Linköping Studies in Health Sciences, Thesis No. 113

Stability of zopiclone in whole blood

- Studies from a forensic perspective

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Linköping 2010

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Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2010

ISBN 978-91-7393-339-1
ISSN 1100-6013

Dala-Gård

Ring the bells that still can ring
Forget your perfect offering
There is a crack in everything
That's how the light gets in

Anthem by
Leonard Cohen

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ABSTRACT

Bio-analytical results are influenced by *in vivo* factors like genetic, pharmacological and physiological conditions and *in vitro* factors like specimen composition, sample additives and storage conditions. The knowledge of stability of a drug and its major metabolites in biological matrices is very important in forensic cases for the interpretation of analytical results. Many drugs are unstable and undergo degradation during storage.

Zopiclone is a short-acting hypnotic drug, introduced as a treatment for insomnia in the 1980s. However, this drug is also subject to abuse and can be found in samples from drug-impaired drivers, recreational drug users and forensic autopsy cases. Zopiclone is analyzed in biological materials using different analytical methods. It is unstable in certain solvents and depending on storage conditions unstable in biological fluids. The aim of this thesis was to investigate the stability of zopiclone in human whole blood and to compare stability between authentic and spiked samples. Interpretation of zopiclone concentrations in whole blood is important in forensic toxicology. The following investigations were performed to study the stability of zopiclone in both spiked and authentic human blood.

First, different stability tests were performed. Spiked blood samples were stored at –20°C, 5°C and 20°C and the degradation of zopiclone was investigated in long- and short-term stability. Authentic and spiked blood samples were stored at 5°C and differences in zopiclone stability were studied. Processed sample stability and effect of freeze/thaw cycles were also evaluated.

Second, influence of pre-analytical conditions on the interpretation of zopiclone concentrations in whole blood was investigated. Nine volunteers participated in the study. Whole blood was obtained before and after oral administration of 2 x 5 mg Imovane®. Aliquots of authentic and spiked blood were stored under different conditions and zopiclone stability was evaluated. In this study, the influence from physiological factors such as drug interactions, matrix composition and plasma protein levels were minimized.

Analyses of zopiclone were performed by gas chromatography with nitrogen phosphorous detection and zopiclone concentrations were measured at selected time intervals. Degradation product of zopiclone was identified using liquid chromatography-tandem mass spectrometry.

The first study showed that zopiclone degrades in human blood depending on time and temperature and may not be detected after long-term storage. The degradation product 2-amino-5-chloropyridine was identified following zopiclone degradation. The best storage condition was at –20°C even for short storage times, because freeze-thaw had no influence on the results. In butyl acetate extracts, zopiclone was stable for at least two days when kept in the autosampler. However, in blood samples stored at 20°C a rapid decrease in concentration, was noticed. This rapid degradation at ambient temperature can cause an underestimation of the true concentration and consequently flaw the interpretation.

The second study showed no stability differences between authentic and spiked blood but confirmed the poor stability in whole blood at ambient temperature. The results showed that zopiclone was stable for less than 1 day at 20°C, less than 2 weeks at 5°C, but stable for 3 months at –20°C. This study, demonstrates the importance of controlling pre-analytical conditions from sampling to analysis to avoid misinterpretation of toxicological results.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Inom forensisk toxikologi undersöks förekomst av droger, läkemedel och gifter i biologiskt material. Resultatet av undersökningarna bidrar till bedömningar i rättsliga utredningar av drogmissbruk, drogpåverkan och dödsorsak. Kunskap om stabiliteten hos kemiska föreningar i biologiska prover under förvaring är av väsentlig betydelse både analytiskt och tolkningsmässigt. Många substanser är instabila och förändras under förvaring. Provmaterial transporteras via post, registreras på laboratoriet och förvaras därefter i kyl. Innan samtliga undersökningar är klara har provet normalt förvarats i en till två veckor. Rättsliga processer kan pågå under en längre tid och det händer ibland att prover måste undersökas på nytt när nya frågeställningar tillkommer. Provmaterialet kan då ha förvarats i flera veckor eller månader.

