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Forensic Toxicological Aspects of Tramadol

Focus on Enantioselective Drug Disposition and
Pharmacogenetics

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To Johan Enmyren!

“Success is not final, failure is not fatal
It is the courage to continue that counts”

According to many a quote by *Winston Churchill*, although according to others derived from an *advertising campaign* for beer, which is somewhat disappointing for someone who does not even like beer. But it is still a good quote though, highly relevant for anyone committed to research!

PREFACE

Dear Reader,

I am so glad that you hold this book in your hands!

Primarily because it means that I made it. I have crossed the finish line following one of the toughest journeys I have ever been on, a journey that provided me with a huge learning experience. On the winding and twisting trails that I have travelled, I learned as much about people as I did of science, and as much about leadership as I did of research. But most of all, it taught me about myself.

You holding this book in your hands also makes me very happy because chances are good that you are either a dear family member or friend, whom I will soon have more time to spend with. Or you are one of my friendly, knowledgeable and inspiring colleagues that I am very grateful to have gotten to know through the years. Or perhaps you are, or are soon to become, a researcher curious of the fascinating tramadol molecules, just like me.

My journey is scientifically depicted in the following chapters of this book. However, herein I would also like to share some of my thoughts and reflections that goes beyond the formulas, laboratory results and papers. Luckily, these will not occupy many pages, since I have realized that they already have been formulated to perfection by others. Accordingly, they are not, as opposed to research, in any way new or innovative. Although, to create a research environment that favours learning and development of aptitudes, they are equally important. On the next page, I will therefore communicate some of my favorite quotes*. To me they either serve as guiding principles in the world of research, or as encouragement and tribute to the PhD-students who do not (or did not) give up in spite of being assigned the mission impossible.

Sincerely,

Pernilla Haage
October 2018

“To me, the rainbow was a profoundly hopeful symbol, separating the white light of appearances into its multiple spectrum and revealing a hidden dimension. It reminded me of my belief that it was the mission of science to pierce through the layers of everyday reality and penetrate to the truth.”
Candace B. Pert

“You can’t learn to play the piano without playing the piano, you can’t learn to write without writing, and, in many ways, you can’t learn to think without thinking. Writing is thinking. To write well is to think clearly. That’s why it’s so hard.” *David McCullough*

“Ambition has its disappointments to sour us, but never the good fortune to satisfy us. Its appetite grows keener by indulgence and all we can gratify it with at present serves but the more to inflame its insatiable desires.”
Benjamin Franklin

“Ambition and the belly are the two worst counselors.” *German Proverb*

“Fear is not your enemy. It is a compass pointing you to the areas where you need to grow.” *Steve Paulina*

“Bad times have a scientific value. These are occasions a good learner would not miss.” *Ralph Waldo Emerson*

“Do not judge me by my successes, judge me by how many times I fell down and got back up again.” *Nelson Mandela*

“Courage isn’t having the strength to go on – it is going on when you don’t have strength.” *Napoleon Bonaparte*

“Disappointments are a result of failed expectations. To have less disappointments, either expect less from other people or demand more from yourself.” *Kevin Ngo*

“There is nothing noble in being superior to your fellow man; true nobility is being superior to your former self.” *Ernest Hemingway*

“Climb the mountain not to plant your flag, but to embrace the challenge, enjoy the air and behold the view. Climb it so you can see the world, not so the world can see you.” *David McCullough*

“Respect for ourselves guides our morals, respect for others guides our manners.” *Laurence Sterne*

“And once the storm is over you won’t remember how you made it through, how you managed to survive. You won’t even be sure, in fact, whether the storm is really over. But one thing is certain. When you come out of the storm you won’t be the same person who walked in. That’s what this storm’s all about.” *Haruki Murakami*

* The wise words were found in various quote compilations, although not always with a proper source. Subsequently, I do apologize for any possible misattributions.

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ABSTRACT

One of the most difficult parts in forensic toxicology is to interpret obtained drug concentrations. Was it therapeutic, toxic or even lethal to the particular individual that the blood sample was drawn from? Concentrations of opioid drugs are especially difficult to interpret, because of large interindividual differences in innate and acquired tolerance.

Tramadol is a complex drug. Not only is it an opioid, it is also a racemic drug with the (+)- and (-)-enantiomers of the parent compound and metabolites showing different pharmacological effects. Further, it is metabolized by polymorphic enzymes, which may affect the amounts of metabolites formed and possibly the enantiomer ratios of the parent compound and its metabolites. It has been speculated that particularly the (+)/(-)-enantiomer ratio of *O*-desmethyltramadol is related to the risk of adverse effects, and it has been shown that the ratio is affected by *CYP2D6* genotype.

The overall aim of the thesis was to evaluate if forensic interpretations of tramadol, regarding toxicity and time since drug administration, may be improved by the use of genotyping and enantioselective concentration determination of tramadol and its three main metabolites.

To simultaneously quantify the enantiomer concentrations of tramadol, *O*-desmethyltramadol, *N*-desmethyltramadol and *N,O*-didesmethyltramadol in whole blood, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated. Genetic variation in *CYP2D6*, *CYP2B6*, *CYP3A4* (encoding the tramadol metabolizing enzymes), *ABCB1* (encoding a transport protein) and *OPRM1* (encoding the μ -opioid receptor) was investigated, using pyrosequencing, xTAG, and TaqMan analysis. The methods were applied to the blood samples of two study populations; 19 healthy volunteers administered a therapeutic, single tramadol dose, and 159 tramadol positive autopsy cases.

The most important finding was the positive correlations between all four enantiomer ratios and time since tramadol administration in the healthy volunteers. All enantiomer ratios except the one of tramadol was also affected by the *CYP2D6* genotype, which was apparent among the autopsy cases as well. Genetic variation in *CYP2D6* and possibly *CYP2B6* was shown to have an impact on tramadol pharmacokinetics, although no association to neither drug related symptoms nor tramadol related causes of death was found. Tramadol intoxications were predominantly characterized by low age (median 26 years) and male sex, often with a

history of substance abuse and with other drugs (at fairly low concentrations) detected in blood.

In conclusion, enantiomer concentration determination combined with genotyping seems promising regarding estimations of time since drug administration, although is of low value concerning interpretations of toxicity in autopsy cases.

SVENSK SAMMANFATTNING

Vad är en dödlig blodkoncentration av det smärtstillande läkemedlet tramadol? Hur lång tid har gått sedan intag av tramadol och tidpunkten för döden? Tramadol skrivs ofta ut för olika typer av smärttillstånd, men har också blivit en populär missbrukssubstans som idag är narkotikaklassad. Det är därför inte ovanligt att rättsläkare och rättstoxikologer ställs inför dessa frågeställningar i sitt arbete med att utreda dödsorsak. Frågeställningarna är dock svåra att besvara, eftersom det finns en stor individuell variation i hur väl tramadol tolereras. Enbart blodkoncentrationen av tramadol är alltså inte alltid tillräcklig för att avgöra om ett dödsfall orsakats av tramadolförgiftning eller inte.

Något som skulle kunna bidra till en förbättrad bedömning i dessa fall är så kallade enantiomerkvoter. Läkemedelstabletten tramadol innehåller nämligen två stycken läkemedelsmolekyler, två enantiomerer. Dessa är exakt likadana, med den enda skillnaden att de är varandras spegelbilder. Detta är dock tillräckligt för att ge enantiomererna olika egenskaper i kroppen, och därmed möjligen också olika biverkningsprofiler. Även de nedbrytningsprodukter som bildas av tramadol i kroppen består av två enantiomerer med olika egenskaper. Koncentrationsförhållandet i blodet mellan två enantiomerer är det som kallas enantiomerkvot.

I avhandlingsarbetet utvecklades en analytisk metod med förmåga att mäta enantiomerkoncentrationerna av både tramadol och tre av dess nedbrytningsprodukter. Metoden användes sedan för att analysera blod från dels friska individer som intagit en förhållandevis låg engångsdos av tramadol, och dels från avlidna som intagit en eller flera okända doser av tramadol. Ett samband mellan alla fyra enantiomerkvoter och tidpunkten efter intag upptäcktes. Ju längre tid som gått efter intaget av tramadol, desto högre blev enantiomerkvoterna. Detta samband skulle, om det studeras vidare, kunna användas för att ungefärligt beräkna den tid som passerat mellan tramadolintag och dödsfall. Det skulle också kunna användas för att beräkna tidpunkten mellan tramadolintag och blodprovstagning i levande individer, och därmed komma till nytta även i arbetet med andra typer av forensiska fall, som exempelvis misstänkta drograttfylleriärenden. Något samband mellan enantiomerkvoter och risk för tramadolrelaterade symptom eller risk för tramadolrelaterade dödsfall kunde dock inte påvisas. Däremot upptäcktes att genetisk variation i en specifik gen som är av betydelse för kroppens nedbrytning av tramadol också är av avgörande betydelse för storleken på enantiomerkvoterna. För

att enantiomerkvoter framöver ska kunna användas för att uppskatta tiden sedan tramadolintag i en individ kommer det därför sannolikt vara nödvändigt att känna till en liten del av individens genetiska uppsättning. Män med förhållandevis låg ålder (median 26 år) var mycket vanligt förekommande bland de 15 dödsfall som av rättsläkare bedömdes ha orsakats av enbart tramadolförgiftning. Ofta hade dessa också en känd missbruksbakgrund och i deras blod detekterades inte sällan andra läkemedel och droger, om än i relativt låga koncentrationer.

LIST OF PAPERS

The thesis is based on the following original papers:

- I. Bastami S ^{*}, **Haage P** ^{*}, Kronstrand R, Kugelberg FC, Zackrisson AL, Uppugunduri S. Pharmacogenetic aspects of tramadol pharmacokinetics and pharmacodynamics after a single oral dose. *Forensic Sci Int* 2014;238:125-32.
- II. **Haage P**, Kronstrand R, Carlsson B, Kugelberg FC, Josefsson M. Quantitation of the enantiomers of tramadol and its three main metabolites in human whole blood using LC-MS/MS. *J Pharm Biomed Anal* 2016;119:1-9.
- III. **Haage P**, Kronstrand R, Josefsson M, Calistri S, van Schaik RHN, Green H, Kugelberg FC. Enantioselective pharmacokinetics of tramadol and its three main metabolites; impact of *CYP2D6*, *CYP2B6*, and *CYP3A4* genotype. *Pharmacol Res Perspect* 2018;e00419.
- IV. **Haage P**, Kronstrand R, Josefsson M, van Schaik RHN, Green H, Kugelberg FC. The use of enantiomer ratios of tramadol and its metabolites in relation to *CYP2D6* genotype in forensic autopsy casework. Manuscript.

* These authors contributed equally.

The papers are henceforth referred to by their designated Roman numerals, and are appended in full at the end of the thesis. Reprints were made with the permission of the copyright holders.

ABBREVIATIONS

<i>ABCB1</i>	Adenosine triphosphate-binding cassette B1 (the gene encoding P-glycoprotein)
AGP	α_1 -acid glycoprotein
AUC	Area under the drug concentration-time curve
C _{max}	Maximum concentration
CYP	Cytochrome P450 enzyme
<i>CYP</i>	The gene encoding a cytochrome P450 enzyme
DDD	Defined daily dosage
DRS	Drug related symptoms
EM	Extensive (normal) metabolizer
ESI	Electrospray ionization
FDA	Food and Drug Administration
FTI	Fatal toxicity index
HPLC	High performance liquid chromatography
IM	Intermediate metabolizer
IR	Immediate-release
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-TOF-MS	Liquid chromatography time-of-flight mass spectrometry
LLOQ	Lower limit of quantitation
MixTox	Mixed intoxications
NDT	<i>N</i> -desmethyltramadol
NGS	Next generation sequencing
NODT	<i>N,O</i> -didesmethyltramadol
NonTox	Nonintoxications
ODT	<i>O</i> -desmethyltramadol
ODV	<i>O</i> -desmethylvenlafaxine
<i>OPRM1</i>	Opioid receptor mu 1 (the gene encoding the μ -opioid receptor)
OtherTox	Other intoxications
P-gp	P-glycoprotein
PM	Poor metabolizer

SAE	Serious adverse event
SFC	Supercritical fluid chromatography
SNP	Single nucleotide polymorphism
SR	Sustained-release
$t_{1/2}$	Half-life, the time it takes for the drug concentration to fall by one half
THC	Tetrahydrocannabinol
$t_{\max}$	Time of maximum concentration
TraTox	Tramadol intoxications
UM	Ultrarapid metabolizer

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I gratefully acknowledge the head of the National Board of Forensic Medicine, and my supervisors, for the opportunity to perform these PhD-studies!

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My special thanks to my dear family, friends and love, for your endless encouragement and for just being you! When I think about our future, the following unsourced quotation cross my mind:

“When I was 5 years old, my mother always told me that happiness was the key to life. When I went to school, they asked me what I wanted to be when I grew up. I wrote down “happy”. They told me I didn’t understand the assignment, and I told them they didn’t understand life.”

Now it is time to increase the frequency of Swedish fika, movie nights, visits in the great outdoors, skiing, bicycle riding and many other activities associated with a taste of pure happiness!

In person (and mostly in my native language) I would especially like to thank/STORT tack till:

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INTRODUCTION

The key term in understanding the concept of this thesis is “enantiomers”. Within the field of organic chemistry and drug development, it is familiar for sure. Although numerous individuals, without any relationship to those fields, are familiar with the disaster that was caused by enantiomers in the 1950s. The thalidomide (in Sweden more known as Neurosedyn) disaster, is a tragic story, but well worth telling. Not only for the understanding of enantiomer existence, but also for the understanding of the pharmacological effects of drugs, and the necessity of laws and guidelines that regulate drug development and drug research today.

The thalidomide disaster

Thalidomide was originally synthesized as an antihistamine at Chemie Grünenthal in 1954. It was, however, launched as a sedative three years later, since the drug showed weak antihistaminic properties but produced marked sedative effects [1]. Sedatives were popular in the 1950s with one in eight prescriptions being for a sleeping pill [2]. Thalidomide also gained popularity among pregnant women suffering from nausea [3], and in 1960 thalidomide was marketed in 20 countries [1]. The same year, an estimated 14.6 tons of pills were sold in Germany free of prescription [1]. The drug was considered safe [1, 3], merely based on the fact that a median lethal dose could not be established [3], since excessive doses of thalidomide did not cause lethality in rodents [1]. A little over a year following the launch of thalidomide, adverse effects like dizziness, trembling hands and polyneuritis (inflammation of several nerves) were reported [2]. However, the only one that seemed to be concerned about the sometimes irreversible neuritis was Dr. Frances Kelsey, working at the U.S. Food and Drug Administration (FDA). She prevented thalidomide from being marketed in the United States, and for that she was later rewarded with the President’s Award for Distinguished Federal Civilian Service from President John F. Kennedy [3, 4]. In 1961, two physicians suspected an association between severe malformations of newborns and the use of thalidomide during the first trimester of pregnancy [1]. The drug was withdrawn in most countries the same year [4]. Several difficulties were however associated with the withdrawal. Initially, profit makers were unwilling to remove the drug from the market, and the 51 different trade names was an aggravating circumstance. Thalidomide was also not concurrently withdrawn in all

countries, why several hundred malformations occurred in Japan when the drug was already withdrawn in other countries. Some people in Sweden also continued to use the drug, provided by local pharmacies, since they were not aware of the national withdrawal [2]. In total, 10 000 children were born with amelia (absence of limbs), phocomelia (reduced limbs), and other malformations such as ear, eye, heart and gastrointestinal abnormalities [1]. Up to 40% of the affected children died within a year, stillborn children and miscarriages not counted. Even a single dose was shown to be associated with an increased risk of malformations [3].

The good and the bad enantiomer

In many medications the active drug, i.e. the component that exerts the pharmaceutical effect, is constituted of one compound. However, the active drug in the thalidomide tablets was constituted of two structurally identical compounds. These two compounds are called enantiomers and only differ in the fact that they are each other's mirror images. The enantiomers are distinguished by the prefixes (+) and (-), and a drug constituted of equal amounts of them is called a racemic drug.

Many years following the thalidomide disaster, it was revealed that the enantiomer responsible for the desirable thalidomide effect, the sedation, was the (+)-enantiomer, while the harmful effects were caused by the (-)-enantiomer. It was therefore proposed that the tragedy could have been avoided if the thalidomide drug had been based on the pure (+)-enantiomer. However, it should become apparent that thalidomide undergo chiral inversion in biological media, meaning that the different enantiomers are converted into each other in the human body. Therefore, the disaster could not have been avoided by using a drug formulation only consisting of the (+)-enantiomer [5].

Due to its anti-inflammatory properties [5], and usefulness in the treatment of serious diseases, there is still a clinical interest in thalidomide. FDA approved the drug for treatment of leprosy in 1998, and later also for multiple myeloma [4]. However, the drug must of course not be used by women of fertile age. It has been proposed that the anti-inflammatory effect is associated with (-)-thalidomide, why a stable analogue of the pure enantiomer potentially could be used as a new drug, with the wanted anti-inflammatory properties but without the sedation [5].

The learned lessons from the past

In the 1950s, many pharmaceutical companies were not dedicated to drug development. Chemie Grünenthal, which developed thalidomide, was for

example a subsidiary of a soap and cosmetics producer. Also, at that time, synthetic drugs were a new concept and no, or few, laws governed the development and marketing of these drugs [2]. The drug manufactures themselves were responsible for the safety testing, and clinical experiments could be performed without informed consent. Automatic approval of the drug was made in the USA, unless FDA could prove the drug unsafe within 60 days, based on the company's own tests. After approval of the drug, all knowledge of it was confidential to the manufactures [6]. The process of drug regulation was, however, more advanced in the USA than in Europe and the rest of the world. From 1938 it was required for the drug manufactures to perform safety tests and to inform about any harmful effects by adequate labelling. Referring to this regulation, Kelsey could prevent the marketing of thalidomide in her country [2]. When the thalidomide disaster was a fact, a series of initiatives were taken worldwide to prevent a similar episode. New laws were passed [3], committees for safety of drugs were formed, and guidelines for the testing of new drugs were written [2]. Many guidelines were found to be based on the same principles, and those became the foundation of the International Conference on Harmonisation (ICH) Guidelines in 1990 [2].

