration line to base semi-quantification on. The RSD on the micro 1 and 2 for 0.01 mg/l was above 25%, as was 0.1 mg/l on the micro 2.



Supplementary Figure 5 Scattering of responses of individual measurements of oxycodone. The measurements are conducted (**a**) on the micro 1 and (**b**) on the micro 2. The measured concentrations are 0.01 mg/l, 0.1 mg/l, 0.5 mg/l and 1.0 mg/l. The individual responses do not overlap between different concentrations. The RSD on the micro 2 for 0.01 mg/l was above 25%.



Supplementary Figure 6 The average responses of the different measured concentrations of oxycodone on the micro 2. This graph also shows the average response of the concentration 0.01 mg/l, even though the RSD was above 25%. The R^2 is 1. The measured concentrations are 0.01 mg/l, 0.5 mg/l and 1.5 mg/l.