Zopiklon introducerades som läkemedel på 1980-talet för behandling av kortvariga sömnbesvär. I Sverige finns zopiklon som den verksamma substansen i sömnmedlet Imovane®. Inom forensisk toxikologi undersöks förekomst av zopiklon när analys av läkemedlet begärs. Zopiklon återfinns i såväl missbruksärenden, drograttfylleriärenden som i obduktionsfall. Zopiklon kan analyseras i olika biologiska material som till exempel helblod, urin, hår och postmortalt blod. Beroende på förvaringsförhållanden, materialets beskaffenhet och pH förändras mängden zopiklon i lösningar och i biologiskt material. Syftet med studierna i denna avhandling var att undersöka stabiliteten för zopiklon i helblod, samt att studera stabilitetsskillnader mellan blod innehållande zopiklon efter tillsats (spikade prover), med blod innehållande zopiklon efter intag av läkemedlet (autentiska prover). Kunskap om stabiliteten för zopiklon i detta material är viktig, eftersom resultat från analyser på helblod utvärderas och tolkas inom forensisk toxikologi.

Två olika studier genomfördes. I den första studien gjordes olika typer av stabilitetstester. Spikade prover förvarades vid −20°C, 5°C och 20°C och koncentrationerna av zopiklon följdes över tid. Stabilitet i provextrakt under förvaring på analysinstrument och stabilitet efter det att prov frysts och tinats undersöktes också.

I den andra studien undersöktes hur provhantering innan analys kan inverka på koncentrationerna av zopiklon i helblod och påverka tolkningen av resultat. I studien deltog nio frivilliga individer. Blodprov togs före och efter intag av 2 x 5 mg av läkemedlet Imovane®. Spikade och autentiska prover förvarades vid −20°C, 5°C och 20°C och koncentrationerna av zopiklon följdes över tid och jämfördes. I denna studie kontrollerades även faktorer som indirekt kan ha en påverkan på substansens koncentration i helblod. Faktorer som materialets beskaffenhet, förekomst av andra droger och mängden av plasmaproteiner kontrollerades.

Den första studien visade att koncentrationerna av zopiklon i helblod sjunker under förvaring beroende på temperatur och tid. Vid provförvaring i rumstemperatur sjönk koncentrationen av zopiklon snabbt. Det har tidigare visats att när pH stiger förändras zopiklonmolekylen och degraderar via kemisk hydrolys till 2-amino-5-klorpyridin. Denna degraderingsprodukt kunde identifieras vid ett enkelt försök på zopiklonspikade prover som inkuberats vid 37°C. Mängden aminoklorpyridin ökade i proportion till minskningen av mängden zopiklon. Mätning av degraderingsprodukten kan komma till nytta vid utredningar av förekomst av zopiklon i fall där provmaterial har förvarats under lång tid, till exempel vid speciella dödsfallsutredningar.

Frysning och tining av prov hade ingen inverkan på koncentrationerna av zopiklon i helblod. Zopiklon var stabilast vid förvaring i frys och eftersom frysning och tining inte påverkade analysresultatet, bör prover kunna förvaras i frys både under kortare och längre tidsperioder.

Zopiklon var stabilt under minst två dagar i provextrakt som förvarats i rumstemperatur på analysinstrumentet. Det innebär att om det uppstår oförutsedda problem under pågående analys, så är det möjligt att analysera provextraktet på nytt inom denna tidsperiod.

Den andra studien visade inga skillnader i stabilitet mellan spikade och autentiska prover och resultaten från stabilitetstesterna i denna studie bekräftade resultaten från den första studien. Zopiklon i helblod visade sig vara stabilt mindre än en dag vid förvaring i rumstemperatur, mindre än två veckor vid förvaring i kyl, men i minst tre månader vid förvaring i frys. Detta innebär att provmaterialets förvaring från provtagning fram till analys måste kontrolleras med avseende på temperaturförhållanden. Analysresultat från prover som förvarats en längre tid måste tolkas med stor försiktighet.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Stability tests of zopiclone in whole blood.
Nilsson GH, Kugelberg FC, Kronstrand R, Ahlner J.
Forensic Sci Int. 2010, 200(1-3):130-135.

II. Influence of pre-analytical conditions on the interpretation of zopiclone concentrations in whole blood.
Nilsson GH, Kugelberg FC, Ahlner J, Kronstrand R.
Forensic Sci Int. 2010, accepted for publication.