The unlearned lessons from the past

In spite of the awareness of the possible difficulties associated with racemic drugs following the thalidomide disaster, such drugs were continuously developed during the twentieth-century. Both pharmaceutical production and laboratory analysis of individual enantiomers were technically challenging at the time. Pharmacological and clinical evaluation of most racemic drugs were therefore based on the two enantiomers together, rather than on the individual enantiomers. However, still today, research studies are conducted and published without any consideration to the fact that a racemic drug, per definition, is actually constituted of two compounds in the same tablet, and should be investigated accordingly [5]. Drawing conclusions from studies not distinguishing between the two enantiomers have by critics been described as “sophisticated nonsense” and “at best misleading”. A need of focusing on the individual drug enantiomers have been stressed, as “it matters to science, readers of publications and last but not the least to the patients” [7].

BACKGROUND

Forensic toxicology

When toxicology is used to investigate questions or issues within the legal system, it is referred to as forensic toxicology [8, 9].

Investigations

There are primarily three categories within the field of forensic toxicology:

1. Postmortem toxicology, also known as death investigation toxicology [8, 9], in which it is examined whether alcohols, or illegal or prescription drugs have caused or contributed to an unexpected or unnatural death [10].
2. Human performance toxicology, in which it is investigated if the actions, performance, or behavior of an individual may be explained by or related to drug intake. It could for example be investigated if impaired driving was caused by alcohol or drug ingestion, or if a victim was drugged to facilitate a sexual assault. It could also be investigated if the perpetrator of a sexual assault or any other violence related crime was under the influence of drugs at the time of the crime [8, 9].
3. Urine drug testing, in which it is examined if any drug has been consumed. The test may for example be performed of convicted offenders [8, 9].

Preferred specimens

Blood is the specimen of choice regarding both postmortem and human performance toxicology, since drug effect and impairment are best interpreted from blood drug concentrations [8]. Also, only from blood samples it may be possible to approximately estimate when the drug was ingested [9].

In postmortem toxicology, whole blood is always analyzed, because it is usually not possible to separate the red blood cells from serum in postmortem samples [10]. Further, samples from a peripheral vein is preferred over samples from a central vein. In living individuals, drug concentrations are usually the same in central and peripheral blood, although postmortem, drugs may diffuse from central organs with high tissue drug concentrations into the central veins [11].

From urine samples only drug exposure can be established [8]. That is however enough to prove a petty drug offence. Urine is even preferred over blood in this regard, since the timeframe of drug detection is longer for urine [9].

Interpretation difficulties regarding opioids

There are high demands on the methods measuring drug concentrations within the field of forensic toxicology, regarding both specificity and accuracy. Otherwise, the results could not be utilized in a court of law [8]. However, the most challenging task in forensic toxicology is to interpret the measured drug concentrations [10]. This is especially true for the autopsy cases. What drug concentration levels should be interpreted as therapeutic, toxic or lethal? A literature search on drug concentrations reported in living individuals, either with a therapeutic or toxic outcome, could possibly provide some insights. However, these concentrations are usually derived from measurements in serum or plasma. This is problematic, since many drugs are unequally distributed between the cells and the fluids that constitute our blood. Thus, a concentration measured in blood may be both lower and higher than it would have been if analyzed in serum or plasma [10, 11]. In addition, there are numerous other factors that potentially could have an impact on the blood concentrations measured postmortem, for example the degree of putrefaction [11].

However, relating drug concentration to drug effect is not straightforward regarding living individuals either. One major reason for this is the large interindividual differences in drug response. Blood concentrations are not always closely related to drug effects. Already in the beginning of the twentieth-century, Sir Archibald E. Garrod coined the term “chemical individuality” and also stated [12]:

”Every active drug is a poison, when taken in large enough doses; and in some subjects a dose which is innocuous to the majority of people has toxic effects, whereas others show exceptional tolerance of the same drug.”

This is a statement being highly relevant still today. Several studies on patients have shown many-fold differences in required daily doses of various prescription drugs. For example, 30-fold differences have been observed in treatments with the antidepressant drug amitriptyline, 60-fold differences in treatments with the anticoagulant drug warfarin, and from a study on cancer patients, a 120-fold difference in the oral dose of morphine was reported [13]. In fact, opioids, which are widely used in the management of moderate to severe pain, are known to cause large interindividual variation in both opioid response, dose requirements and minimum effective blood level [14]. It is, however, hard to assess how large the interindividual differences are. It has been estimated that only one-in-

four of postoperative patients receive adequate analgesic treatment [15, 16]. It has also been reported in cancer patients that one month following the start of an opioid treatment only one-third experience decreased pain. One –fifth experience increased pain [17]. Others report that it is one-third, or less, of morphine treated patients that experience inadequate analgesia or intolerable adverse effects [18]. Nevertheless, due to the difficulties in predicting opioid effect, analgesic drugs are often titrated based on the self-reported pain level of the patient [19, 20]. Consequently, there is a risk of both adverse effects and undertreatment [20]. Genetic factors are thought to explain a considerable part of the interindividual differences [13, 14, 20], and are estimated to account for approximately 12-60% of the observed variability in opioid response [15]. However, there are also more cautious opinions on this subject, emphasizing that there are many factors besides genetic variation that potentially could give rise to individual differences [21].

The one and only Swedish forensic toxicology laboratory

There is only one forensic toxicology laboratory in Sweden; the Department of Forensic Genetics and Forensic Toxicology at the National Board of Forensic Medicine. The laboratory is commissioned by the police, prosecutors, the courts, and the prison and probation service to perform drug analyses in biological material, and in many cases to aid in the interpretation of the results. Specimens collected for forensic toxicological analyses are sent from all over the country to the laboratory located in Linköping.

Regarding the postmortem toxicological analyses, the commission passes through the Department of Forensic Medicine at the National Board of Forensic Medicine. The department is constituted of six units that are spread across Sweden. The forensic pathologists examine the deceased and determine which toxicological analyses that should be performed. However, there is close collaboration between the forensic pathologists and the forensic toxicologists, both regarding analyses to perform and interpretation of the results. Especially in cases where no evident cause of death is found following the initial investigations of the deceased.

In addition to identifying and quantifying alcohols and drugs, the Department of Forensic Genetics and Forensic Toxicology performs genetic investigations. These are mainly used to identify deceased individuals and to conduct paternity, maternity and kinship tests. Social welfare committees, courts and the Swedish Migration Agency are the major contracting authorities. There is also a pharmacogenetic interest at this department, which could lead to improved interpretations of the

forensic toxicological analyses in the future. However, that requires that the relationship between specific genetic variants and drug related effects, such as impaired performance or toxicity, is found.

Research, being the key in the search for such relationships, is therefore of the outmost importance to be able to deliver evidence based reports on unique questions and issues, with the legal certainty that is required.

To overcome some of the interpretation difficulties associated with the lack of proper reference data, the forensic pathologist Henrik Druid and the forensic toxicologist Per Holmgren initiated the Toxicolist project in the 1990s [22]. The Toxicolist project has grown larger ever since, with the purpose of creating a forensic toxicology database, comprising postmortem reference values for a large number of drugs. When establishing the reference concentrations for a certain drug, all postmortem positive cases from years past are independently reviewed by at least two persons. The cases are then discussed and a consensus is reached on the degree of drug involvement in the cause of death for each case. Subsequently, the cases are divided into A, B and C cases, corresponding to single drug intoxications (intoxications only caused by the investigated drug), multiple drug intoxications (intoxications caused by the investigated drug and at least one other compound), and controls (nonintoxications in which the individuals clearly was not incapacitated by drugs immediately before death), respectively. To ensure highest possible quality in the resulting reference values, a large proportion of cases, with any obscure circumstances, are excluded. For the majority of drugs, the median concentration in group A is higher than the one in group B, which in turn is greater than the one observed in group C. However, in accordance with the experience of opioid treatments within clinical practice, showing various responses and difficulties in dosing, hugely overlapping opioid concentrations between the A, B and C groups have been found in the Toxicolist project. Consequently, interpretations of opioid findings are still difficult. The need of other opioid toxicity blood markers than the traditional ones are therefore highly warranted. Finding them would likely have implications of both clinical and forensic toxicology.

In the present thesis, enantioselective drug disposition combined with various genotypes were investigated as possible factors contributing to improved forensic interpretations of the opioid drug tramadol.

Essential pharmacological terms

For the study and understanding of interindividual differences in drug action and toxicity, there are some general terms that one needs to be familiar with.

Pharmacokinetics

Pharmacokinetics is considered the part of pharmacology explaining “what the body does to the drug”. Pharmacokinetics comprises the processes of drug absorption, distribution, metabolism, and excretion [23], and so describes the dose-concentration relationship [13].

When a certain drug dose has been administered to an individual, and blood concentrations are repeatedly measured following the drug intake, the blood concentrations of the study subject can be plotted over time to create a concentration-time curve. The peak of the curve corresponds to the highest blood concentration achieved following the drug intake and is called $C_{\max}$; the maximum concentration. The time when $C_{\max}$ occurs is called $t_{\max}$; the time of maximum concentration. **AUC** is a general abbreviation for the area under the curve, but in this context refers to the area under the drug concentration-time curve. Thus, AUC is a measure of the total systemic exposure to the drug. Somewhat simplified, the shape of the concentration-time curve before the peak is a function of the absorption process, while the shape of the curve after the peak is a function of the metabolism and excretion processes [24].

There is always a significant difference in blood concentrations between patients receiving the same dose regimen. The factors contributing to this variability are many and of both genetic and non-genetic origin [13].

Absorption

Orally administered drugs are primarily absorbed to the blood circulation from the upper part of the small intestine [13]. Efflux transporters in the gastrointestinal tract may limit the drug absorption, while influx transporters may promote it [25].

There can be considerable differences in the rate and extent of drug absorption both between individuals and within the same individual on different occasions. One reason for this is variations in the gastric emptying time and in the motility of the small intestine. Food may also have a large impact on the drug absorption, depending on the size and components of the meal, and of the physiochemical properties of the particular drug. Drugs that are highly soluble and highly permeable are usually less affected by concomitant food intake, especially when administered as an immediate-release (IR) formulation [13].

The fraction of ingested drug that reaches the systemic blood circulation is designated **bioavailability** [24].

Metabolism

After the drug has been absorbed from the intestine, but before it reaches the systemic blood circulation, it is transported to the liver via the portal vein [24]. In the liver, enzymes may modify the drug and form drug metabolites. The process is called first-pass metabolism. Most opioids are subject of extensive first-pass metabolism in the liver, which decreases their bioavailability [25]. Different enzymatic pathways are involved in the metabolism of different drugs. The most common reactions performed by the liver enzymes are oxidation, reduction, hydrolysis, and conjugation. The three former are often referred to as phase I reactions, simply because they occur first, and conjugations are referred to as phase II reactions. The enzymes exerting the oxidation and reduction of many drugs belong to the superfamily of so called cytochrome P450 enzymes, which is abbreviated CYPs. The superfamily is further divided into families, indicated by the numbers 1, 2, or 3, and into subfamilies, designated by the letter A-E. One specific enzyme is indicated by yet another number. CYP2D6 is a well-known example of a metabolic enzyme belonging to the CYP superfamily. It only comprises about 2% of the total liver content of CYP enzymes, although it is involved in 25% of the liver drug metabolism [24]. Interindividual differences in drug metabolism can be caused by variations in the functionality of these liver enzymes. An altered functionality, increased or decreased, of a liver enzyme may be the result of genetic variation. Once more, CYP2D6 is an appropriate example. Numerous genetic variations have been identified in the *CYP2D6* gene. Based on the combination of these genetic variants carried, individuals may be classified into poor, intermediate, extensive (normal), or ultrarapid metabolizers. These groups are abbreviated PMs, IMs, EMs, and UMs, respectively [20]. As the names imply, PMs have inactive CYP2D6 enzymes and UMs have CYP2D6 enzymes with an increased activity. The consequence of inactive CYP2D6 enzymes, following administration of a drug whose metabolism is highly dependent on these enzymes, is increased blood concentrations of the drug, and possibly adverse effects. On the contrary, for individuals with enzymes showing a superior activity, the blood concentrations of the drug will be lower than expected, and the therapeutic effect of the drug may be lost [26]. In a Caucasian population there are about 5-10% PMs, 10-15% IMs, 65-80% EMs, and 5-10% UMs [27]. The proportion of UMs is somewhat lower in Sweden, about 1-2%. In non-Caucasian populations, the frequency of UMs may instead be much higher, such as in Ethiopia (29%) and in Saudi Arabia (21%) [26].

Liver enzymes can also be induced or inhibited by coadministered drugs or by environmental factors [26].

Distribution

Once a drug has been absorbed to the systemic blood circulation, it is distributed throughout the body, and will reach the organ or tissue being its site of action. The distribution involves the crossing of several cell membranes, which may occur by passive diffusion or by influx- and efflux transporters. The concentration of the drug in the intracellular fluid of any cell is determined by the net difference of drug influx and efflux [13]. The **volume of distribution** is a term reflecting the fraction of a drug in plasma versus the fraction in the tissues, and it varies widely between drugs. The larger the volume of distribution, the larger is the drug fraction in the tissues [24].

Excretion

Excretion is defined as the loss of intact drug from the body. Most drugs are predominantly excreted via the kidneys. However, as already mentioned, drugs may also be converted to metabolites in the liver, and the metabolites are subsequently excreted from the body. The term elimination of a drug therefore refers to the process of both excretion and metabolism. In general, metabolism constitutes the major elimination route of drugs [24]. Drug transporters in the kidneys are involved in the renal excretion of drugs and their metabolites [25].

A frequently used pharmacokinetic term concerning the elimination of a drug is its half-life, $t_{1/2}$. It corresponds to the time it takes for the drug concentration to fall by one half [24].

Pharmacodynamics

Pharmacodynamics is the part of pharmacology explaining “what the drug does to the body”, i.e. the drug action [23] or the concentration-response relationship [13]. Because of the recent progress in analytical techniques it is relatively easy to measure a blood drug concentration, in comparison to measure a drug response [13]. However, it could be underlined that it is the drug concentration at the target site, being the brain for many opioids, which is of the greatest significance to the drug effect. Although, for obvious reasons it is not possible to measure brain concentrations (at least not in living individuals). Blood concentrations, instead of brain levels, are therefore used in relation to drug effect.

Pharmacodynamic interindividual variation may be caused by different receptor binding affinity, receptor density, and receptor activity [25]. Psychological factors can also be of major importance regarding interindividual differences in drug response. The placebo effect is a well-known phenomenon, although scientifically not completely understood. It means that a patient administered an inactive compound, while believing

it is the active drug, actually experiences improvements in disease symptoms. The opposite is known as the nocebo effect, meaning that a patient administered the active drug, but who disbelieves in the treatment, does not experience any improvements or may even experience a deterioration in the symptoms [13].

Pharmacogenetics

As apparent from the above, proteins acting as drug transporters are of importance both in the absorption, distribution, and elimination of drugs. Proteins being liver enzymes are instead critical in drug metabolism, while proteins constituting drug targets, for example receptors, are essential for the drug to exert its effects. In conclusion, biological proteins are considerably involved both in the pharmacokinetics and pharmacodynamics of drugs. If a protein is encoded by a gene subject to sequence variations, the protein might be affected in its function and in turn affect drug action. Pharmacogenetics is thus a term describing how genetic variation may affect the pharmacokinetics and pharmacodynamics of certain drugs [25].

Proteins are built from several amino acids in a specific order, dictated by the genetic code. The genetic code is written using only four letters; A, T, G and C, corresponding to so called deoxyribonucleotides in the DNA-molecule. The protein machinery read the genetic code in sequential groups of three. Different three letter combinations thus encode different amino acids [28, 29]. Polymorphisms are genetic variations in the DNA code that are present in more than 1% of the population. Single nucleotide polymorphisms (SNPs) are as the name implies a polymorphism concerning only one nucleotide [30]. However, the impact on the protein produced may be large, sometimes resulting in a protein with altered function, or even a nonfunction. How large or small the consequences depends on if the exchanged nucleotide may change the three letter combination, so that it is read as a different amino acid or as a stop signal, and if these modifications may change the protein structure and thereby its function [31].

The frequency of SNPs is high in the human genome. In fact, SNPs have been identified in 93% of all known genes. All proteins involved in the pharmacokinetics and pharmacodynamics of a drug could consequently be subject of genetic variation. Much research has been focused on polymorphisms in genes encoding drug metabolizing enzymes, such as *CYP2D6*, while polymorphisms in genes encoding transporters and receptors have been less investigated [13].

Chirality

Stereoisomerism

Isomers are compounds with the same molecular formula [32], and these may be divided into structural isomers and stereoisomers. Structural isomers have different structural formulas, meaning that the atoms are arranged differently in the molecule (different configuration). Stereoisomers, also called spatial isomers, on the other hand, only differ in the spatial orientation of the atoms. So the atom-to-atom linkages and bonding distances are the same, but from a three-dimensional point of view there is a difference [33, 34]. Stereoisomerism in drugs is often due to what is called chirality [35]. Chirality is dependent on the presence of an asymmetric center, also called chiral center, in the drug molecule, which is often a carbon atom with four different substituent groups [7, 33, 36]. The word chiral originates from the Greek language, where *cheir* means handedness [36]. Hands are often used to exemplify the nature of chirality. The left and right hand may namely be considered as isomers, since they have the same formula, consisting of 1 palm and five different fingers. They are also stereoisomers, because the fingers are arranged in the same way in relation to the palm. And they may be considered as enantiomers, because the left and right hand are mirror images of each other that cannot be superimposed. That is, if the hands are placed upon each other, when palms are facing the same direction, they do not match [34] (**Figure 1**). Stereoisomers not being enantiomers are called diastereoisomers. They are also nonsuperimposable, but not mirror images of each other [33]. Both enantiomers and diastereoisomers occur with a drug having more than one chiral center. If a certain drug has two chiral centers, there will be two pairs of enantiomers. The enantiomers in one of those pairs become

Figure 1. The enantiomers of the chiral drug tramadol are, in similarity with the left and right hand, mirror images of each other that cannot be superimposed. (Hand drawn by Maria Norlund, colleague at the National Board of Forensic Medicine).

diastereoisomers in relation to the enantiomers in the other enantiomer pair [37].

Enantiomer nomenclature

There are several ways of designating the enantiomers constituting an enantiomer pair. While diastereoisomers differ in physical and chemical properties [7], enantiomers are identical in those aspects [33]. There is, however, one exception; they rotate plane-polarized light in opposite directions. One way of naming the enantiomers is based on this property. Enantiomers that rotates plane-polarized light to the right are called dextrorotary, abbreviated (d), or (+)-enantiomers. Those who rotate the light to the left are known as levorotary, (l), or (-)-enantiomers [7, 33, 36]. Equimolar mixtures of the enantiomers that form an enantiomer pair is called a racemic mixture or a racemate [34]. Racemates, with the prefix ($\pm$) or (d,l), does not have any optical activity [7, 36], since the rotation caused by the (+)- and (-)-enantiomers are equal in magnitude but opposite in direction [33]. This nomenclature of enantiomers is the oldest and originates from the work of Pasteur, a French chemist and biologist. Already in 1848, he discovered two different crystal forms of sodium ammonium tartrate, which he managed to separate. He also found that those two forms rotated plane-polarized light differently [33, 36]. The d and l prefixes must not be confused with the D- and L-terms, which instead constitutes the Fisher nomenclature used for carbohydrates and amino acids. The molecule is written in what is called Fisher projection, with the most oxidized carbon at the top. If the substituent (OH) at the bottom chiral center points to the right it is termed the D-stereoisomer, while if it points to the left it is called the L-stereoisomer [33]. Enantiomers may also be designated R or S, according to the Cahn-Ingold-Prelog convention describing the absolute configuration of the molecule. To determine if a certain enantiomer has the R- or S-configuration, all substituents to the chiral centre are prioritized according to their atomic number. The highest atomic number corresponds to the highest priority. When the chiral centre is oriented so that the lowest priority substituent is pointing away from the viewer, the order of the other substituents, from the highest to the lowest priority, may be either in a clockwise or counter clockwise direction. If it is clockwise, the molecule is considered to have the R-configuration, where R is an abbreviation for the Latin word *rectus*, meaning right. If the direction is counter clockwise, the molecule is instead considered to have the S-configuration, from the Latin word *sinister*, meaning left [7, 36]. A racemate is designated as R,S [36]. So, while the (+)/(-) system describes the rotation of plane-polarized light, both the D/L and R/S systems refer to the spatial orientation of the substituents at the chiral center. However,

there is no correlation between any of the systems, so an enantiomer may be named using more than one system, for example S(+) or S(-) [7, 33, 34].

Nature's homochirality; the maintenance of life

Biological macromolecules, such as nucleic acids, enzymes, receptors and transporters are chiral molecules, as are the amino acids and carbohydrates that forms them [7, 38]. In ordinary chemical production of chiral compounds, the L- and D-stereoisomers are formed in equal amounts, which is a racemic mixture. As opposed to artificial molecules, related biological chiral compounds almost exclusively exist in only one of the two forms. For example, most amino acids are L-stereoisomers, while most carbohydrates are D-stereoisomers [36, 38, 39]. This phenomenon is called homochirality [38]. It is proposed that equal amounts of L- and D-amino acids were present on earth before life evolved. It is, however, not known why that situation changed and why all living organisms now are constituted of mainly L-amino acids [40]. What biased the formation of one enantiomer over the other, and how was that bias sustained? Those questions have bemused scientists for more than a century. Already in 1898 F.R. Japp, being President of the Chemical Section of the British Association for the Advancement of Science, declared [41]:

“The absolute origin of compounds of one-sided symmetry found in the living world is a mystery as profound as the origin of life itself...the production of a single enantiomorph cannot conceivably occur through the chance play of symmetric forces.”

Although the cause of homochirality is not clear, the necessity of it is easier to understand. With different spatial orientation, L- and D-amino acids give rise to different three-dimensional protein structures [38]. Consequently, polymers consisting of different amino acid stereoisomers would not be folded in the same way as the protein structures that we know today [40]. Since protein structure is closely related to protein function, homochirality is essential for the molecular functions of organisms [38] and to the maintenance of life [40].

Diverse drug action of the two enantiomers

Even though the enantiomers of a chiral drug have identical physiochemical properties, they may interact differently with other chiral molecules in the body, such as transporters, enzymes, and receptors. Consequently, the enantiomers may demonstrate distinguished pharmacokinetics and pharmacodynamics, such as diverse absorption, tissue distribution, plasma protein binding, metabolism or elimination, as well as different therapeutic and adverse effects [7, 33, 36]. Accordingly, biological systems recognize the chiral drug as two different compounds.

In the same way that a right hand fits into a right hand glove, but not a left hand glove, the two enantiomers of a drug may, or may not, interact with the binding- and catalytic site of a certain biological protein. The phenomenon can schematically be explained by a three-point interaction between the enantiomers and the binding site of the target protein, as shown in **Figure 2**.

Figure 2. The three-point interaction, explaining why one enantiomer in a drug enantiomer pair may exert an effect and not the other. For a drug effect to be induced in this case, all three drug substituents shown as geometrical figures must interact with the corresponding regions of the receptor binding site illustrated by the blue plane. Only one of the enantiomers has substituents spatially orientated to fit the binding site. (Illustration by Johan Enmyren ♥, in accordance with the wishes of Pernilla Haage)

The target protein in this case is a receptor, and for a drug to exert an effect, the substituents of the drug illustrated by the geometrical figures must interact with the corresponding regions of the receptor binding site. Because of the differences in spatial orientation of the substituents, only one of the two enantiomers have the ability to induce a receptor mediated effect. In spite of having the same substituents, the other enantiomer cannot align with the receptor, no matter how it is rotated in space [36, 42]. The inactive enantiomer at this receptor may, however, interact with other receptors, and evoke other therapeutic or adverse effects. But it may also completely lack pharmacological effects. With a chirally less discriminating receptor than the one shown in **Figure 2**, there is also a third possibility. Both enantiomers may have the ability to bind the target receptor, so that the enantiomers exert the same effect with almost the same magnitude, or one of the enantiomers may exert the same effect although with less magnitude compared to the other enantiomer [36, 43, 44]. It is also possible that one of the enantiomers have antagonistic properties regarding the target receptor [44]. It should be underlined that, as well as for other effects, adverse effects may not be induced only by one of the enantiomers. Consequently, it is not always the case of a “good” and a “bad”

enantiomer (also referred to as eutomer and distomer, respectively) such as with thalidomide. Also in the case of only a single enantiomer possessing the desirable effect, both enantiomers may cause toxicity [36]. The three-point interaction to explain enantiomer differences in drug action was postulated by Easson and Stedman in 1933 [5]. During the preceding decades the first evidence of pharmacological differences between drug enantiomers had been shown [5, 33]. It was for example found that endogenous adrenaline, being levorotatory, was twice as potent as racemic adrenaline, being synthetically produced. The dextrorotatory enantiomer was, on the contrary, much less potent [5]. As important for drug action as receptor interactions and chiral pharmacodynamics, are the processes involved in chiral pharmacokinetics [36]. Drug metabolism is generally regarded as the key factor in causing stereoselective drug disposition [43, 45]. Several metabolic enzymes have been shown to preferably metabolize either the R- or S-enantiomer of several drugs [45]. Any changes of metabolic capacity of an enzyme, due to induction or inhibition, may therefore affect the drug disposition of the enantiomers, and consequently the pharmacological effect [43, 45]. However, not all pharmacokinetic differences between enantiomers are to be explained by their metabolism, and it has been emphasized that also investigations of the interaction between enantiomers and their transporters should be performed. There are much less studies in this field compared to the one of stereoselective drug metabolism. In vitro and animal models have demonstrated enantioselective activity of a transporter named P-glycoprotein (P-gp), although this activity may be concentration dependent and different between species [46].

In 1992, the FDA published a guideline on chiral drugs, stating that development of single enantiomer drugs were generally to be preferred over racemates, if toxicity could be eliminated with preserved pharmacological effect [32]. To a larger extent than previously, newly developed drugs are now achiral drugs or constituted of single enantiomers [32, 33]. However, there are numerous chiral drugs already on the market [32, 33], and much of the knowledge on their pharmacokinetics and pharmacodynamics are derived from non-chiral assays [45]. This is due to that most conventional analytical techniques are not able to discriminate between the enantiomers [43], and once again the reason is the identical physical and chemical properties of the enantiomers, as long as they are not facing a chiral environment [33]. Diastereoisomers on the contrary, differ in those aspects and can therefore easily be separated [7]. Data generated from achiral assays are at best of limited value, but at worst highly misleading when relating total drug concentrations in blood to therapeutic or adverse effects [45]. This is especially true when the enantiomers of a chiral drug have considerably different

pharmacodynamic effects, and when the enantiomers are diversely metabolized by enzymes that can either be induced or inhibited, or that are polymorphic [43]. Developing and utilizing stereoselective assays, and investigate the pharmacokinetics and pharmacodynamics of such chiral drugs are therefore important [43, 45].

Tramadol

The "safe" opioid alternative

Tramadol is a commonly prescribed opioid analgesic, administered as a racemic mixture of (+)- and (-)-tramadol. There are two chiral centers in the tramadol molecule, resulting in four diastereoisomers. However, only two of the diastereoisomers are administered as the racemic drug, being the enantiomer pair of 1R,2R (+) and 1S,2S (-) [47]. Throughout the rest of the thesis, the (+)/(-) system will be utilized to describe the enantiomers of tramadol.

Tramadol was discovered already in 1962, but was not approved by the FDA until 1995 [48]. Pain relief is exerted through both opioid and nonopioid pathways, involving μ -opioid receptor activation as well as inhibition of serotonin and noradrenaline reuptake [48, 49]. The analgesic effect of tramadol is therefore only partially blocked by naloxone. Because of the dual mechanism of action tramadol may be regarded an atypical opioid. The mechanism of serotonin and noradrenaline reuptake is similar to the action of some antidepressants, and preclinical studies have confirmed an antidepressant effect of tramadol. In comparison to other opioids, tramadol is a relatively weak μ -opioid receptor agonist [48]. For quite some time the drug was therefore considered to be less toxic and with less abuse potential than other opioids. However, it is now apparent that tramadol use may be associated with both abuse [50] and serious side effects [51, 52]. In Sweden, tramadol and its metabolite *O*-desmethyltramadol (ODT) are scheduled as narcotic compounds since December 2007 and May 2011, respectively.

Trends in tramadol use

Worldwide, the use of opioid analgesics more than doubled between the time periods of 2001-2003 and 2011-2013. Substantial increases were found in North America, western and central Europe, and in Oceania [53]. Some people consider this well-known phenomenon as a prescription opioid epidemic [54]. The increases in Europe are likely explained by the use of tramadol, fentanyl, and oxycodone [55].

Figure 3. Opioid trends in Sweden, illustrated by (a) the number of positive autopsy cases between 1995 and 2017, and (b) the prescription expressed as million defined daily dosages (DDDs), and (c) the number of prescribed patients, respectively, between 2006 and 2017. The total number of postmortem toxicological investigations is approximately the same every year. Data in (b) and (c) were obtained from The National Board of Health and Welfare.

Between the years of 2006-2015 the annual sales of tramadol increased by 62% in France. Overall though, the consumption of weak opioids decreased by 53%, due to the reduced use of dextropropoxyphene [55]. In Italy, a 269% increase in the amounts of tramadol sold to community pharmacies between the years of 2000 and 2010 has been shown. The amounts sold to hospitals were however rather stable during this period, showing an increase of only 3.6% [56]. When investigating the number of community-dispensed prescriptions of any analgesic in Scotland between 1995 and 2010, only a modest increase was found. However, there was a substantial increase in the prescription of strong opioids. Tramadol is often regarded a rather weak opioid, although in this study it was classified as a strong opioid, and it did also show the largest prescription increase in the group of strong opioids. Dextropropoxyphene was the most commonly prescribed weak opioid in 1995, although was not dispensed at all in 2010. The drug was withdrawn in Scotland 2007 because of an association with overdose fatalities [57]. When opioid consumption was examined in the five Nordic countries between 2002 and 2006, an increase for tramadol was observed in all countries with the exception of Sweden. The increase was largest in Norway, being 98%. Dextropropoxyphene was not prescribed at all in Iceland during the time period, and a decreased consumption was shown in all other countries, in Sweden by 53% [58]. Some interesting patterns was revealed when recently comparing the trend in the number of opioid positive autopsy cases with the trends concerning prescription of the same drugs in Sweden (**Figure 3**). Consistent with previous reports was a decreased use and prescription of dextropropoxyphene. For tramadol as well, there was a decrease in the prescribed defined daily dosages (DDDs) and the number of prescribed patients from 2011. Although, the same downward trend was not observed regarding findings in autopsy cases. This difference may reflect an abuse, and an illegal market, of tramadol. The trends in prescription versus the trend in autopsy cases were also different for fentanyl, showing increases in the number of autopsy findings but rather stable prescription rates. This may as well reflect an abuse, although it must be emphasized that the National Board of Forensic Medicine switched to a more comprehensive screening method in 2011, wherefore increases in the number of findings also may reflect an improved drug detection rate.

Dosing and pharmacokinetic parameters

Tramadol is available in many formulations and may be administered both by oral, sublingual, intravenous, intramuscular, subcutaneous, and rectal delivery.

To minimize adverse effects such as nausea and vomiting, tramadol may be slowly titrated. The starting oral dosage for moderate chronic pain is 25 mg

daily for three days, followed by gradual increases to 50 mg every four to six hours. Doses of 100 mg may also be administered every four to six hours, although the total daily dosage should not exceed 400 mg [59].

Following oral administration, absorption is almost complete and occurs rapidly. $C_{\max}$ is reached in about 2 h, and is approximately 0.3 mg/L following a single dose of 100 mg. Both plasma concentration and AUC increase linearly in the dose range of 50 mg to 400 mg. At maximum dosage the steady-state $C_{\max}$ is about 0.35 mg/L. The bioavailability is 70% after a single dose, and 90-100% following multiple administration. The increase is likely caused by saturation of the liver enzymes performing the phase I reactions. Distribution also occurs rapidly, and 20% of the drug is bound to plasma proteins. The volume of distribution is 306 L, indicating a high tissue affinity. The $t_{1/2}$ is about 5-6 h for tramadol and 7-9 h for ODT. The excretion occurs almost exclusively via the kidneys. Approximately 30% of the drug dose is excreted as unchanged drug (tramadol) in the urine and about 60% is excreted as metabolites. The remaining drug dose is excreted in the feces [48, 60-63].

For oral administration, both IR and sustained-release (SR) formulations of tramadol are available on the market. Because of the relatively short $t_{1/2}$ of tramadol, IR formulations must be administered frequently throughout the day, often four to six times. With SR formulations, which release the active compound over a longer period of time, the number of administrations per day can be reduced [61, 64]. Two dosage regimens, constituted of either a 50 mg IR dose four times daily at 6 h intervals, or a 200 mg SR formulation at 24 h, have been pharmacokinetically compared. The investigation comprised both the 24 h interval (corresponding to a single dose of the SR formulation) and multiple dose administration for five days. With regard to the total exposure of tramadol and ODT, measured as AUC, the dosage regimens were equivalent concerning both the single dose administration and the repeated administration during five days. However, the $C_{\max}$ of tramadol and ODT was significantly lower with the SR formulation regarding both the single dose administration and the repeated administration. The incidence of adverse effects was similar in the two dosage regimen groups [64]. Blood concentrations of 0.1-0.8 mg/L and 1-2 mg/L are generally considered therapeutic and toxic, respectively, while concentrations above 2 mg/L may be lethal [65, 66].

Adverse effects

The most frequent adverse effects following tramadol ingestion are nausea, dizziness, drowsiness, fatigue, sweating, vomiting, and dry mouth [61]. Two adverse effects, being more severe, that also may occur are seizures (convulsions) and serotonin syndrome. These seem to be associated with tramadol overdoses and with coadministration of other drugs, particularly

antidepressants. However, they may also occur following exclusive tramadol use. Serotonin syndrome is caused by an excessive amount of the neurotransmitter in the central and peripheral nervous system, and may be lethal. The syndrome is characterized by neuromuscular hyperactivity (such as tremor, hyperreflexia or rigidity), autonomic hyperactivity (for instance resulting in fever, tachycardia or tachypnea), and altered mental status (that for example may be indicated by agitation or confusion) [51].

During a couple of months in 2007, 114 tramadol intoxicated subjects were admitted to a hospital poison center in Iran. The most common adverse effects were nausea (76.3%), dizziness (62.3%), vomiting (43.9%) and headache (38.6%). However, also seizure (35.1%), anxiety (27.2%), and unconsciousness (23.4%) were frequent. The doses associated with seizures were relatively high, although in one case it occurred following a 300 mg dose [67]. In another study, the lowest dose associated with seizure was 200 mg [68]. Unconsciousness was subdivided into grade I-IV. Most of the affected individuals (61.5%) showed grade I unconsciousness. This was thought to be explained by the weak μ -opioid effect of tramadol compared to many other opioids. Eight unconscious cases (7.0%) were admitted to the intensive care unit where they were intubated and ventilated. Two of these cases, attempting suicide, died from cardiopulmonary arrest. The doses ingested were 5000 mg and 8200 mg, respectively [67].

In the US, exposures of prescription opioids were investigated by reviewing cases reported to poisoning centers. Serious adverse events (SAEs) resulting from opioid exposures were subdivided into death, hospitalization, or major medical effect. The latter referred to life-threatening symptoms or significant residual disability. Tramadol, although being the least potent opioid analgesic investigated, was the third most prevalent drug reported to the poison centers concerning SAEs. The inhibitory effect of tramadol on serotonin and noradrenaline reuptake was suggested as a possible explanation, resulting in adrenergic hyperactivity, seizures, and serotonin syndrome. During the seven year study period between 2010 and 2016, seven tramadol related deaths were reported, and the number of major medical effect and hospitalization was 526 and 3070, respectively [54].

The fatal toxicity index (FTI) is a measure of relative toxicity. It is calculated by dividing the number of fatal intoxications caused by a particular drug by the consumption of the drug over the same time period and area. In the years of 2005, 2009, and 2013, the highest mean opioid FTIs in Finland concerned methadone, dextropropoxyphene, oxycodone, tramadol, and morphine in descending order. If only evaluating the year of 2013, the three opioids with the highest FTIs (expressed as deaths per million defined daily doses) were oxycodone (7.18), tramadol (6.77), and fentanyl (4.61),

respectively. FTIs at or above 1.0 may be attributed to especially high toxicity. In 2005 the tramadol FTI was 3.91. Some caution should however be taken when interpreting the results, since many fatal intoxications are caused by several coadministered drugs. In this study, only the most important drug finding in each case, as judged by the forensic pathologist, was taken into account [69].

Metabolism

Several tramadol metabolites, which in similarity with tramadol itself are composed of (+)- and (-)-enantiomers, have been identified. The major ones are *O*-desmethyltramadol (ODT), *N*-desmethyltramadol (NDT) and *N,O*-desmethyltramadol (NODT) [70]. The metabolic enzymes CYP2D6, CYP2B6 and CYP3A4 are responsible for the metabolite formation [71] (Figure 4).