ABBREVIATIONS

CV	Coefficient of variation
CYP	Cytochrome P450
GABA	γ-aminobutyric acid
GC	Gas chromatography
GHB	Gamma-hydroxybutyric acid
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LSD	Lysergic acid diethylamide
MS	Mass spectrometry
NPD	Nitrogen-phosphorus detector
SEM	Standard error of the mean
SD	Standard deviation
THCCOOglu	11-nor-Δ^9-carboxy-tetra-hydrocannabinolic glucuronide
THCCOOH	11-nor-Δ^9-carboxy-tetra-hydrocannabinolic acid

INTRODUCTION

Pre-analytical conditions

Laboratory activities are commonly classified in pre-, intra- and post-analytical processes. The pre-analytical phase includes request, sample collection, transport, registration, preparation and aliquoting, storage, freezing and thawing [1]. The intra-analytical phase covers the measurement procedures while the post-analytical phase includes processing, verifying, interpreting and reporting of the results. In the past, the development of analytical technology and quality specifications has been the major focus. However, in clinical chemistry it was noticed that many problems occurred in the pre-analytical phase [2,3] and attention was directed to the pre-analytical process in laboratory medicine as well as in forensic toxicology [4-6].

Toxicological laboratory analysis results are influenced pre-analytically by *in vivo* factors like genetic, pharmacological and physiological conditions and *in vitro* factors like specimen composition, sample additives and storage conditions. Pharmacokinetic and pharmacogenetic studies have shown that factors such as age, gender, ethnic origin, body weight, liver and kidney function, plasma/blood ratio and polymorphism of drug metabolizing enzymes as well as drug interactions must be considered when interpreting results [7-10]. Analytical methods must be carefully validated for drug measurement and quantifications. Method validation includes several analytical parameters; selectivity, linearity, accuracy, precision, limit of quantification, limit of detection, recovery, robustness and nowadays even parameters affected by specimen composition such as matrix effects and stability [11-13].

Drug stability

Stability has been defined as “The chemical stability of an analyte in a given matrix under specific conditions for given time intervals” [14]. In forensic toxicology, the analyte can be a drug, metabolite and/or a degradation product

in a biological matrix. Examples of biological matrices are whole blood, serum, plasma, urine, hair, oral fluids and tissues.

The knowledge of stability of a drug and its major metabolites in biological matrices is very important in forensic cases for the interpretation of analytical results [6,15,16]. In forensic investigations the pre-analytical stability processes start at the time of sampling and proceeds until the time of analysis. Frequently, there is a delay of a few days between sampling, drug screening and drug quantification. In forensic toxicology supplementary analysis or reanalysis is sometimes necessary because of the legal process. In such cases it is not uncommon that samples are stored weeks or months before the final drug quantification is done. In post-mortem forensic cases the storage of the body between the time of death and the time of sampling during the autopsy also has to be considered. A drug, which is present in a biological sample, may decompose during storage and may not be detected when the sample is analyzed.

The presence of drugs and poisons are tested in biological materials like blood, urine and hair [17,18]. The identification and the quantification of drug and metabolite concentrations in blood are valuable for the assessment of drug abuse in connection with crime and sometimes for establishing the cause of death. The time of sampling is important, especially if there is any suspicion of drug influence in the crime. Urine samples are useful in cases of drug misuse or abuse because the drug is present in urine for a longer time and in higher concentrations than in blood. Analysis of hair segments may define historical drug use or changes in drug habits. In Sweden the specimens of venous whole blood are taken by a nurse or physician, urine samples by the police and post-mortem samples (e.g. femoral blood, urine, vitreous humor, liver, brain, kidney and lung) by a forensic pathologist. After sampling all specimens are sent to one central laboratory for toxicological analysis. During the transport the samples are stored at ambient temperature for a period of about 20-24 h. However, the blood samples contain 100 mg sodium fluoride and 25 mg potassium oxalate as preservatives and the urine samples contain 1% sodium fluoride as a preservative. Before analysis, the samples are stored in a refrigerator.

The best storage temperature for most of the drugs is at 4°C for short-term storage and at –20°C for long-term storage [17]. For practical reasons it is most common to keep blood samples at 4°C even for long-term storage. In Sweden the forensic laboratory has to keep blood samples in a cold place for one year to enable reanalysis if necessary.

Many substances are unstable in biological samples and undergo degradation during storage. Instability can depend on physical (e.g. type of tubes and preservatives, light, temperature), chemical (hydrolysis, oxidation) or metabolic processes (enzyme activities and/or metabolic production) [6,15,17]. In the area of analytical toxicology, the stability of drugs of abuse in biological specimens has been extensively studied (see section "Stability investigations of drugs").