Figure 4. Three main metabolites are formed from tramadol by the CYP2D6, CYP2B6, and CYP3A4 enzymes, respectively; *O*-desmethyltramadol (ODT), *N*-desmethyltramadol (NDT), and *N,O*-desmethyltramadol (NODT). Asterisks indicate the two chiral centers.

Liver enzymes may be induced or inhibited by other coadministered drugs and several compilations have been published on the substrates, inhibitors, and inducers, respectively, of the metabolic enzymes [72, 73]. However, there is less information on the magnitude of pharmacokinetic changes due to drug interactions, even though it is clear that they may be large. Paroxetine is an antidepressant and also a very potent CYP2D6 inhibitor. The effect of coadministration with tramadol has been investigated in sixteen healthy CYP2D6 EMs, in a study by Laugesen et al [74]. The

participants received either 20 mg daily paroxetine or placebo during three consecutive days, before being administered a single oral dose of 150 mg tramadol. Pretreatment with paroxetine resulted in an increased AUC of (+)- and (-)-tramadol with 37% and 32%, respectively. The AUC of (+)- and (-)-ODT decreased with 67% and 40%, respectively [74].

Enantiomer pharmacodynamics

Both the parent compound, tramadol, and some of its metabolites contribute to the therapeutic as well as the adverse drug effects. It is known that (+)-ODT is the enantiomer with the highest affinity and potency for the μ -opioid receptor, but also (+/-)-NODT and (-)-ODT have higher affinity for this receptor than the tramadol enantiomers [75]. However, (+)-ODT is considered the enantiomer exerting most of the μ -opioid effects [75].

Regarding the second mechanism of action, the neurotransmitter reuptake inhibition, there are also potentially important differences between the two enantiomers. The (+)-enantiomer of tramadol is known to inhibit serotonin reuptake to a greater extent than (-)-tramadol, which instead is more potent in inhibiting noradrenaline reuptake. The pharmacodynamic effects of the tramadol enantiomers may be summarized as follows:

(+)-tramadol: serotonin reuptake inhibition $\approx$ μ -opioid receptor binding > noradrenaline reuptake inhibition >> δ -opioid receptor binding >> κ -opioid receptor binding [76].

(-)-tramadol: noradrenaline reuptake inhibition >> serotonin reuptake inhibition >> μ -opioid receptor binding > κ -opioid receptor binding > δ -opioid receptor binding [76].

Tramadol pharmacogenetics

CYP2D6

Several studies have consistently showed that CYP2D6 PMs, in comparison to EMs, achieve higher AUCs of (+)- and (-)-tramadol combined with reduced AUCs of the ODT enantiomers, particularly of (+)-ODT [77-79]. UM, on the contrary, are expected to present with higher AUCs of (+)-ODT than EMs, although few comparative studies have been performed. The UM phenotype is generally not caused by SNPs, as opposed to the PM phenotype. It is instead characterized by one or several duplications of functional or decreased-functional alleles of the *CYP2D6* gene. The largest number of copies found is 13. However, the *53 allele might cause ultrarapid metabolism of tramadol without duplication, although this allele has only been detected in a small percentage of South Asians [62].

PMs have been shown to require higher dosages of tramadol than EMs to achieve sufficient pain relief, and their need of rescue medication is also increased. Some patients being PMs do not experience any effect of tramadol at all [62]. As a corollary, it is hypothesized that the opioid related adverse effects following tramadol administration is related to the concentrations of (+)-ODT; the higher the concentration, the higher the risk of side effects and toxicity [62, 71]. Hence, PMs are expected be less prone to adverse effects, while UMs are expected be more prone [62].

CYP2B6 and CYP3A4

The CYP2B6 and CYP3A4 enzymes are, in similarity with CYP2D6, involved in the tramadol metabolism, for what reason polymorphisms in the *CYP2B6* and *CYP3A4* genes may have a significant impact on it. Several SNPs have been found in these genes and some of them have been shown to cause increased or decreased metabolic capacity of many drugs [80, 81]. The potential impact of these polymorphisms on the pharmacokinetics of tramadol and its metabolites has however not been investigated.

ABCB1

The polymorphic adenosine triphosphate-binding cassette B1 (*ABCB1*) gene encodes the transmembrane transporter P-gp. P-gp is located in the blood-brain barrier and gut and works as an efflux pump of a variety of drugs. Thus P-gp reduces the access of drugs into the blood circulation and into the brain. The protein is also present in several other organs, such as the heart, lungs and kidneys [82]. The most common SNPs in the coding sequence are 1236 C > T, 2677 G > T/A, and 3435 C > T [83], of which the latter is the most studied. It has been associated with both increased and decreased expression of P-gp [82]. In a study of healthy volunteers administered 100 mg tramadol, no statistically significant increases in C_{max} or AUC of the parent compound were observed with an increased number of the variant allele [84]. In a follow-up study on postoperative patients, the SNP 3435 C>T did not influence the analgesic efficacy of tramadol (although CYP2D6 phenotype did) [85]. However, polymorphisms within the *ABCB1* gene have been shown to affect treatment with other opioids. For example, the combined evaluation of the SNP 118 A>G in the μ -opioid receptor gene (*OPRM1*, see below) and the *ABCB1* SNP 3435 C>T could in one study predict morphine pain relief in humans. Best responders were individuals with the *OPRM1* AA genotype combined with the *ABCB1* TT genotype [86]. Since the three most common SNPs are in strong linkage disequilibrium [83], some studies have investigated haplotypes instead of, or in addition to, the individual SNPs. An increased chance of requiring higher methadone doses has been observed with the TT-TT-TT haplotype,

while individuals with the CT-GT-CT haplotype had an increased chance of requiring lower doses [83].

Large increases in brain concentrations have been shown for several drugs, among them methadone, in knock-out mice. Loss-of-function mutations in the *ABCB1* gene of collie dogs have also caused severe neurotoxic effects following treatment with the opioid loperamide. However, in humans, no loss-of-function mutations have been described [82].

It has not been established if tramadol is a substrate of P-gp, an in vitro study and one in vivo study in rat have suggested that it is not [87, 88]. However, venlafaxine, being structurally very similar to tramadol [89], is a P-gp substrate in mice [90]. Also, CYP3A4 and P-gp have overlapping substrate specificities [82], and methadone, primarily metabolized by the same enzymes as tramadol (CYP3A4, CYP2D6 and CYP2B6) is also a P-gp substrate in humans [83].

OPRM1

The three opioid receptors μ , δ , and κ are the most common. They are all G protein-coupled receptors, being structurally similar in the transmembrane and cytoplasmic domains, but different in the extracellular domains. Consequently, distinct opioids may have different selectivity for the three receptors. The majority of prescription and illicit opioids are selective for the μ -opioid receptor. Polymorphisms in the gene encoding the μ -opioid receptor, being the *OPRM1* gene, could therefore constitute a significant part of the interindividual differences in opioid response [13, 91]. More than 3000 genetic variations have been identified in *OPRM1*, although many of them are rare [91]. Still, several hundred of them are more frequently found (i.e. polymorphisms), of which the 118 A>G is the most common. Studies of this polymorphism in vivo have shown that it causes reduced gene expression, binding affinity, and signal transduction. However, studies investigating opioid requirements in pain patients have found both higher and lower requirements in the carriers of the SNP in question [20, 91]. Thus it seems like the effect of 118 A>G is different depending on the type of opioid administered [20]. For tramadol treatment there is only limited data on the influence of the SNP. There is one study investigating the impact of 118 A>G on experienced pain severity before and after, respectively, the administration of a combined tramadol and acetaminophen therapy. The study showed that patients with AG or GG genotypes experienced less pain relief compared to patients with the AA genotype. The need of rescue analgesia was also increased in the former individuals [92].

Difficulties in forensic interpretations of tramadol

As previously discussed, the major difficulty concerning opioid interpretations within the field of forensic toxicology is the fact that drug effects are not closely related to blood concentration. Further evidence pointing in the same direction are the significantly overlapping concentrations between different types of forensic cases received by the National Board of Forensic Medicine, i.e. suspected cases of driving under the influence of drugs (DUID), suspected cases of petty drug offence (PDO), and the postmortem (PM) cases studied in this thesis, respectively (**Figure 5**). It is apparent that the approximate lethal concentration of above 2 mg/L [65, 66] is frequently found also in living individuals.

Figure 5. Total tramadol concentrations in suspected cases of driving under the influence of drugs (DUID, $n = 1563$) and of petty drug offence (PDO, $n = 600$) received by the National Board of Forensic Medicine in Sweden from 2011 until present. The concentrations are compared to those of postmortem cases (PM, $n = 159$) included in the thesis, being selectively included between 2014 and 2016. Eight data points are outside the presented concentration range, all assigned to the PM group.

The results regarding impaired drivers are in accordance with previous studies, showing tramadol levels up to 4.10 mg/L [93], 5.36 mg/L [94], and 7.8 mg/L [95], respectively. Twenty-four percent of the tramadol positive drivers in the latter study presented with concentrations higher than expected from normal therapeutic use [95].

One of the major reasons for this phenomenon is likely the tolerance that may develop when opioids are repeatedly used. The degree of tolerance cannot be measured and therefore is an element of uncertainty in the interpretation of drug concentrations. A history of drug abuse may indicate tolerance. Although the tolerance may be lost if an individual temporary

interrupts its substance abuse, which can constitute a risk of overdosing if the individual reassume its drug use at the same dosage as when being tolerant. Sometimes a drug analysis in a hair sample can provide useful information about the continuity of drug use, since administered drugs present in the blood circulation also gets trapped into the hair. For how long time the use of a drug can be traced depends on the length of the hair [96, 97].

It must be underlined that since not only tramadol, but also its metabolite ODT exerts both pharmaceutical and adverse effects, the concentration of the metabolite should also be taken into account. Further, with the enantiomers of the same compound also showing differences in these aspects, it may be valuable to investigate the enantiomer ratios of tramadol and ODT. Although in current practice are neither enantioselective analysis nor genotyping performed.

In addition to the actual drug concentrations and the possible tolerance of an individual, a third consideration when interpreting tramadol concentrations is other coadministered drugs that may have potentiating or attenuating effects. This applies to both pharmacokinetic and pharmacodynamic effects. Many coadministered drugs present in the blood will be detected and quantified during the postmortem toxicological investigation, although interpretation is not always straightforward. There is little information in literature regarding all possible drug combinations that occurs in the investigation of autopsy cases.

The above mentioned factors; the concentration of tramadol, ODT and its enantiomers, tolerance and coadministered drugs apply to forensic interpretations of both living individuals and autopsy cases. However, regarding the latter, there are also interpretation difficulties associated with the nature of the postmortem blood.

Blood drug concentrations may significantly change between the moment of death and the time of autopsy. Drug degradation and postmortem redistribution are two of the reasons, which may be more pronounced in decomposed cases [10]. Elevated drug levels in heart blood following death is common, and the concentrations are usually increasing with time. Cardiac blood is therefore not an appropriate specimen for forensic interpretations [8]. However, the drug concentration ratio between central blood (such as heart blood) and peripheral blood (such as femoral blood) is sometimes utilized as a measure of redistribution. The ratios for tramadol and ODT were found to be 1.40 and 1.28, respectively, in a study of 15 suspected fatal tramadol intoxications. These values were not considered indicative of significant redistribution [98]. It has also been suggested that lipophilic drugs with a volume of distribution above 3-4 L/kg are more prone to redistribution [10, 98]. However, it is highly probable that some degree of tramadol postmortem redistribution occurs

when permeability of cell membranes increases following the moment of death. From that perspective, forensic interpretations based on enantiomer ratios may be preferable over those based on achiral concentrations. Because even if redistribution does occur, the magnitude is expected to be the same for the two compounds constituting an enantiomer pair, since they have the same physiochemical properties and the mechanism of redistribution is passive diffusion.

The time between tramadol administration and death is very seldom known in autopsy cases, but it may be of importance for the forensic interpretation knowing if the drug intake occurred in close proximity to the death or not. The metabolite to parent compound ratios are sometimes utilized as indicators of acute intake. The underlying idea is that only small amounts of the drug metabolite have had the time to be formed in an acute intake, consequently causing low blood concentration ratios. However, the extent of scientific evidence for such an approach varies for different compounds. A clear association between the metabolite to parent compound ratio in plasma and the time since cannabis smoking has been reported [99], and the urinary metabolite to parent ratio has been investigated for estimations of the time since buprenorphine administration, showing promising results [100]. However, there was little data available on the ratio between metabolite and parent compound in relation to tramadol intake before the studies in this thesis were conducted. Because the formation of ODT is dependent on the *CYP2D6* genotype, it was speculated that the ODT/tramadol ratio may not be an equally useful indicator of time lapse.

The ODT/NDT ratio has been utilized as an indicator of acute intake in some forensic investigations concerning tramadol related deaths. The approach started with a case report by Moore et al, finding greater ODT than NDT concentrations in a postmortem case of tramadol intoxication. It was concluded that this was in contrast to many other postmortem reports, and since the death in the present case was known to have occurred within a 3 h window it was suggested that an ODT/NDT ratio above 1 indicated an acute death [101]. In a report concerning two postmortem cases attributable to acute tramadol intoxication, the ODT/NDT ratios were found to be 2.22, and 0.24, respectively. Subsequently, the authors concluded that the hypothesis by Moore et al. was supported in one of the two cases, but they also emphasized the possible impact of drug coadministrations and *CYP2D6* polymorphisms on the metabolite concentrations. These factors, and also enantioselective metabolism, were raised as interesting to take into account when further investigating tramadol related deaths [102]. However, in spite of an awareness of the likely impact of other factors, the ODT/NDT ratio is continuously utilized

[65] and discussed [103] as a potential indicator of acute tramadol intoxication.

Enantiomer ratios may be valuable in the estimation of the time lapse between tramadol administration and death, although is much less investigated. In one healthy volunteer orally administered 100 mg tramadol, the enantiomer ratios of tramadol, ODT, NDT and NODT were found to change over time [104].

Taken together, there is a need of investigating the enantiomer ratios of tramadol and its metabolites in relation to both toxicity and time since tramadol administration, since they may improve forensic interpretations.

AIMS OF THE THESIS

The overall aims were to evaluate if forensic interpretations of tramadol, regarding toxicity and time since drug administration, may be improved by:

- Adding genotyping (pharmacogenetics) to the routine practice
- Introducing enantioselective concentration determination

The aims were addressed in four studies with the following specific objectives:

- To develop and validate a bioanalytical method for the enantioselective determination of tramadol and its three main metabolites ODT, NDT, and NODT in whole blood.
- To evaluate six blood concentration ratios in relation to the time since drug administration; ODT/tramadol, ODT/NDT, (+)/(-)-tramadol, (+)/(-)-ODT, (+)/(-)-NDT, and (+)/(-)-NODT.
- To investigate the impact of genetic variation in *CYP2D6*, *CYP2B6*, *CYP3A4* and *ABCB1* on tramadol pharmacokinetics.
- To assess the pharmacokinetic impact on drug related symptoms (DRS) and tramadol related death.
- To study the characteristics of autopsy cases in which death was caused solely by tramadol intoxication according to the forensic pathologist.

STUDY POPULATIONS

The studies on the populations described below were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

Healthy volunteers

The overall aim of the thesis refers to an improved interpretation in forensic casework. To achieve that, investigations not only of forensic samples, but also of a well-defined population, under controlled conditions and performed in the same specimen (whole blood) are central. The first step was therefore to obtain such a reference population by performing a study on healthy volunteers, administered a single tramadol dose.

From advertisement to informed consent

The advertising was made at Linköping University and at Linköping University Hospital, through written information sheets on the background, aims and design of the study. Details on how to contact the study coordinators were provided. Individuals interested in participating in the study were sent further written information, and were later also informed in dialogue, with the opportunity to ask any questions. Subsequently, informed consent was confirmed in writing by each participant.

Inclusion and exclusion criteria

The inclusion criteria were a minimum age of 18 years, and good health as assessed by a physician. The assessment was based on general condition, heart sounds, blood pressure, and the health form that each participant completed and signed. The form included questions on height and weight, intolerance, allergy, disease, and present or previous use of any prescribed or nonprescribed drug, illegal drugs and naturopathic drugs included. Any ongoing drug treatment was an exclusion criterion, with the exception of contraceptive medication. Additional exclusion criteria were pregnancy, breast-feeding or present participation in any other scientific study, which were also asked about in the health form.

Conduct of the study

Twenty healthy volunteers were included in the study, and randomized into two equally sized dosage groups of either 50 mg or 100 mg. The randomization was made by an online random generator. Nine males and ten females, aged between 19 and 34 years (median 25), completed the process of drug administration and sampling at the Human Lab of Linköping University Hospital. These so called experimental days were performed with no more than two participants at the same day, at 10 different occasions.

Following a blood pressure measurement, the participants were orally administered their predetermined dose of an IR formulation of tramadol (Tramadol HEXAL, Sandoz) at about 08 a.m. The subjects were not told which dose they received, although requirements for blinding were not fulfilled. Neither were the researchers or the medically responsible individuals blinded. Blood samples were drawn from a peripheral venous catheter in the forearm prior to dosing and at 16 occasions following the drug administration. The exact times of blood sampling were noted, being as close as possible to 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 24, 48, and 72 hours after tramadol intake.

Before arriving at the Human Lab, the participants were allowed to eat breakfast of their own choice. During the experimental day they were offered two lunch alternatives from a nearby restaurant, and provided fruit, some biscuits, tea, coffee, juice, and water in optional amounts. Alcohol was, however, not allowed, neither the day before, or during, the day of the tramadol administration.

In the end of the experimental day, about 10 hours following drug administration, the participants were given a form concerning DRS. The participants were asked to grade their experiences of nausea, dizziness, headache, vomiting, dry mouth, sweating, and fatigue on a scale between zero to five, where zero was no symptoms at all and five was the worst imaginable symptoms. The subjects were also requested to report any other perceived DRS.

For the drawing of the 24, 48, and 72 h blood sample, respectively, the participants returned to the Human Lab.

In addition to blood samples, also urine and hair samples were collected at several occasions during the study period. Analysis of these samples were, however, not part of this thesis.

Additives and storage of blood samples

The collection tubes contained sodium fluoride as a preservative and potassium oxalate as an anticoagulant. (In paper I it is erroneously stated that sodium-heparinized collection tubes were utilized).