Design and evaluation of stability experiments

Stability investigations mainly comprise studies of the influence of long-term and/or short-term storage under the same conditions that laboratory samples are normally collected, stored and processed. But in connection with method validation also in-process stability, freeze-thaw stability and processed sample stability are included. Accounts and recommendations of stability experimental designs and stability evaluations are available [12,13,19], but generally accepted guidelines have not yet been established [15,20].

Several different types of stability tests including stock standard solution stability are required for complete evaluation [11-13,19]. Long-term stability studies usually cover a storage period that is expected for ordinary laboratory samples and under the same storage conditions used routinely. In-process or bench-top stability is the stability at ambient temperature over the time needed for sample preparation. During reanalysis, samples have to be frozen and thawed; therefore stability tests over multiple freeze/thaw cycles are recommended. Processed stability tests are needed to investigate stability in prepared samples e.g. sample extracts in auto sampler conditions.

Stability testing by comparing quality control samples at two concentration levels before (comparison samples/reference samples) and after (stability samples) exposing to test conditions has been suggested [12,13,19]. The reference samples can either be freshly prepared or stored below –130°C. After storage at selected temperature and time intervals in the study, reference and stability samples are analysed together and the results are compared. Stability acceptance has been recommended for concentration ratios between reference samples and stability samples of 90 and 110%, with 90%-confidence intervals within 85-115% [19]. The mean of the stability can also be tested against a lower acceptance limit corresponding to 90% of the mean of the reference samples using a one-sided *t* test [13].

Various experimental designs and different procedures for data evaluation exist in stability investigations. Mostly, stability tests are conducted by adding (spiking) the drug (analyte) at different concentrations to a pooled drug-free matrix (e.g. whole blood, plasma, serum and/or urine), aliquoted and stored at the same time and in the same way as ordinary samples. The concentrations are measured at selected time intervals and compared to detect any degradation trend [21-32]. Among reported investigations, also studies on authentic material from volunteers dosed with the drug or from laboratory cases have been performed [25,32-36].

Stability investigations have been evaluated in several different ways by statistical parametric tests like *t* test [26], paired *t* tests [32,35], analysis of variance (ANOVA) [28,36] or by nonparametric tests like Kruskal-Wallis and Mann-Whitney [31]. Analytes have been regarded as stable if difference between initial concentration (C_0) and concentration at a given time (C_t) does not exceeded the critical difference, $d = C_0 - C_t < SD$ of the method of analysis [30,34]. Stability has also been evaluated on a percentage base with regard to analyte decrease or increase during storage [22,23,29,33,36].

Stability investigations of drugs

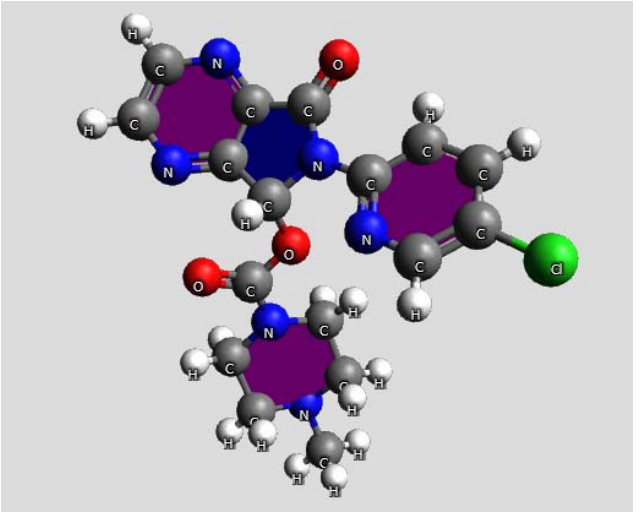
Specific stability studies on several forensically relevant drugs in human blood or tissues have been done; such as *cocaine, its metabolites and its degradations product* in whole blood, post-mortem blood or plasma [24,29], *benzodiazepines, antidepressants, analgesics and/or hypnotics* in whole blood, plasma or post-mortem blood [21,22,35,37,38], *morphine and/or its glucuronides and/or buprenorphine* in whole blood, plasma or post-mortem blood [23,25], *11-nor-Δ^9-carboxy-tetra-hydrocannabinol glucuronide (THCCOOglu)* in plasma [30], *toluene and acetone* in liver, brain and lungs [31], *3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA) and 3,4-methylene-dioxyamphetamine (MDA)* in whole blood [26], *carbon monoxide* in post-mortem blood [39], *gamma-hydroxybutyric acid (GHB)* in blood [36] and *ethanol* in whole blood and plasma [32]. In urine, drug stability during storage has been investigated for drugs like *11-nor-Δ^9-carboxy-tetra-hydrocannabinol* acid *(THCCOOH), amphetamine, methamphetamine, ephedrine, morphine, codeine, cocaine, benzoylecgonine and/or phencyclidine* [27,33,34], *lysergic acid diethylamide (LSD)* [40], *the LSD* metabolite *2-oxo-3-hydroxy lysergic acid diethylamide (O-H-LSD)* [28] and *GHB* [36].