Directly after sampling and mixing, the blood samples were placed in the refrigerator at the Human Lab. Samples were transported to the National Board of Forensic Medicine two times a day, once in the middle of the day and once in the evening. The transport took about 15 minutes and was performed in current outdoor temperature. Following arrival, the samples were aliquoted so that there were separate sample tubes for the achiral (paper I) and enantioselective (paper III) analysis. The samples were stored in -20°C until all blood samples from the same individual had been obtained, whereupon they were stored in -80°C pending analysis.

Ethical considerations

Single, therapeutic doses of the registered and frequently prescribed drug tramadol were administered. In the risk-benefit assessment, no advantages for the study participants were identified, since they were in no need of a pain relieving treatment. On the other hand, risks were considered slight, with dizziness and nausea being the most plausible adverse effects. Medical staff was responsible for the safety of the participants. A nurse was present during the whole experimental day, supervising the participants, and did also perform the blood sampling. A physician dispensed the tramadol dose, and discharged the participants at the end of the day, when regarding them in good condition. In case of any incident in the time between, the physician in charge was rapidly available. Since driving was not allowed by the participants, their return home was arranged. The participants were insured through the Swedish Legal, Financial and Administrative Services Agency.

No names or other personal information were indicated on the blood tube labels, or on the appurtenant records. Instead, every participant was assigned a number, which was used throughout the whole process, from randomization of dosages to blood sampling and analysis. Before publication of Paper I, the participants were re-numbered; 01-10 for the participants administered the 50 mg dose, and 11-19 for the individuals administered the 100 mg dose.

The study was approved by the Regional Ethical Review Board in Linköping (No: 2011/337-31).

Autopsy cases

Inclusion and exclusion criteria

Forensic postmortem samples were included in the study during the time period between September 1, 2014 and September 1, 2016. Inclusion criteria was tramadol positive femoral blood, as assessed in the

postmortem toxicological investigation. During the first 12 months, the only exclusion criterion was an amount of blood less than 4 g following the completed toxicological investigation. During the second year, the exclusion criteria was extended, and did also comprise samples with an intermediate or extensive CYP2D6 phenotype. The purpose was to accumulate samples with the more rare phenotypes. In total, 159 samples were included in the study.

Classification based on cause of death

The autopsy cases were classified into four groups, based on the involvement of tramadol in the cause of death:

TraTox = tramadol intoxications. Even though other drugs may have been detected in the blood, tramadol alone was interpreted as having caused the death.

MixTox = mixed intoxications. Tramadol was interpreted as being contributory to the cause of death.

OtherTox = other intoxications. Tramadol was considered only an incidental finding.

NonTox = nonintoxications. Death was caused by disease or by unnatural causes other than drug intoxication.

The classification was made by a forensic pathologist, aided by an experienced forensic toxicologist regarding a few cases.

Additives and storage of blood samples

When the forensic toxicological investigation was completed, aliquots of 2-3 g were collected in consecutive batches and stored in -80°C. Before aliquotation, the blood was stored in a refrigerating room (4°C). The additive used for sampling of postmortem blood was potassium fluoride.

Ethical considerations

The individuals, whose blood samples were included in the study, had all been subject to a forensic toxicological investigation. Since the blood samples were drawn postmortem, it was not possible to obtain informed consent. However, the blood samples that were analyzed for research purposes were the same as those collected for the routine investigations. The purpose was solely to improve future postmortem toxicological investigations. No additional information or samples were obtained, and no personal data was extracted from the databases containing the already existing information. Neither was any personal data revealed on the labels of the tubes with aliquoted blood. The researchers involved in these procedures were employed at the Nation Board of Forensic Medicine.

The study was approved by the Regional Ethical Review Board in Linköping (No: 2016/139-31).

METHODS

Herein are presented the fundamentals of utilized methods. The challenges associated with the development and maintenance of the enantioselective method are also depicted. However, further details essential to reproduce any study are given in the original papers.

Concentration Determination

Two methods for the quantitation of tramadol and its metabolites have been utilized in the thesis, both of them based on the technology of liquid chromatography tandem mass spectrometry (LC–MS/MS).

LC-MS/MS

In LC-MS/MS the high performance chromatographic separation of compounds in liquid are combined with the highly selective identification and accurate quantitation of the same compounds as ions in vacuum. As the description implies, the difficulty with the technique is to obtain the compounds in vacuum from the liquid phase. Therefore the so called interface, where the ionization and transportation takes place, is a very important part of an LC-MS/MS instrument. However, optimization of the LC part is often necessary to favour the interface procedures.

Schematically, the LC part is composed of an autosampler, pumps delivering a mobile phase (mixture of A and B) and a column (stationary phase), as shown in **Figure 6**. Depending on the characteristics of the mobile phase and the column, there is a distinction between normal phase chromatography and reversed phase chromatography. In normal phase the column is of polar (hydrophilic) character, and the mobile phase is of nonpolar (hydrophobic) character. In reversed phase chromatography, which is currently more frequently utilized, it is the other way around. The column is nonpolar and the mobile phase is polar. The character of the compound or analyte that are to be quantified, determines for how long time the analyte will reside in the column. The more hydrophobic analyte, the longer it will reside in a nonpolar column. The various strengths of the hydrophobic interaction for different analytes in a sample constitute the basis for separating the same analytes. By changing the mobile phase composition, the strength of the hydrophobic interaction between analyte and column can be changed.

Figure 6. The constituent elements of a liquid chromatography (LC) system, in which drugs present in a sample are separated using a mobile phase and a stationary phase (column). The separation precedes the detection in the tandem mass spectrometry (MS/MS) part. ACN = acetonitrile, Aq = aqueous solution (Adapted from illustration by Svante Vikingsson, colleague at the National Board of Forensic Medicine)

There are different kinds of LC-MS/MS interfaces, but they all rely on analyte ionization. For the methods used herein an electrospray ionization (ESI) interface was utilized. ESI occurs at atmospheric pressure and is a relatively soft ionization technique that keeps the analyte molecules intact. Very simplified, the interface is composed of three parts (**Figure 7**). The first is the nebulizer, from which the mobile phase containing the analytes are sprayed into the second part, which is the chamber. The spraying of the mobile phase, rather than a flow of mobile phase into the chamber, is accomplished by a high voltage supported by a heated nitrogen gas (nebulizer gas). One of the purposes with the nebulizer gas is to facilitate evaporation of the mobile phase, and it is therefore important to use mobile phases that are volatile. The high voltage is applied between the nebulizer tip and the mass analyser inlet, being the third part. The droplets of mobile phase leaving the nebulizer therefore becomes charged. When the droplets decrease in size due to the evaporation, charge repulsion will divide the droplets until only charged analytes remain. These ionized analytes can then be captured by the mass analyser inlet and be further transported by the MS/MS system. Gas and neutrals will be separated from the analyte ions before they reach the vacuum part, where identification and quantitation are performed based on the mass to charge ratio (m/z) of the analytes.

The ionization can be performed in either positive ion mode, creating protonated analyte molecules, or in negative ion mode, creating deprotonated analyte molecules.

Figure 7. Ionization of drug molecules in the interface between the liquid chromatography (LC) and tandem mass spectrometry (MS/MS) part. The mobile phase containing the analytes are sprayed from the nebulizer into the chamber, in which a high voltage is applied, creating ionized molecules that can be captured by the mass analyser inlet. (Adapted from illustration by Svante Vikingsson, colleague at the National Board of Forensic Medicine)

Once again simplified, the MS/MS part consists of two quadrupoles in a row with a collision cell in between, as shown in **Figure 8**. The quadrupoles can either be operated in scan mode, collecting data on all ions, or in selected ion monitoring (SIM) mode, only collecting data on preselected ions. The latter is strongly recommended for quantitation applications. To accurately quantify an analyte it must also be accurately identified. For this purpose, the multiple reaction monitoring (MRM) mode is preferable. The first quadrupole (Q1) is then utilized to select a specific precursor ion, i.e. the analyte ion of interest. The selection is as earlier mentioned based on the m/z , so that only ions with a mass corresponding to the one of the analyte ion are allowed to pass through Q1 and enter the collision cell (Q2). In Q2 the analytes are introduced to a neutral gas. When the analytes collide with the gas, their kinetic energy may be converted into internal energy, and consequently break chemical bonds in the analyte molecule. Thus, fragment ions are generated. The third quadrupole (Q3) only permits preselected fragment ions to pass through it, in order to reach the detector. In the case of tramadol analyses, Q1 is set to allow passing of analyte ions (MH^+) with a molar mass of 264, and Q3 is often set to allow passing of fragment ions with a molar mass of 58. The whole process is called a transition, abbreviated 264/58. The quadrupoles can be set to also collect data from other transitions, measured in cycles. So to further strengthen the identification of tramadol, a second transition, measuring the same precursor ion, but a different fragment ion, may be used.

Figure 8. The quadrupoles (Q) in the mass spectrometry (MS/MS) part only permits passing of analytes with a specific mass to charge ratio (m/z). When a preselected drug molecule passes through Q1 it is fragmented using gas in the collision cell (Q2). Preselected ion fragments can then, following transit in Q3, reach the detector. Thus, both identification and quantitation of a certain drug are performed. (Adapted from illustration by Svante Vikingsson, colleague at the National Board of Forensic Medicine)

Achiral versus enantioselective quantitation

The method utilized in paper I quantitated the achiral concentrations of tramadol and ODT, that is, a quantitation without distinguishing between the two enantiomers in each enantiomer pair. It was used in routine practice at the time, and thus was already developed and validated when this project started. The method of enantioselective quantitation, on the contrary, was both developed and validated during the thesis work. A summary of the methods and their characteristics are shown in **Table I**. The characteristics of the achiral tramadol method are similar to those of many other LC-MS/MS methods that are used routinely at the National Board of Forensic Medicine. The reasons for the enantioselective method being different in many aspects are explained in the following section.

The development of the enantioselective tramadol method

Because enantiomers have the exact same mass and also do not differ neither in physical or chemical properties, they are impossible to separate and quantitate using a traditional LC-MS/MS approach. To succeed in doing so, the enantiomers need to encounter a chiral environment, just like

they do in the human body. There are generally three ways to accomplish such an environment in the LC system, utilizing either a chiral column, a chiral mobile phase or a chiral derivatization reagent. To use a chiral column is the most common approach.

When the development was initiated, the majority of previously described methods for chiral separation of tramadol and its metabolites were performed with high performance liquid chromatography (HPLC) with fluorescence detection, or LC-MS/MS, in normal phase mode with a stationary phase composed of polysaccharides. The mobile phases were based on hexane, alcohols and diethylamine, to achieve chiral resolution and proper peak shape [105-110]. Rudaz et al [111] used capillary electrophoresis with a chiral selector in the buffer and electrospray ionization MS for enantioselective determination of tramadol and as much as five metabolites. However, when the method was applied to a plasma sample collected 2 h following a 100 mg oral dose only tramadol and ODT were detected, implying low sensitivity.

The strategy of the present development was to utilize a sensitive LC-MS/MS method with chiral chromatography in reversed phase mode and with atmospheric pressure ionization in positive mode. Initially, chiral stationary phases composed of various polysaccharides were tested, due to their wide chiral recognition and high loading capacity. The mobile phases were composed of either methanol or acetonitrile in an ammonium bicarbonate buffer. However, even though separation of the enantiomers was achieved, it was with less selectivity and more asymmetric peaks, compared to what had been shown previously.

Ardakani et al [47] and Campanero et al. [112] separated the enantiomers of tramadol, ODT and NDT with a chiral stationary phase composed of either α_1 -acid glycoprotein (AGP) or cellulose using reversed phase HPLC with fluorescence detection. However, the mobile phases utilized were based on a phosphate buffers, which are not compatible with an LC-MS/MS approach.

The use of chiral AGP, but also columns composed of other glycoproteins, and mobile phases with possible substitutes for triethylamine and phosphate buffer were therefore investigated. The use of the AGP column and a mobile phase consisting of acetonitrile in an ammonium acetate buffer showed to be appropriate. Further optimization regarding pH, buffer concentration and the amount of acetonitrile resulted in a final mobile phase composition of 0.8% acetonitrile in an ammonium acetate buffer of 20 mM with pH 7.2. Most strikingly, small changes in the amount of acetonitrile had great impact on the chromatographic separation of the enantiomers. In comparison to the mobile phase A composition that are

Table I. Characteristics of the utilized achiral and enantioselective tramadol method.

	Achiral	Enantioselective
Analyte extraction	Protein precipitation	Liquid–liquid extraction
Column	C18 (1.8 μ m)	AGP (5 μ m)
Column guard	Filter (0.2 μ m)	AGP (5 μ m)
Mobile phase A	0.05% formic acid in 10 mM ammonium formate	0.8% acetonitrile in 20 mM ammonium acetate, pH 7.2
Mobile phase B	0.05% formic acid in methanol	None
Post-column infusion	None	0.05% formic acid in acetonitrile
Elution	Gradient	Isocratic
Run time	8 minutes	30 minutes
Interface	Electrospray with positive ionization	Electrospray with positive ionization
Transition tramadol	264/58, 264/246	264/58
Transition ODT	250/58, 250/232	250/58
Transition NDT	Not included	250/44
Transition NODT	Not included	236/44
Transition tramadol- ¹³ C-D ₃	268/58	268/58
Transition ODT-D ₆	256/64	256/64
Identification criteria	Relative ion intensity, relative retention time	Peak pattern
Calibration range	10-3000 ng/g	0.25-250 ng/g
LLOQ tramadol	20 ng/g	0.125 ng/g
LLOQ ODT	10 ng/g	0.50 ng/g
LLOQ NDT	Not included	0.125 ng/g
LLOQ NODT	Not included	0.125 ng/g
Manual wash	No	Yes

When two transition ratios are given, the first corresponds to the quantifying transition and the second to the qualifier transition. Concentrations refers to the sum of (+)- and (-)-enantiomers regarding the achiral method, and to each enantiomer regarding the enantioselective method. LLOQ = lower limit of quantitation, AGP = α_1 -acid glycoprotein

used in the achiral method (**Table I**), it can also be stated that a pH of 7.2 is remarkably high. Generally, utilizing positive ionization mode, it is an advantage using acidic mobile phases. Further, the recommended pH interval for the AGP column is 4-7 according to the manufacturer. However, a high pH was found to improve the resolution of the enantiomer pairs, and this is theoretically logical. The isoelectric point of AGP is 2.7, so at a pH above 2.7 the negative charge of the glycoprotein will increase with increasing pH. The pK_a value of tramadol is approximately 9.4, meaning that at pH 7.2 the tramadol molecules will be almost completely ionized. Consequently, the conditions are optimal for ionic bonding between the analytes and the AGP, which will retain the analytes further in the column and therefore facilitate improved selectivity. However, the major limitation with such an approach is that the ionization in the interface will not be facilitated. In addition to the high pH, also the high water content in the mobile phase may deteriorate the ionization in the interface. To improve sensitivity and maintain stable signals during the runs it became clear that a post-column infusion with 0.05% formic acid in acetonitrile was required. In contradiction to the enantioselective method, the achiral method utilized two mobile phases. The mobile phase B with methanol may facilitate the ionization, but also has an additional advantage. This advantage is related to the term elution, which according to **Table I** is a gradient for the achiral method. This means that the amount of mobile phase A and B, respectively, that is pumped into the LC system changes over the analysis time, so that the percentage of mobile phase B gradually increases. Before injection of a new sample, the mobile phase composition is restored, with a lower percentage of the B phase. The procedure reduces the total analysis time for each sample, and also contributes to a wash effect of the system following each sample. There are several reasons to why gradient elution cannot be applied to the enantioselective method. Firstly, the amount of organic content has a huge impact on the chiral chromatography, and secondly, the AGP column also do not tolerate an organic content of more than 20%. But not even if these obstacles were possible to overcome, the chiral columns have a too long equilibrium time to allow gradient elution. Isocratic elution is therefore utilized, meaning that the mobile phase composition remains unchanged during the analysis time. The large difference between the methods concerning the run time for each sample, 8 minutes versus 30 minutes, is also, in part, due to these facts.

As indicated in **Table I**, the achiral method utilized two transitions for each analyte measured, one used for quantitation and one used as a qualifier transition. Thus, relative ion intensity between the two fragment ions was used to further increase the selectivity regarding the identification. For the enantioselective method, only one quantifying transition was used for each

analyte, because qualifier transitions could not be obtained for all three metabolites measured, due to poor fragmentation. An additional identification criteria in the achiral method was the relative retention time between the analyte and the internal standard. Such an approach was not investigated regarding the enantioselective method, since retention times were not robust. Consequently, identification in the enantioselective method was merely based on the obtained peak pattern. However, the interactions between the enantiomers of an analyte and the chiral column following thorough optimization is much more selective than the interaction between the analyte and a traditional achiral C18 column, and thus contributes to the identification. Interaction of venlafaxine with the present chiral system exemplified the phenomenon. Tramadol and venlafaxine are very structurally similar, and *O*-desmethylvenlafaxine (ODV) has the same molecular mass as tramadol. However, when utilizing the present method, the chromatographic peaks of venlafaxine and ODV were well separated from those of tramadol and its metabolites. In total, 82 compounds were investigated regarding selectivity, and only two additional drugs resulted in chromatographic peaks. One of them, ketobemidone, was found to have the ability of interfering with (+)-ODT. Nevertheless, all samples studied in the thesis project had previously been analyzed with the achiral method, also preceded by a liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) screening method regarding the autopsy cases. According to this screening performed in the postmortem toxicological investigation, ketobemidone was not detected in any of the autopsy cases studied. The total tramadol and ODT concentrations (the sum of the (+)- and (-)-enantiomers) measured with the achiral and enantioselective method, respectively, were also highly similar.

The differences in calibration range between the two methods (**Table I**) reflects the purposes with them. Generally, methods within the field of forensic toxicology utilizes wide ranges to be able to detect and quantify both therapeutic and toxic concentrations. However, to be able to study tramadol pharmacokinetics following a relatively low single dose, an optimal calibration function in a lower concentration range was prioritised. This is also reflected in the lower limit of quantitation (LLOQ) of the enantioselective method. However, as a consequence of the lower calibration range, a high proportion of the autopsy samples required dilution before enantioselective analysis.

Before analyzing any sample with either LC-MS/MS method, the analytes were extracted from whole blood using protein precipitation before achiral analysis, and liquid-liquid extraction before enantioselective analysis. Generally, the latter results in more clean extracts and was chosen because of the high sensitivity requirements on the enantioselective method, and to

protect the vulnerable AGP column. As apparent from **Table I**, the chiral column was also protected by a guard column, while the achiral column was only preceded by a filter.