Depending on conditions (e.g. time, temperature and/or pH) changes in drug concentrations were observed for many drugs in these experimental studies. For example, GHB in post-mortem blood and urine (metabolic production) [36], THCCOOH in urine, THCCOOglu in urine and plasma (decarboxylation, enzymatic and chemical hydrolysis) [30,34], acetone in liver, brain and lungs (reduction) [31] and cocaine in whole blood and post-mortem blood (enzymatic and chemical hydrolysis) [24]. In post-mortem blood, degradation was noticed for e.g. the benzodiazepine metabolite 7-amino-nitrazepam and for the hypnotic drug zopiclone [35].

Zopiclone

Zopiclone is a short-acting hypnotic drug, a central nervous system depressant, with muscle relaxant and anticonvulsant properties. The drug was introduced for treatment of insomnia in the 1980s. In Sweden, zopiclone is a common finding in samples from drug-impaired drivers, users of recreational drugs and forensic autopsy cases [16,41].

Zopiclone is considered a non-benzodiazepine from the cyclopyrrolone class. It contains an asymmetric carbon atom and since all the four substituents in the molecule are different it possesses chirality. Each form (left- or right-handed) of the chiral compound, the two mirror images of the molecule, are called enantiomers or optical isomers. Zopiclone is a racemic mixture composed of the (+)-enantiomer with S-configuration and the (−)-enantiomer with R-configuration [42]. The chemical structure of zopiclone is shown below (**Fig. 1**).



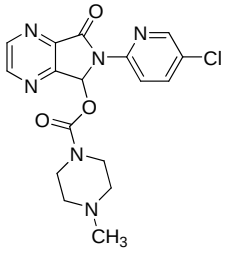


Fig. 1. Zopiclone, 6-(5-chloro-2-pyridyl)-7-(4-methyl-1-piperazinyl)carbonyloxy-6,7-dihydro(5H)pyrrolo-(3,4-b)pyrazin-5-one. Empiric formula: $C_{17}H_{17}ClN_6O_3$. Molecular weight: 388.81 g/mole.

The racemic mixture of zopiclone is sold under various brand names such as Imovane® (e.g. Sweden, Norway), Imozop® (Denmark), Zimovane® (e.g. United Kingdom, Ireland) and Limovan® (e.g. Spain). The S(+)-enantiomer eszopiclone is available under the brand name Lunesta® (e.g. USA). The drugs are used for short-term insomnia therapy. The short-term effects of insomnia are characterized as difficulty in falling asleep, frequent nocturnal awaking and/or early morning awaking. The usual dose of Imovane® is 5 or 7.5 mg at bedtime [43].

Pharmacokinetics

After oral administration of the racemic mixture, zopiclone is rapidly absorbed from the gastrointestinal tract, with a bioavailability of approximately 80% [44]. Plasma protein binding of zopiclone was reported as 45% [45] in one study and 80% in another [46]. Both albumin and α 1-acid glycoprotein contribute to protein binding but also other proteins can be involved (e.g. globulins, lipoproteins) [46]. Zopiclone is rapidly and widely distributed to body tissues including the brain, and is excreted in urine, saliva and breast milk. Zopiclone is metabolized by decarboxylation, oxidation, and demethylation. In the liver zopiclone is partly metabolized to an inactive N-demethylated (13-20% of dose) and an active N-oxide metabolite (9-18% of dose) (**Fig. 2**) [44].