The amount of acetonitrile in the reconstitution solution following liquid–liquid extraction was optimized. With a column not countenancing an organic content larger than 20% an acetonitrile amount as low as possible was eligible. However, to achieve clean enough extracts a 50:50 mixture of mobile phase and acetonitrile was required. This could potentially reduce the lifetime of the column, although since the injection volume was only 2 µl it was not expected to have such a great impact.

Maintenance of the enantioselective tramadol method

To maintain chromatographic performance the column and guard column were washed with 10% acetonitrile in water for about 30 minutes following each assay. The guard column alone was further washed with 15% isopropanol in water for about 30 minutes and subsequently with 10% acetonitrile in water for about 15 minutes. Also the interface was washed following each run, using isopropanol and water (50/50), and the mobile phase system was flushed with acetonitrile and water (50/50).

Noteworthy is the fact that the optimal amount of ACN in the mobile phase showed to be different for different batches of AGP-columns. There was also differences regarding selectivity between different batches, especially regarding the enantiomers of NDT.

Genotyping

Three techniques were utilized in the thesis for the genotyping analyses; pyrosequencing, TaqMan and xTAG technology. The pyrosequencing methods were performed either at the National Board of Forensic Medicine or at the University in Linköping, Sweden, while the methods based on the other techniques were performed at the Erasmus University Medical Center in Rotterdam, The Netherlands. All genotyping methods were well-established and no development was consequently performed for the purpose of the present studies.

Pyrosequencing

The following genes, alleles (*) and polymorphisms were investigated using in-house applications based on the pyrosequencing technique:

- *CYP2D6*; *3 (2549 A>del), *4 (1846 G>A), *5 (whole gene deletion), *6 (1707 delT), and *CYP2D6xN* (multiple gene copies)
- *ABCB1*; 1199 G>A, 1236 C>T, 2677 G>T/A, and 3435 C>T

- *OPRM1*; 118 A>G
- *CYP2B6*; *4 (785 A>G), *5 (1459 C>T), *6 (516 G>T and 785 A>G), *7 (516 G>T, 785 A>G and 1459 C>T) and *9 (516 G>T)

TaqMan analysis

The following genes, alleles (*) and polymorphisms were investigated using TaqMan analysis:

- *CYP2D6*; *2 (2850 C>T), *3 (2549 delA), *4 (1846 G>A), *6 (1707 delT), *7 (2935 A>C), *8 (1758 G>T), *9 (2613 delAGA), *10 (100 C>T), *12 (124 G>A), *14 (1758 G>A), *17 (1023 C>T), *29 (3183 G>A), *41 (2988 G>A)

The OpenArray PGx Express Panel (Applied Biosystems, Foster City, CA) was utilized.

- *CYP2D6xN*, including *5 (whole gene deletion), was analyzed utilizing the CYP2D6 exon 9 assay Hs00010001_cn (Applied Biosystems).
- *CYP3A4**22 present in intron 6 (15389 C>T) was investigated using the genotyping assay C_59013445_10 (Applied Biosystems).

xTAG technology

The following *CYP2D6* alleles (*) and polymorphisms were investigated using the xTAG CYP2D6 Kit v3 (Luminex, Austin, TX):

- *2 (-1584 C>G, 1661 G>C, 2850 C>T, 4180 G>C), *3 (2549 delA), *4 (100 C>T, 1661 G>C, 1846 G>A, 2850 C>T, 4180 G>C), *5 (whole gene deletion), *6 (1707 delT, 4180 G>C), *7 (2935 A>C), *8 (1661 G>C, 1758 G>T, 2850 C>T, 4180 G>C), *9 (2613 delAGA), *10 (100 C>T, 1661 G>C, 4180 G>C), *11 (883 G>C, 1661 G>C, 2850 C>T, 4180 G>C), *15 (137_138 insT), *17 (1023 C>T, 1661 G>C, 2850 C>T, 4180 G>C), *29 (1659 G>A, 1661 G>C, 2850 C>T, 3183 G>A, 4180 G>C), *35 (-1584 C>G, 31 G>A, 1661 G>C, 2850 C>T, 4180 G>C), *41 (1661 G>C, 2850 C>T, 2988 G>A, 4180 G>C), and *CYP2D6xN*

CYP2D6 genotype-phenotype prediction

There is no true consensus on how to predict the phenotype from the genotype, that is how to classify CYP2D6 PMs, IMs, EMs, and UMs based on their genotype [62, 113, 114]. The predictions in the present studies were based on the guidelines of the Dutch Pharmacogenetics Working Group (DPWG).

The phenotype of an individual can also be determined by actual phenotyping, meaning administration of a probe drug that is primarily

metabolized by CYP2D6. The concentrations of the probe drug and one or several metabolites are then measured in the urine, to obtain parent compound to metabolite ratios that are subsequently used for the phenotype determination [115].

Genotyping and phenotyping both have their pros and cons regarding phenotype determination. However, only genotyping is possible when investigating postmortem cases. Stated phenotypes in the present study populations always correspond to the predicted phenotypes (from the genotypes).

RESULTS AND DISCUSSION

Interpretation of time since tramadol administration

Six different ratios, which potentially could be used to estimate the time of tramadol administration, were investigated in the thesis.

The *O*-desmethyltramadol/tramadol ratio

It was shown in paper I that the ODT/tramadol ratio is positively correlated with the time following drug intake in healthy volunteers (**Figure 9**). However, large interindividual variations were found, regarding both the initial measurable ratio at 1.0 h and the incline of the linear correlation. Some of the variation could be explained by the *CYP2D6* genotype. The mean ratio was at every time point higher in *CYP2D6* EMs than in IMs. Similarly, the ratios were higher in the IMs compared to the only *CYP2D6* PM with detectable amounts of ODT in the blood. In fact, the ratio was almost constant during 10 h following tramadol ingestion in the PM. The same result was shown when the ODT/tramadol ratio was calculated based on the AUC and $C_{\max}$. The mean AUC ratio was 0.41, 0.24, and 0.09 in EMs, IMs, and the PM, respectively, and the corresponding $C_{\max}$ ratio was 0.33, 0.20 and 0.07, respectively. Because of the differences in ODT/tramadol ratios between the *CYP2D6* phenotype groups, it is apparent that an estimation of the time lapse between drug administration and blood sampling is not valid without *CYP2D6* genotyping. However, that does not mean that an estimation on individual basis is valid only because genotyping has been performed. As evident by **Figure 9**, there are individuals with the IM phenotype showing ODT/tramadol ratios over time that are very similar to the ones of both PMs and EMs.

The enantiomer ratios of tramadol and its metabolites

Healthy volunteers

In paper III it was shown that the (+)/(-)-enantiomer ratios of tramadol, ODT, NDT and NODT were positively correlated with the time following drug administration in healthy volunteers (**Figure 10**). For tramadol and NDT, the mean increases in enantiomer ratios over time were close to

linear during the first 24 h. For this reason, they were considered the best candidates for estimations of time since tramadol intake.

An additional advantage with the enantiomer ratio of tramadol in this regard was that it seemed to be independent of *CYP2D6* genotype. With only two *CYP2D6* PMs studied, it was not possible to conclude that the genotype is of no importance regarding the tramadol ratio, although if it was confirmed it would allow estimations of time without genotyping. For the three other enantiomer pairs, the mean (+)/(-)-ratios were significantly lower in *CYP2D6* PMs compared to in the other phenotype groups. In similarity with the ODT/tramadol ratio, genotyping would consequently be required in order to avoid always interpreting the intake of PMs as acute or recent, no matter the real time lapse between drug intake and blood sampling.

The additional advantage with the enantiomer ratio of NDT, compared to the one of the other enantiomer pairs, was the considerably larger increase over time observed for EMs and IMs. During 24 h following drug administration these phenotype groups showed a three-fold increase in enantiomer ratio; from about two to about seven. Large differences in ratio between points close in time would theoretically facilitate the estimation.

However, as with the ODT/tramadol ratio there was large interindividual variation regarding the enantiomer ratio of both tramadol and NDT, which also seemed to increase with time (**Figure 11a,b**). As apparent from the graph a (+)/(-)-NDT ratio of four may in one individual correspond to a drug intake about 5 h ago, and more than 15 h ago in another individual. Similarly, a ratio of six may correspond to about 12 h and about 20 h since tramadol administration, respectively.

Regarding the (+)/(-)-tramadol ratio, some individuals did not show an increase at all over time.

The large interindividual differences not explained by investigated genetic variation are important to emphasize, because they affect the possibility of utilizing the ratios for time estimation within the forensic field. The smaller the interindividual differences, the better the ratio for estimation. Obviously, accurate estimations are crucial to guarantee legal certainty, which is of outmost importance in living individuals risking prosecution. In autopsy cases, the estimation of time since intake will not result in any legal penalties (given the drug is self-administered), nevertheless, with too large interindividual variation the ratio will not be useful in the death investigation. Before eventually applying enantiomer ratios in time estimations, they must also be evaluated in relation to different dosages, regular dosing and steady-state, as well as in relation to the possible impact of concurrent drug treatment.

Figure 9. The *O*-desmethytramadol (ODT) to tramadol ratio was found to be positively correlated with the time since drug administration in healthy volunteers. However, large interindividual variations were observed, partially explained by CYP2D6 phenotype. Two poor metabolizers (PMs) participated in the study, although only one of them showed measurable amounts of ODT in the blood.

EMs = extensive (normal) metabolizers, IMs = intermediate metabolizers

Figure 10. Positive correlation between the mean enantiomer ratios of tramadol, *O*-desmethytramadol, *N*-desmethytramadol and *N,O*-didesmethytramadol, and the time since tramadol administration in CYP2D6 poor (PMs), intermediate (IMs) and extensive metabolizers (EMs), respectively. Only one of the PMs presented with measurable amounts of (+)-*O*-desmethytramadol and (+)-*N,O*-didesmethytramadol.

It was possible to calculate the tramadol enantiomer ratio also at 48 h in 11 individuals (**Figure 11b**). Four of these individuals were administered the 50 mg dose and seven of them were administered the 100 mg dose. For some of the individuals a change in linearity became evident, with a steeper incline between 24 h and 48 h than between 0.5 h and 24 h. Thus, estimations of time since tramadol intake may be difficult to perform after 24 h. Nevertheless, with a longer detection time, the tramadol enantiomer ratio offers the opportunity to prove a tramadol intake a longer time after the occasion of administration.

Autopsy cases

Regarding the autopsy cases, there is no knowledge about the true time lapse between tramadol ingestion and death. Hypothetically though, the time lapse would in general be shorter among the subjects dying from tramadol intoxication, than among the subjects dying from tramadol unrelated causes. Although, there will of course be individuals in the latter group, that ingested tramadol in close relation to the death, even though something else than the drug caused the death. And there might also be tramadol intoxications with a more detained course of death.

With the large interindividual differences observed in healthy volunteers, no attempt of estimating the specific time between drug intake and death was made. Instead, it was investigated if it may be possible to discriminate between a recent (< 5 h) and a past (≥ 24 h) intake, based on the enantiomer ratio of NDT. The mean enantiomer ratio of NDT was approximately three at 5 h following drug administration in healthy volunteers, and about seven at 24 h.

In agreement with the hypothesis, all postmortem cases with an enantiomer ratio above seven ($n = 17$) were assigned to either the group of OtherTox or NonTox. Thus, in no death interpreted as being partly or exclusively explained by tramadol, did the enantiomer ratio of NDT indicate a long time lapse between drug administration and death. The autopsy cases showing ratios above seven were also associated with relatively low total tramadol levels (the sum of the (+)- and (-)-enantiomer), with a mean parent compound concentration of $0.4 \mu\text{g/g}$ and a median concentration of $0.1 \mu\text{g/g}$. This could possibly be due to that the metabolism of the parent compound had been ongoing for a while, resulting in decreased tramadol levels.

The causes of death among the postmortem cases that presented with (+)/(-)-NDT ratios below three ($n = 23$) were both related and unrelated to tramadol ingestion. However, in nine of these cases, assigned to the groups of TraTox and MixTox, a remarkably high total tramadol concentration of $> 14 \mu\text{g/g}$ was measured in blood. No other postmortem cases presented with tramadol concentrations in the same range.

A similar pattern was shown for the enantiomer ratio of tramadol in postmortem cases. The subjects with the highest (+)/(-)-tramadol ratios were found in the groups of OtherTox and NonTox, and were associated with low total tramadol concentrations. Extremely high concentrations, found in individuals assigned to the groups of TraTox and MixTox, were associated with low enantiomer ratios.

From the combined results obtained from the studies on healthy volunteers and autopsy cases it could be concluded that high enantiomer ratios indicates a past drug intake. However, it should be emphasized that high enantiomer ratios also could be the result of repeated drug administrations, reflecting steady-state. The effect of such circumstances on the enantiomer ratios over time has not been investigated. Low enantiomer ratios may indicate a recent or acute intake, but could also be caused by other factors.

Figure 11. Concentration ratios in healthy volunteers. Large increases in the enantiomer ratio over time was observed for NDT (a) in CYP2D6 extensive (EMs) and intermediate metabolizers (IMs). However, large interindividual variation was apparent. Individual differences were also found concerning the (+)/(-)-tramadol ratio, which in some individuals could be measured for as long as 48h (b). A mean ODT/NDT ratio (c) above 1 was found at all time points in CYP2D6 EMs and IMs, while it was below 1 at all time points in poor metabolizers (PMs).

The ODT/NDT ratio

As previously mentioned, an ODT/NDT ratio above 1 has been utilized and discussed as an indicator of acute intake in autopsy cases [65, 101-103]. However, originating from a single postmortem case report, it has not been investigated how the ratio changes over time or if the ratio is affected by genotype. Based on the data generated from the healthy volunteers, a linear correlation between the present ratio and time following intake could not be shown (**Figure 11c**). Furthermore, every participant being a CYP2D6 EM or IM presented with an ODT/NDT ratio above 1 at all time points, while the CYP2D6 PMs showed ratios below 1 at all time points. Accordingly, when the ratio was investigated in postmortem cases, statistically significant differences were found between the CYP2D6 phenotype groups. The median values in CYP2D6 PMs, IMs, EMs, and UMs were 0.058, 0.28, 1.27, and 2.70, respectively. Consequently, if aiming at estimating the time between tramadol ingestion and death, the ODT/tramadol ratio, or, even better, the enantiomer ratios of tramadol and its metabolites, are more appropriate to use than the ODT/NDT ratio. However, the previous utilization of the ODT/NDT ratio in this regard, highlights the difficulties associated with the use of case reports as reference data.

Interpretation of tramadol toxicity

The pharmacogenetic impact on tramadol pharmacokinetics

Genotypes causing increased AUC, $C_{\max}$ or (+)/(-)-enantiomer ratios of tramadol or ODT are hypothesized to be of special concern regarding the toxicity of the drug. In healthy volunteers, the relationship between several SNPs in different tramadol related genes, and the pharmacokinetics of the drug was investigated. For the autopsy cases, in which AUC and $C_{\max}$ are not possible to study, only the impact of CYP2D6 genetic variation on enantiomer ratios has been examined so far.

CYP2D6

CYP2D6 PMs were expected to show lower $C_{\max}$ and AUC of the active ODT metabolite, as well as decreased (+)/(-)-ODT ratios, compared to IMs and EMs. UMs, on the contrary, were expected to show higher levels of the same parameters. However, in the population of healthy volunteers, no UMs and only two PMs were present (**Table II**).

Table II. Compilation of the data regarding the 19 healthy volunteers administered a single tramadol dose. The AUC values were dose-adjusted

No.	AUC TRA	AUC (+)-TRA	AUC (-)-TRA	AUC ODT	AUC (+)-ODT	AUC (-)-ODT	ABCB1 haplotype	CYP2D6 phenotype	CYP2D6 genotype	Dose (mg)	DRS
04	4632	2383	2249	1049	574	475	CT-GT-TT	IM	*1/*5	50	0
15	4330	2201	2129	1031	570	461	CT-GG-CC	IM	*1/*4	100	13
19	3989	2288	1701	304	65	239	CC-GA-CC	PM	*4/*4	100	0
05	3986	2135	1851	226	<LOQ	226	TT-TT-TT	PM	*4/*5	50	4
16	3795	2084	1711	410	202	208	CT-GT-CT	IM	*4/*41	100	25
06	3641	2002	1638	1116	570	546	TT-TT-TT	IM	*1/*4	50	4
13	3442	1854	1588	1233	660	573	CT-GT-TT	IM	*1/*3	100	3
14	3336	1810	1526	1005	509	496	CC-GG-CC	EM	*1/*35	100	4
11	3078	1607	1471	481	237	244	TT-TT-CT	IM	*1/*5	100	16
07	3044	1654	1390	657	343	314	CT-GG-CC	IM	*2/*5	50	5
09	2978	1620	1358	1365	706	660	CC-GG-CT	EM	*1/*1	50	0
12	2918	1624	1294	903	456	447	TT-TT-TT	IM	*4/*35	100	0
10	2644	1478	1165	947	464	483	CT-GT-TT	EM	*2/*41	50	5
17	2442	1344	1099	734	386	349	TT-TT-TT	EM	*1/*9	100	2
18	2235	1242	993	1353	697	656	CC-GG-CT	EM	*2/*35	100	3
02	2192	1199	993	916	502	415	CC-GG-CT	EM	*1/*1	50	2
01	2016	1096	920	913	518	395	CC-GG-CT	EM	*1/*2	50	2
08	2009	1086	922	603	308	294	CC-GG-CT	EM	*1/*41	50	8
03	1954	1074	881	1133	510	623	CT-GG-CC	IM	*1/*5	50	1

AUC = area under the drug concentration-time curve, TRA = tramadol, ODT = O-desmethyiltramadol, DRS = drug related symptoms, LOQ = limit of quantitation

Accordingly, when statistically evaluated in paper I, no significant associations were found between CYP2D6 phenotype and tramadol or ODT pharmacokinetics, neither regarding $C_{\max}$ or AUC. In paper III, the same individuals were studied, although with a different approach. The newly developed enantioselective method was applied, dose-dependent pharmacokinetic parameters were dose-adjusted, and results were essentially descriptively presented. It was found that the two CYP2D6 PMs showed considerably different AUCs of all three metabolites, compared to the individuals being IMs and EMs. The metabolic profiles were characterized by small AUCs of the ODT and NODT enantiomers, and extensively large AUCs of the NDT enantiomers. The (+)-enantiomers of ODT and NODT were affected to a larger extent than the (-)-enantiomers. The PM administered the lower dose never even achieved (+)-ODT or (+)-NODT concentration levels above LLOQ. It was indicated that CYP2D6 IMs and PMs achieved larger AUCs of the (+)- and (-)-enantiomers of tramadol than EMs, although these results were not statistically significant. Nevertheless, all results were in accordance with previously published studies [77-79], with the exception of those regarding the NODT enantiomers, to our knowledge not formerly studied in different CYP2D6 phenotype groups.

The large impact of the CYP2D6 phenotype on the enantiomer ratios of ODT, but not of tramadol, in the healthy volunteers was declared in the previous section. Similar results were obtained when investigated in the study population of autopsy cases (paper IV), in which individuals also being UMs were represented. It was found that PMs showed significantly lower median enantiomer ratios of ODT, in comparison to all the other phenotype groups. Also the IMs significantly differed from EMs and UMs in this regard. However, there was no difference between EMs and UMs. As for the healthy volunteers, no influence of the CYP2D6 phenotype on the enantiomer ratio of tramadol was observed.

The concordance between the well-defined study population of healthy volunteers and the one of autopsy cases implies that the genotype not only affects the enantiomer ratio of ODT, it is of major importance. It is otherwise unlikely that the genotype would have such a significant impact on the ratio also in autopsy cases, in which there are numerous confounding factors that cannot be adjusted for, e.g. the time since drug administration. Even though enantiomer ratios have not been investigated in postmortem blood previously, the results are in agreement with the findings published by Levo et al. They showed a correlation between the number of functional *CYP2D6* alleles and the tramadol/ODT ratio in autopsy cases [116].

Based on these results, it may be concluded that if the (+)/(-)-enantiomer ratio of ODT is related to adverse effects as hypothesized, there is an

increasing risk of toxicity with the number of active *CYP2D6* alleles. Although, it could not be established if there are any differences between EMs and UM in this regard.

CYP2B6* and *CYP3A4

As shown in **Figure 4** the *CYP2B6* and *CYP3A4* metabolic enzymes forms the NDT metabolite, which is not known to have a pharmacological effect. One could therefore speculate that genetic variation in the genes encoding these enzymes is of no toxicological interest. However, a reduced or increased metabolism by these enzymes could affect the *CYP2D6* metabolic pathway by indirect means, affecting the amounts of the parent compound available to the *CYP2D6* enzyme. Such a complementary function of the two metabolic pathways probably accounts for the observed large AUCs of NDT in the two *CYP2D6* PMs.

Two individuals were homozygote of *CYP2B6* variant alleles, with the genotypes **5/*5* and **6/*6*. These subjects showed the smallest AUCs of the NDT enantiomers in the study population. Apart from the *CYP2D6* PMs they also showed the smallest AUCs of the NODT enantiomers. Compared to individuals carrying one or two *wild-type CYP2B6* alleles, although sharing the same *CYP2D6* phenotype (EM), the AUC of both NDT and NODT was reduced by approximately 50%. However, the AUCs of tramadol and ODT were not considerably affected. Because the significance of *CYP2B6* polymorphisms in tramadol pharmacokinetics has not been carefully investigated previously, and the present study comprised only a few participants, the results must be confirmed in further investigations. If the results hold true, genotyping of *CYP2B6* in forensic cases concerning toxicity aspects is likely to be of minor significance.

None of the individuals was homozygote for *CYP3A4*^{*22}, and only one was heterozygote. Consequently, the impact of this particular polymorphism on tramadol pharmacokinetics could not be evaluated.

ABCB1

The *ABCB1* protein P-gp, present in the cell membranes of both the gastrointestinal tract, the liver and the blood-brain barrier, could significantly affect the pharmacokinetics, given that tramadol really is a substrate. Significant associations between the AUC of tramadol and three of the investigated SNPs (1236 C>T, 2677 G>T/A, 3435 C>T) were found in paper I. Highest AUC values were shown for the homozygotes of the variant alleles. However, the association was only observed in the 50 mg dosage group, which implies that further studies on the issue are required.

In paper I, the $C_{\max}$ and AUC parameters were not dose-adjusted, in spite of linear tramadol pharmacokinetics. The *ABCB1* SNPs were also individually investigated. Although, since the three SNPs are reported to be in strong linkage disequilibrium, results may also be presented based on haplotypes. In **Table II**, these haplotypes, along with the dose-adjusted AUCs of the tramadol and ODT enantiomers, are presented. A comparison between the haplotypes TT-TT-TT and CC-GG-CC in relation to pharmacokinetic parameters would have been of outmost interest. However, as shown in **Table II**, four individuals had the TT-TT-TT haplotype but only one the CC-GG-CC haplotype. There were, however, three individuals that presented with the CT-GG-CC haplotype, and one with the CC-GA-CC haplotype. Thus haplotypes with at least two homozygote wild-type positions, of which one always corresponded to the most studied 3435 C>T SNP. Comparing the total tramadol (the sum of both enantiomers) AUC of the TT-TT-TT individuals with the AUC of the individuals with either the CC-GG-CC, CT-GG-CC, or CC-GA-CC haplotype, yielded a p-value of 0.7 using the Mann-Whitney test. Investigating differences between the groups regarding only the AUC of (+)- or (-)-tramadol, utilizing the same test, also yielded nonsignificant results. Subsequently, no statistically significant findings were observed, although studies based on a larger population must confirm it. However, before investigating the issue further it must be determined if tramadol truly is a human P-gp substrate.

The pharmacokinetic impact on drug related symptoms

Three participants, subject 15, 11 and 16, reported quite significant adverse effects with total DRS scores of 13, 16 and 25, respectively (**Table II**). Subject 15 did not score any individual symptom higher than 3. Subject 11 scored nausea, sweating and fatigue as 4. Subject 16 was the individual most apparently affected by the drug, both fainting (at 1 h and 15 min following drug intake) and vomiting (at around 4 h following drug administration and also later in the evening) during the experimental day. This participant scored both nausea and dizziness as 5, meaning worst imaginable symptoms. Vomiting, sweating and fatigue were scored as 4. All three individuals were administered the 100 mg dose and were CYP2D6 IMs.

The general hypothesis in literature regarding adverse effects following tramadol administration is that the frequency and intensity is related to the concentrations of (+)-ODT. The higher the concentration, the higher the risk of side effects and toxicity [62, 71]. However, there was no correlation between $C_{\max}$ or AUC of the potent (+)-ODT and DRS score, nor between the (+)/(-)-ODT enantiomer ratio of the same parameters and DRS score. With the exception of the CYP2D6 PMs showing the lowest AUC values, the

two subjects with the highest DRS scores were the ones with the lowest AUC of (+)-ODT (**Table II**). Neither was there a correlation between pharmacokinetic parameters of any other enantiomer pair and DRS score. However, the two individuals with the highest DRS scores showed a metabolic profile that differed from all the others. Small AUCs of the ODT enantiomers and large corresponding values of the NDT enantiomers were seen in combination with significant AUCs of the NODT enantiomers. In fact, these two participants were the only individuals showing AUCs of the NODT enantiomers exceeding the AUCs of the ODT enantiomers.

The pharmacokinetic impact on tramadol related death

Obviously, some pharmacokinetics parameters, being the total tramadol and ODT blood concentrations, have a large impact on the risk of tramadol related death. Therefore they are used in the interpretation of tramadol related versus unrelated deaths today. Accordingly, paper IV showed that the total tramadol and ODT concentrations (the sum of the (+)- and (-)-enantiomers) were significantly higher in the deceased with tramadol related causes of deaths (TraTox and MixTox), compared to the ones with tramadol unrelated causes of death (OtherTox and NonTox). However, for reasons explained in the introduction, only the concentrations of tramadol and ODT are not always in themselves evidence of a tramadol related or unrelated death. With the indications in literature of a relationship between the (+)/(-)-enantiomer ratios of ODT and adverse effects, the research question formulated was if an examination of enantiomer disposition in the deceased could improve these interpretations. Given that there is an association between enantiomer ratios and toxicity, higher ratios are to be expected in the blood of postmortem cases with tramadol related causes of death. However, no differences were found in the enantiomer ratios of ODT between the groups of TraTox, MixTox, OtherTox, and NonTox, respectively. Consequently, the results did not support the hypothesis. Although since the grouping of the postmortem cases was based on an interpretation, neither can the results be regarded evident enough to reject the hypothesis. For the enantiomer ratio of tramadol, only the group of OtherTox showed somewhat higher ratios compared to the others. Knowing about the relationship between enantiomer ratios and time following drug intake, it seems more probable that these higher ratios reflect a longer time lapse between drug intake and death, than that they would reflect tramadol intoxicated individuals assigned a fallacious cause of death. However, further studies are required to adequately investigate the potential relationship between enantiomer ratios and toxicity, preferably in tramadol treated patients in which the CYP2D6 function as well as the time lapse between drug ingestion and blood sampling are known.

The pharmacogenetic impact on tramadol pharmacodynamics

OPRM1

Regarding DRS and adverse effects, one must not forget that genetic variation potentially affecting tramadol pharmacodynamics might be equally important to genetic variation affecting tramadol pharmacokinetics. In the present study, the most common SNP in the *OPRM1* gene, 118 A > G, was investigated in the healthy volunteers. However, only two individuals presented with the AG genotype, and no one with the GG genotype. Consequently, no conclusions could be drawn regarding its pharmacodynamic impact. It is, however, noteworthy that the individual presenting with most DRS was one of the two individuals with the AG genotype. Since the SNP is hypothesized to result in reduced opioid effects, this finding was in contradiction to what was expected. But the finding also underlines the need of investigating the potential impact of the serotonin and noradrenaline system on DRS and adverse effects.

CYP2D6

In accordance with our results, a previous study on healthy volunteers found smaller differences than expected in (+)-ODT concentrations between EMs and UMs. However, almost 50% of the UMs experienced nausea following the single 100 mg dose, compared to only 9% of the EMs [117]. Severe adverse effects such as respiratory depression [118] and cardiotoxicity [119] has also been reported in two individuals being UMs. It should be mentioned though, that the individual in the former case suffered from renal impairment and that the individual in the latter case ingested an excessive tramadol dose.

A higher frequency of unresponsiveness and rescue medication due to a lower degree of pain relief have been shown in PMs. Analgesic treatment has therefore been the subject of recommendations in clinical practice, proposing an alternative drug for PMs and IMs and a decreased tramadol dose for UMs [71]. Some review authors have however expressed a need of more studies before a relationship between *CYP2D6* metabolizer status and tramadol adverse effects might be established [120, 121].

In 2007 Gan et al. published a study based on a different hypothesis, that PMs instead are the ones that more frequently experience adverse effects. No PM was present in the population of Malaysian patients, although a higher incidence of adverse effects was found among IMs than among EMs and UMs. Vomiting was however unrelated to *CYP2D6* genotype, and more frequent among females [122]. The three individuals reporting the highest DRS scores in the present study were all women and *CYP2D6* IMs.

Although, in contradiction to the hypothesis by Gan et al, the PMs in the present study reported relatively low total DRS scores, 4 and 0, respectively (**Table II**).

Nevertheless, these inconsistent results, and the fact that the frequency of adverse effects can be significantly different in two phenotype groups in spite of small pharmacokinetic differences, implies that CYP2D6 phenotype may affect drug effect via a mechanism that is unrelated to drug pharmacokinetics. Such a mechanism has been proposed. *CYP2D6* is not only expressed in the liver but also in several brain regions, in which the enzyme contributes to metabolism of endogenous neuroactive substrates. The different personality of PMs that has been shown in some studies, such as higher responsibility, orderliness and psychic anxiety, are thought to be explained by the CYP2D6 brain function. It has been hypothesized that the role of CYP2D6 in the brain may also be related to therapeutic drug responses, such as placebo or nocebo effects [123].

Characteristics of tramadol intoxications

Without appropriate reference data, interpretations of tramadol related versus unrelated causes of death are difficult to perform. Drug concentrations and characteristics of case reports, in which circumstances strongly suggest tramadol intoxication, may be valuable. However, with the limitation of being, by definition, a single case, the findings may not be generally applicable to other cases. In the tramadol postmortem literature, a list of nearly all cases classified as intoxications exclusively by tramadol have been summarized (**Table III**) [65, 94, 101, 102, 124-129]. It seems like it has become common practice for authors publishing a case report to fill in this list, in addition to describing the new case. By these means an international reference data compilation can be created. A certain amount of caution concerning interpretations based on these data must however be exercised, since the data is derived from different laboratories with different procedures and methods used. Nevertheless, with a comprehensive list, future conclusions on the characteristics of tramadol intoxications may be drawn.

The three cases presented by Häkkinen et al. [128] were not case reports in the sense of describing the circumstances of the cases. Although tramadol and ODT concentrations were reported and it was stated that no other compounds were detected. It is apparent that few intoxications with tramadol alone have been reported. The fifteen TraTox cases (**Table IV**) that may now be added to the previous list almost double the number of cases. Further, enantioselective quantitation and *CYP2D6* genotyping have not been investigated in previous cases. However, what has been highlighted also previously is the fact that few cases present with only tramadol in their blood, even though the other compounds are not found

at toxic concentrations. Tramadol was exclusively detected in the femoral blood of only a few individuals assigned to the group of NonTox. The most prevalent coadministered drugs in the group of TraTox were diazepam/nordazepam (33.3%), paracetamol (26.7%), tetrahydrocannabinol (THC, 26.7%), venlafaxine/ODV (26.7%), and pregabalin (26.7%), respectively. Accordingly, central nervous system (CNS) depressants, especially benzodiazepines, have commonly been reported in association with tramadol related deaths [66, 128]. Benzodiazepines have also been associated with fatal buprenorphine intoxications in Finland, where buprenorphine is the most abused opioid [130]. It has also been shown that opioids is a frequently concomitant finding in deceased pregabalin abusers [131]. Based on the rather common findings of both benzodiazepines, THC and pregabalin in the TraTox cases, it could be speculated that tramadol has been abused in many of these cases, rather than used as medical treatment. The assumption is further reinforced by the fact that more than half of the TraTox cases had a known history of alcohol and/or drug abuse. They were also at a young age, the median was 26 years, being the lowest in the four investigated cause of death groups, and the range was 21-51 years. Based on the circumstances of the cases, the manner of death was considered suicide in 20% of the cases, and accidents in 33% of the cases. However, the manner of death was unclear in as many as 47%. Thus it cannot be evaluated how large proportion of the tramadol intoxications that were intentional versus unintentional. A previous study performed at the department indicated that individuals with a history of substance abuse may be at risk of unintentional tramadol related deaths [52]. A large majority of the TraTox cases in the present study was men.

Table III. Lethal tramadol intoxications reported in literature

Ref.	No.	Age (sex)	MoD	Coadministrations	Conc. (µg/ml)	TRA	ODT	NDT	NODT
[124]	01	49 (M)	Suicide	7-aminoflunitrazepam	NQ	13			
[101]	02	23 (F)	Suicide	Negative		15.1	6.8	5.7	
[125]	03	26 (M)	-----	Negative		9.6			
[126]	04	67 (M)	Accident	Negative		3.7			
[94]	05	43 (M)	-----	Lorazepam	0.04	1.6	0.44	0.09	
[94]	06	37 (F)	-----	Atropine	NQ	4.21	0.56	0.79	
				Oxycodone	0.2				
				Trazodone	0.3				
				Mirtazapine	<0.05				
				Meprobamate	<2.5				
				Carisoprodol	<2.5				
				Nordazepam	<0.05				
				Clonazepam	<0.05				
				Hydroxyzine	0.33				
				Benzoyllecgonine	<0.05				
[94]	07	28 (M)	-----	Venlafaxine	0.23	7.9	3.1	1.3	
				O-desmethylvenlafaxine	0.14				
				Trazodone	0.08				
				Mirtazapine	<0.05				
				Ethanol	0.02g/100 mL				
				Caffeine	NQ				

Table III. Continued

Ref.	No.	Age (sex)	MoD	Coadministrations	Conc. (µg/ml)	TRA	ODT	NDT	NODT
[94]	08	43 (M)	-----	Amtriptyline	0.5	13.9	0.5	9.7	
				Nortriptyline	0.85				
				Caffeine	NQ				
[127]	09	28 (M)	-----	Negative		5.2			
[102]	10	17 (M)	Suicide	Negative		7.7	1.3	0.6	
[102]	11	75 (F)	Suicide	Norfluoxetine	109 µg/L	48.3	2.4	10.1	
[128]	12	-----	-----	Negative		1.6	0.6		
[128]	13	-----	-----	Negative		2.4	0		
[128]	14	-----	-----	Negative		8.1	1.5		
[65]	15	48 (F)	Suicide	Carbamazepine	3.2	61.83	14.13	134.25	3.71
[129]	16	48 (M)	Suicide	Negative		23.9	0.2		
[129]	17	17 (M)	Suicide	Negative		5.8	0.6		

MoD = manner of death, NQ = not quantified, TRA = tramadol, ODT = O-desmethyiltramadol, NDT = N-desmethyiltramadol, NODT = N,O-didesmethyiltramadol

Table IV. Lethal tramadol intoxications in the thesis study population

No.	Age (Sex)	MoD	Coadministrations	Conc. (µg/g)	TRA	ODT	NDT	NODT	CYP2D6 phenotype	CYP2D6 genotype
01	42 (M)	Suicide	Paracetamol	4	2.1	0.4	0.3	0.2	EM	*1/*2
			Venlafaxine	0.8						
			O-desmethylvenlafaxine	1.9						
			Dihydropropiomazine	0.03						
			Alprazolam	0.002						
02	23 (M)	Accident	Pregabalin	3.1	4.0	0.9	0.3	0.2	EM	*2/*2
			Metoclopramide	0.06						
			Oxycodone	0.05						
			Tetrahydrocannabinol	0.0032						
03	23 (M)	Unclear	Pregabalin	2.1	2.0	0.3	0.8	0.4	EM	*1/*41
			Venlafaxine	0.4						
			O-desmethylvenlafaxine	0.3						
			Promethazine	0.07						
			7-aminoclonazepam	0.06						
			Olanzapine	0.03						
			Zopiclone	0.02						
			Zopiclone	0.17	1.6	0.3	0.2	0.2	EM	*1/*41
04	49 (M)	Unclear	Pregabalin	4.6	11	1.5	0.7	0.4	EM	*1/*9
			Alprazolam	0.04						
			Codeine	0.01						
			Hydrocodone	0.008						
			Tetrahydrocannabinol	0.0005						
06	35 (M)	Accident	Ethanol	0.13 ‰	11	1.1	2.5	0.7	EM	*1/*2
07	23 (M)	Accident	Pregabalin	4.3	58	21	3.4	10	EM	*1/*1
			Tetrahydrocannabinol	0.0004						
08	21 (M)	Accident	Paracetamol	4	11	0.9	6.1	0.3	IM	*1/*4
			Alimemazine	0.1						
			Desmethyllalimemazine	0.2						
			Venlafaxine	0.1						