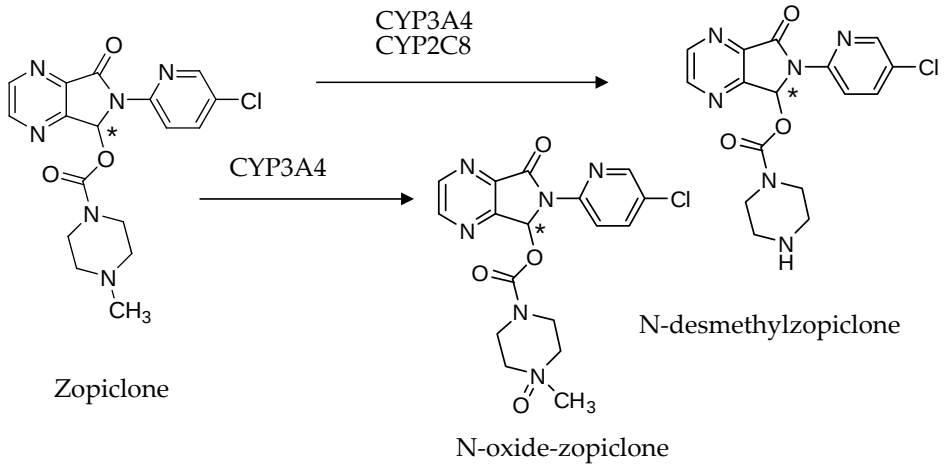


Fig. 2. Pathways of zopiclone metabolism in humans (*position of the asymmetric carbon).

The cytochrome P-450 (CYP) enzymes CYP3A4 and CYP2C8 are involved in the metabolism of zopiclone. Both metabolites have a correlation to CYP3A4 activity but the N-desmethylzopiclone formation also has a correlation to CYP2C8 activity [47]. Approximately 50% of the administered dose is decarboxylated to inactive metabolites of which some is excreted as carbon dioxide via the lungs. Less than 7% of the administrated dose is renally excreted as unchanged zopiclone. In urine, the N-desmethyl and N-oxide metabolites account for 30% of the initial dose. Elimination half-life ($t_{1/2}$) of zopiclone is in the range of 3.5 to 6.5 hours [44].

There is no gender difference in zopiclone pharmacokinetics and patients with liver or renal dysfunction show only minor modification of the pharmacokinetic parameters, but the plasma half-life of zopiclone increases with age [44,45].

All the pharmacokinetic processes, absorption, distribution, metabolism and excretion, are influenced by chirality. Plasma concentration of S(+)-zopiclone becomes higher than that of its antipode R(−)-zopiclone after oral administration of the racemic mixture and the urine concentration of R(–)-N-desmethylzopiclone and R(−)-N-oxide-zopiclone become higher than that of their antipodes [48].

Drug interactions occur when the efficacy or toxicity of a medication is changed by concomitant administration of another substance. Inhibitors of CYP3A4 can increase plasma zopiclone concentrations [49,50] while CYP3A4 inducers decrease the plasma concentration of zopiclone [51].

Pharmacodynamics

$GABA_A$ receptors mediate inhibitory synaptic transmission in the central nervous system and are the targets of neuroactive drugs used in the treatment of insomnia. $GABA_A$ receptors are pentametric membrane proteins that operate as GABA (γ-aminobutyric acid)-gated chloride channels. Agonists increase the chloride permeability, hyperpolarize the neurons, and reduce the excitability. The receptors are made up of seven different classes of subunits with multiple variants (α1-α6, β1-β3, γ1-γ3, ϱ1-ϱ3, δ, ε and θ) that are differentially expressed throughout the brain. Most $GABA_A$ receptors are composed of α-, β- and γ-subunits [52]. Zopiclone has a high affinity for the benzodiazepine binding site and acts at γ2-, γ3-bearing $GABA_A$ receptors, including α1β2γ2 and α1β2γ3, but relative to benzodiazepines, produce comparable anxiolytic effects with less sedation, muscle relaxation, or addictive potential [53,54].

It has been suggested that zopiclone has a pharmacological profile different from benzodiazepines either because of a differential affinity for different $GABA_A$ receptor subtypes or partial agonistic properties. Further, it has been found that zopiclone behaves as a partial agonist at the $GABA_A$ receptor with a lower intrinsic activity relative to benzodiazepines. S(+)-zopiclone has more affinity for benzodiazepine sites than R(−)-zopiclone, but both enantiomers are active at $GABA_A$ receptors [54-57].

The advantage of racemic zopiclone could be seen within 30 minutes, by ease of falling asleep, increased sleep duration and decreased number of awakenings during the night [43]. Drugs that act on the central nervous system can have adverse effects on performance and behaviour of the individual. Residual effects on psychomotor and cognitive functions are dependent on dose and half-life of the drug [58].

Forensic cases

At the clinically recommended dose of 7.5 mg, the peak plasma concentration of 0.06 mg/L was reached within 1-2 h [43]. Blood concentrations of about 0.1 mg/L were seen following therapeutic use [59] and toxicity might occur at serum levels above 0.15 mg/L [60]. During the past few years there have been an increasing number of reports about the abuse and misuse of zopiclone. One study showed a prevalent misuse of zopiclone when its degradation product 2-amino-5-chloropyridine in urine samples was detected [61]. The zopiclone distribution in cases of petty drug offences in Sweden in the 2000 to 2009 period is shown in **Table 1**.

Table 1. Concentrations of zopiclone in whole blood samples from cases of petty drug offences in Sweden with zopiclone detected (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

Year	Number of zopiclone cases	Mean value (μg/g)	Median value (μg/g)	Highest value (μg/g)
2000	3	0.03	0.03	0.03
2001	7	0.06	0.03	0.19
2002	12	0.18	0.16	0.50
2003	10	0.08	0.07	0.15
2004	6	0.05	0.05	0.06
2005	15	0.10	0.04	0.50
2006	22	0.12	0.06	0.90
2007	26	0.09	0.06	0.28
2008	28	0.10	0.05	0.70
2009	37	0.08	0.05	0.30

Zopiclone and other sedative hypnotic drugs are detected frequently in cases of people suspected of driving under the influence of drugs [16]. Because of the adverse effects of increased reaction time, which may affect driving performance, the drug is not suitable when skilled tasks are performed. Zopiclone concentrations in blood from drivers over a period of about six years showed that 80% had higher blood concentrations than those expected from therapeutic doses [62]. A connection between road-traffic accidents and zopiclone use has been reported concluding that users of zopiclone should be advised not to drive [63]. The zopiclone distribution in cases of drug-impaired drivers in Sweden in the 2000 to 2009 period is shown in **Table 2**.

Table 2. Concentrations of zopiclone in whole blood samples from cases of drug-impaired drivers in Sweden (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

Year	Number of zopiclone case	Mean value (μg/g)	Median value (μg/g)	Highest value (μg/g)
2000	34	0.10	0.05	0.30
2001	59	0.09	0.07	0.44
2002	55	0.11	0.07	0.53
2003	58	0.11	0.08	0.45
2004	52	0.08	0.04	0.34
2005	59	0.12	0.09	0.41
2006	62	0.11	0.06	0.50
2007	64	0.10	0.07	0.50
2008	89	0.14	0.08	1.0
2009	108	0.10	0.08	0.40

Fatalities resulting from poisoning with zopiclone combined with alcohol or other drugs have also been described [64-66] and cases of fatal zopiclone overdose with zopiclone concentrations of 1.4-3.9 mg/L in the blood have been reported [67]. An overdose after ingestion of 90 mg of zopiclone has shown that this amount could be a minimum lethal zopiclone dose (in femoral blood the zopiclone concentration was 0.254 mg/L) [66]. In Sweden, zopiclone is one of the most frequently identified drugs in post-mortem femoral blood [41]. The zopiclone distribution in forensic autopsy cases in Sweden in the 2000 to 2009 period is shown in **Table 3**.

Table 3. Concentrations of zopiclone in femoral blood samples from forensic autopsy cases in Sweden (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

Year	Number of zopiclone cases	Mean value (μg/g)	Median value (μg/g)	Highest value (μg/g)
2000	157	0.18	0.09	1.6
2001	165	0.29	0.09	3.1
2002	194	0.27	0.08	8.2
2003	200	0.26	0.08	5.6
2004	147	0.27	0.08	4.2
2005	226	0.34	0.08	8.6
2006	228	0.36	0.11	4.7
2007	274	0.28	0.08	13.5
2008	264	0.40	0.10	19.0
2009	277	0.27	0.09	4.7

Analytical methods

Zopiclone is a white to light yellow crystalline solid and is slightly soluble in water, soluble in most organic solvents (e.g. ethanol, acetonitrile, dichlormethane) [44]. Analysis of zopiclone in biological specimens is complicated by its instability in certain solvents as methanol, acid or basic conditions [59,68,69]. Standard solution should be prepared in acetonitrile and extraction must be done at neutral pH to ensure stability [59,70].