Table IV. Continued

No.	Age (Sex)	MoD	Coadministrations	Conc. (µg/g)	TRA	ODT	NDT	NODT	CYP2D6 phenotype	CYP2D6 genotype
09	41 (M)	Unclear	Lamotrigine 7-aminoclonazepam Diazepam Nordazepam	2.5 0.16 0.09 0.06	3.8	0.9	0.5	0.4	IM	*1/*4
10	26 (M)	Unclear	7-aminoclonazepam Diazepam Nordazepam Alprazolam	0.11 0.08 0.04 0.03	1.5	0.3	0.4	0.2	IM	*1/*4
11	33 (M)	Suicide	Meclonazepam	NQ	3.9	0.6	0.4	0.2	IM	*1/*4
12	51 (F)	Suicide	Paracetamol Diazepam Nordazepam Dihydropropiomazine Methylphenidate Ritalinic acid Ethanol	6 0.05 0.1 0.07 0.001 0.2 1.26 %	69	0.4	2.4	0.1	IM	*5/*9
13	21 (F)	Unclear	O-desmethylenlafaxine Diazepam Nordazepam Zopiclone Mirtazapine Tetrahydrocannabinol	0.1 0.04 0.07 0.03 0.02 0.0027	1.9	0.5	0.2	0.2	UM	(*2/*41) xN
14	49 (M)	Unclear	Paracetamol Alimemazine Desmethylinemazine Paroxetine	30 0.1 0.3 0.11	8.8	0.7	7.7	0.4	-----	-----
15	23 (M)	Accident	Carbamazepine Diazepam Nordazepam Flubromazolam	2.6 0.09 0.11 0.02	2.3	0.4	0.5	0.2	-----	*1/*4 or *3/*4

MoD = manner of death, NQ = not quantified, TRA = tramadol, ODT = O-desmethylntramadol, NDT = N-desmethylntramadol, NODT = N,O-didesmethylntramadol, xN = multiple gene copies

Limitations regarding the study design

Healthy volunteers

The most beneficial improvement in the study design would have been to increase the size of the study population. To study the disposition of the parent compound, metabolites and their enantiomers, a number of 19 healthy volunteers may be considered sufficient. However, to determine the influence of genetic variation on the same parameters, a larger sample size is required. Alternatively, the individuals could have been genotyped before inclusion in the study, to enrich the number of individuals with the sometimes rare but highly interesting genotypes and phenotypes. One could hypothesize that the individuals being at highest risk of adverse effects and toxicity would be the ones with the CYP2D6 UM phenotype, combined with the *ABCB1* haplotype TT-TT-TT and the *OPRM1* AA genotype. Most optimally, if working according to such a hypothesis, would be to compare such individuals to those being CYP2D6 PMs, combined with the *ABCB1* haplotype CC-GG-CC and the *OPRM1* GG genotype.

Because drug interactions may affect both the tramadol pharmacokinetics and pharmacodynamics, concurrent drug treatment was an exclusion criteria. However, not only prescription drugs and herbal medicines constitute a risk of interactions, but also dietary components. Both herbal extracts, being ingredients in some comestibles, as well as natural products, such as grapefruit, grapefruit juice, and cruciferous vegetables, may modify drug metabolism by CYP enzymes. Also cigarette smoking has been shown to affect some of these enzymes. Nevertheless, drug interactions do not happen with every drug metabolized by the affected enzyme [132]. For tramadol, there is little data reported regarding the potential influence of dietary components on the drug metabolism. Almost any nutrition was admissible to the study volunteers. However, if to preclude dietary impact on the pharmacokinetics, the participants should have been served the same specific diet during, and possibly also in conjunction to, the experimental day.

There are several limitations with the utilization of DRS scores. To facilitate comparisons in DRS score between individuals showing different pharmacokinetic parameters and genotypes, the higher dose (100 mg) should have been administered to all study participants. When utilizing two dosage groups, the individuals should optimally have been blinded to avoid placebo or nocebo effects. Knowing or suspecting that they received a low or relatively high dose, might have had an impact on their perception of DRS. The self-reported questionnaire also used an arbitrary scale, which may have been an additional limitation. Pharmacodynamic effects in relation to genotype could have been investigated using more traditional

methods, such as the cold pressure technique [117]. However, the overall aim of the project referred to the tramadol pharmacokinetics, rather than pain relieving effects and such pharmacodynamic parameters, which were therefore not investigated. Further, with an interest in toxicity, DRS is not an appropriate measure. The adverse effects which may occur following an intake of a single, therapeutic dose are generally not serious. Although such a deterioration of the general condition that was experienced by one of the participants may have consequences for physically weakened people, such as the elderly. Toxicity should better be examined among clinical patients on tramadol treatment, in which doses administered are generally higher and continuously ingested. However, such an approach also introduces many confounding factors, since the patients may suffer from diseases and often are prescribed several other drugs in addition to tramadol.

Autopsy cases

Tramadol positive autopsy cases are, from one point of view, ideal to study if wanting to acquire knowledge about the toxicity of the drug. In a large enough postmortem population, all concentration ranges – therapeutic, toxic and lethal are represented. Comparing enantiomer disposition and genotype in these groups, could lead to the discovery of potential risk factors for tramadol toxicity. The Department of Forensic Genetics and Forensic Toxicology at the National Board of Forensic Medicine receive and analyze blood samples obtained at autopsies from all over the country. To obtain a large autopsy study population is therefore significantly less challenging than obtaining a study population of healthy volunteers. What is challenging, on the contrary, is to group the deceased individuals based on the involvement of tramadol in their cause of death. For several reasons, it is not possible to certainly know which individuals that died from tramadol toxicity. As opposed to the healthy volunteers, neither the administered tramadol dose, the time of the dosing in relation to blood sampling (death), the intention of the drug intake or any experienced adverse effects are known. Additionally, the population significantly differ regarding age, health condition and diseases, tramadol tolerance, and previous and concurrent drug use and abuse. So, in summation, the largest limitation concerning the autopsy cases was the grouping into tramadol related and unrelated cause of death groups, since it was based on the type of interpretation that we aimed at improving. Unfortunately, this is a limitation that is difficult to circumvent, and again emphasize the necessity of confirming results regarding toxicity and time since drug ingestion in a study population of pain patients.

Methodology

Methodology limitations mostly concern paper I, both regarding the measurement of pharmacokinetic parameters and the genotype determination.

Several advantages using the enantioselective tramadol method over the achiral routine method were evident. With the former, four enantiomer ratios associated with time following drug intake were yielded. Depending on the time interval of interest, all four of them may be used in combination to estimate the time of intake. Possibly, the use of combined ratios may compensate for some of the interindividual variability in individual ratios. With the current routine method, only one ratio was yielded, being CYP2D6 dependent.

In addition, higher sensitivity was obtained with the enantioselective method. All four enantiomer ratios could be calculated already from 0.5 h and up to 24 h following drug intake, while the ODT/tramadol ratio, using the achiral method, could be measured between 1.0 h and 10 h. The sensitivity also has large implications on the calculation of total AUC. With the achiral method, large extrapolated areas resulted in the application of the AUC_{0-10h} instead of the $AUC_{0-\infty}$.

CYP2D6 genotype was determined using three different techniques in the healthy volunteers. All of them had the *3, *4, *5, and *6 alleles in common, as well as a copy number analysis. The four alleles mentioned are the most common nonfunctional ones in Caucasian populations [133]. Combined with the copy number determination, the pyrosequencing method thus results in a fairly good *CYP2D6* genotyping, although just in Caucasians. However, the individuals that form the Swedish population have different countries of origin. Therefore the pyrosequencing method is not optimal, neither regarding analysis of the autopsy cases, nor the healthy volunteers. If other origin than the Caucasian one, at least a few generations back, had been an exclusion criterion in the study of volunteers, the pyrosequencing method would, however, have been more suitable. The pyrosequencing method was applied to the study population of healthy volunteers in paper I, but only served as a screening method in the population of autopsy cases. Mostly to be able to enrich the number of CYP2D6 PMs and UMs regarding the second year of inclusion.

With the methods based on TaqMan analysis and xTAG technology, a more comprehensive genotyping could be performed. These methods included many common alleles, although only the TaqMan analysis included the *12 and *14 alleles, and the xTAG kit exclusively included the *11, *15 and *35 alleles. Both the former and the latter are alleles resulting in an inactive CYP2D6 enzyme, except for *35 which results in a fully active enzyme. The xTAG method was used for the genotyping of the healthy volunteers in

paper III. Compared to the results obtained in study I, there were no discrepancies observed. That is no differences in the alleles that both methods should be able to identify. However, as discussed in paper I, two individuals being CYP2D6 IMs, with the pyrosequencing determined genotypes $*1/*4$ and $*1/*3$, respectively, showed remarkable differences in the AUC of ODT. The pharmacokinetic differences were confirmed with the enantioselective quantitative method in paper III. However, the xTAG genotyping technology provided a genetic explanation, revealing that the $*1/*4$ genotype in fact was a $*41/*4$ genotype. With $*41$ being a decreased functional allele, pharmacokinetic differences could be expected.

Most of the autopsy cases were genotyped using the TaqMan analysis, although 20 samples were analyzed with the xTAG method.

The greatest general constraint concerning the genotyping was the inability to discriminate the allele duplicated in heterozygote samples. Thirteen autopsy cases were assigned the UM phenotype based on their genotype. Ten of them presented with two active alleles, and in such cases it is not necessary to know which of them being duplicated to accurately predict the phenotype. In three of the cases, the genotype was either $*2/*17$ or $*2/*41$. Allele $*2$ is an active allele and $*17$ and $*41$ are both decreased functional alleles, and thus the phenotype may be interpreted as either EM (in case of $*17$ or $*41$ duplicated) or as UM (in case of $*2$ duplicated). It is mainly the $*2$ allele that has been reported as duplicated [134], although without confirmation in the present cases there is a risk of misinterpretation. However, the ODT/TRA ratios and (+)/(-)-ODT ratios did not imply an erroneous interpretation, since they were at or above the median of the whole UM group.

Regarding the statistics utilized, more appropriate parameters should have been used for the comparison of DRS scores between the two dosage groups in paper I. It was stated (page 129) that “subjects given 100 mg tramadol reported higher scores in the DRS form compared to subjects given 50 mg, a mean score of 7.3 ± 8.7 and 3.1 ± 2.6 , respectively”, and mean values and standard deviations of the DRS scores were also presented in Table 3 (page 131). However, these calculations require a continuous variable, and not a discrete variable as is the case with the DRS scores. If to be analyzed statistically, a nonparametric test must therefore be utilized. The median value in both the 50 and 100 mg dosage group was 3, and there were no statistically significant differences between the groups when assessed with the Mann-Whitney test.

CONCLUSIONS

The development and validation of an enantioselective method for the quantitation of tramadol, ODT, NDT and NODT enabled a sensitive and selective analysis of whole blood from healthy volunteers and from autopsy cases.

All four (+)/(-)-enantiomer ratios were positively correlated with the time following drug administration. Linear correlations were found for the enantiomer ratios of tramadol and NDT, therefore considered the most appropriate for estimations of the time since drug intake. The additional advantage with the tramadol enantiomer ratio was that it was found to be the only one unrelated to *CYP2D6* genotype, hence not showing lower ratios for the *CYP2D6* PMs. The largest increase in enantiomer ratio over time was observed for NDT, which was also considered an advantage in estimations of the time since tramadol administration. However, before the ratios may be applied to forensic cases in order to distinguish between a recent and past drug intake, interindividual differences must be further investigated and also the potential impact on repeated administration. Nevertheless, the combined use of the enantiomer ratios of tramadol and NDT are good candidates for the task, and preferable compared to the use of the ODT/tramadol ratio and the ODT/NDT ratio.

Polymorphisms in the *CYP2D6* gene had great impact on the tramadol pharmacokinetics for those classified as PMs. Their metabolic profiles were characterized by small AUCs of the ODT and NODT enantiomers, particularly for the (+)-enantiomers. The AUCs of both NDT enantiomers were instead extensively large compared to the ones of IMs and EMs. The enantiomer ratios of ODT were significantly associated with *CYP2D6* genotype also in the autopsy cases, showing increasing median values for PMs, IMs, and EMs, respectively. However, no differences were observed between EMs and UMs. An impaired function of the *CYP2B6* enzyme, resulting in decreased AUCs of NDT and NODT, was indicated.

No association between pharmacokinetic parameters, (+)/(-)-enantiomer ratios included, and neither DRS nor tramadol related death was found. Further, the highest enantiomer ratios in autopsy cases were associated with low total tramadol concentrations, implying that these ratios reflect time since drug administration rather than toxicity. However, larger studies, preferably also including other kinds of study populations, are necessary to fully clarify the pharmacokinetics, pharmacodynamics and pharmacogenetics of tramadol.

The type of coadministered drugs, the frequent history of substance abuse, and the young age in the group of tramadol intoxications indicated that the drug had been abused rather than used in medical purpose.

In conclusion, the enantiomer ratios of tramadol and its metabolites, in combination with *CYP2D6* genotyping, are promising regarding improved forensic interpretations of time since tramadol ingestion. The estimations are likely to become useful concerning both postmortem toxicology and human performance toxicology. However, from the current state of knowledge, the enantiomer ratios and genotyping will not aid in the interpretation of toxicity.

FUTURE PROSPECTS

Pharmacogenetics

To further deepen the understanding of genetic influence on tramadol metabolism, genotyping of not only *CYP2D6*, but also of *CYP2B6* and *CYP3A4* could be performed in the autopsy cases. It would be of special interest to study homozygotes of *CYP2B6* *5 and *6, to elucidate if the polymorphisms seem to cause reduced enzyme function, as indicated in paper III.

Likewise, additional genotyping would be of interest from a pharmacodynamic perspective. The SNP analysis of *OPRM1* that was performed in healthy volunteers (paper I) could also be applied to the autopsy cases. Furthermore, genotyping of *SLC6A4* may shed some light over pharmacodynamic interindividual differences. *SLC6A4* encodes the serotonin transporter (5-HTT), responsible for the reuptake of serotonin from the synaptic cleft back into the presynaptic neuron. Two polymorphisms in strong linkage disequilibrium, a 43-bp insertion/deletion and the SNP 3609 A>G, result in high, intermediate or low expression of 5-HTT. Low 5-HTT expression has been associated with a higher degree of analgesia to the opioid drug remifentanyl [135].

Investigation of another study population

The impact of regular dosing and steady-state must be investigated before the enantiomer ratios of tramadol and its metabolites may be fully utilized in the estimation of time since drug intake. Analyzing the relationship between enantiomer ratios and time in patients regularly administered tramadol would therefore be highly valuable.

Investigation of other specimens

Several urine samples were collected from the healthy volunteers administered a single tramadol dose (paper I). However, only the samples from two individuals have been analyzed with the current routine method for urine tramadol quantitation. It was found that a more sensitive analysis, utilizing enzymatic hydrolysis, was required to follow the changes in concentration for a longer time period. Urine samples were collected up to 14 days, although tramadol could only be detected up to 72 h with the

present method. Data on urine concentrations of tramadol has been scantily reported in literature, but is of importance in the forensic field to demonstrate intake of an illegal compound. It is therefore of interest to know for how long time tramadol may be detected in urine following a single drug intake. A method development for the quantitation of tramadol, ODT, and NDT in human urine using β -glucuronidase hydrolysis has been described in literature. When the method was applied to six healthy volunteers orally administered 50 mg tramadol, the parent compound and ODT could be detected up to 7 days [136]. Before development of a new method at the National Board of Forensic Medicine, stability of tramadol in urine samples needs to be investigated.

If aiming at further investigate tolerance, the postmortem toxicological investigation could be complemented with hair analyses in a prospective study.

Also brain tissue may be obtained at autopsy to investigate the concentration of the tramadol and ODT enantiomers at the site where they exert their effect. In such a study could potentially also the impact of genetic polymorphisms in drug transporters at the blood brain barrier be evaluated. However, quite extensive method development would be required, both regarding the tissue sampling (from which area should the sample be taken), tissue drug extraction and the quantitative method. Further, obtaining postmortem samples that are not routinely taken for the purpose of the toxicological investigation is associated with several ethical considerations.

Potential improvements of methodology

A limitation using pyrosequencing technology is the rather low potential to perform multiplex analyses. With newer techniques, such as the next generation sequencing (NGS), it is possible to analyze numerous genes and polymorphisms in the same run. During the work with the thesis it was also apparent that the pyrosequencing application for copy number analysis was not adequately accurate and precise concerning postmortem samples. Eventually, a more reliable NGS method, also being able to discriminate the *CYP2D6* allele being duplicated, could be developed at the National Board of Forensic Medicine.

Since the enantioselective tramadol method developed had its limitations regarding long run times and robustness, other chiral separation techniques may be considered before implementing the method in routine practice. The supercritical fluid chromatography (SFC) has become increasingly popular during the last years and could possibly be an alternative since shorter equilibration and analysis times in general are

considered advantages compared to HPLC. However, no separation technique is superior over another regarding every application. Furthermore, a reversed phase setup is difficult to implement in SFC [137, 138].

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Papers

Nothing is as highly treasured as an acorn! At least not if you are a prehistorical squirrel named Scrat. Scrat is a wonderful character in the animated ICE AGE movies, of which I have become a huge fan. My favouritism to Scrat is mostly due to that his endless struggles in the chase for acorns very much reminds me of my own struggles in the chase for scientific papers!

I would say we have fought equally hard, me and Scrat, and with about the same luck. And yes, I can relate to the ice age as well. Many are the things, activities and beloved friends and family members that I have forsaken during the last years, not to mention the hottest summer in living memory! Although, soon having collected the fourth, and last, PhD-acorn, I will be released from the rat race that in Swedish is known as “ekorrhjul” (squirrel wheel)!

The acorn is illustrated by awesome drawer **Maria Norlund**, also working as a chemist at the National Board of Forensic Medicine in Linköping.

Papers

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