Several analytical methods, such as high performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) and liquid chromatography tandem mass spectrometry (LC-MS-MS), have been developed for the quantification of zopiclone in whole blood and plasma [69,71-79]. Some assays (HPLC, LC-MS, LC-MS-MS) have also been developed to separate and determine zopiclone and its metabolites (N-desmethylzopiclone and N-oxide-zopiclone) in urine [71,77,80,81]. One method (HPLC) has been reported to detect zopiclone and its metabolite N-desmethylzopiclone in plasma [78]. Stereo specific methods [LC, capillary electrophoresis (CE), radioimmunoassay (RIA) and HPLC] have been developed to separate the enantiomers [70,82-84]. Two methods (HPLC, GC-MS) for detection of the zopiclone degradation product 2-amino-5-chloropyridine have been described [85,86].

Biological specimens

It has been confirmed that physical processes e.g. temperature have an effect on zopiclone stability and it has been shown that zopiclone undergoes degradation by chemical hydrolysis at basic pH by ring opening and conversion to 2-amino-5-chloropyridine [68,85,86].

Specific long-term stability investigation showed a 21% decrease of the zopiclone concentration in post-mortem femoral blood after storage for twelve months at –20°C [35].

Data on zopiclone stability have also been carried out from stability experiments in connection with method development and validation. *Long-term stability* tests have shown that zopiclone in spiked human plasma is stable for one month [69,78] and for six months [70] when stored at –20°C or lower. *Freeze-thaw stability* tests have indicated zopiclone stability in spiked human plasma for three freeze-thaw cycles [69,78]. *Short-term* or *in process stability* tests have shown no evidence of degradation in plasma quality control samples stored at room temperature for 24 h [78]. No loss of zopiclone could be detected following storage of blood samples (plasma) at 4-8°C for less than 6 h, but the concentration of zopiclone in blood was reduced by 25% and 29% for the (–)- and (+)-enantiomers, respectively, after 20 h at ambient temperature [70]. *Processed sample stability* or sample extract reanalysis has shown that zopiclone is stable for up to 24 h in water-methanol extract [78] but unstable in ethanol extracts [69].

Zopiclone in whole blood is analyzed in forensic toxicology and a detailed stability investigation of zopiclone in this matrix is necessary. Both albumin and α-1-acid glycoprotein are involved in zopiclone protein binding [46]. Protein binding might protect drugs from *in vitro* breakdown, but no relationship between concentration decrease during storage and drug binding has been confirmed [21]. However, considering individual differences in plasma protein concentrations or possible plasma protein binding competition between different drugs *in vitro*, stability studies on authentic samples might be important.

AIMS OF THESIS

The overall aim of this thesis was to investigate the stability of zopiclone in human whole blood and to study stability differences between authentic and spiked samples. Depending on handling, storage and specimen conditions, the zopiclone concentration is likely to change. Since interpretation of zopiclone concentrations in whole blood are important in forensic toxicology, detailed knowledge of the zopiclone stability in whole blood matrices is essential for reliable analysis and interpretation of results.

Specific aims

Paper I

To investigate the stability of zopiclone in human blood during storage under different conditions, stability differences between authentic and spiked blood, freeze-thaw and processed sample stability.

Paper II

To compare stability between authentic and spiked blood samples from the same donor and, in particular, to investigate the influence of short-term pre-analytical storage conditions.

MATERIALS AND METHODS

Study designs

Long- and short-term stability

Pooled human drug-free whole blood was used as matrix for spiked samples and this was obtained from the local blood bank at the University Hospital in Linköping. To 1 g blood, zopiclone was added from stock standard solutions to give target concentrations of 0.2 μg/g (low level) and 0.5 μg/g (high level), respectively. These levels were chosen on the basis of concentrations found in authentic blood samples and being high enough to follow decrease over time. After the initial measurement of five replicates at each level, the remaining tubes were stored at –20°C, 5°C or 20°C. The initially measured mean concentration was used as starting value. To investigate the effect of time and temperature, the zopiclone concentration was measured in duplicate at selected times during twelve months.

Authentic and spiked blood was used to investigate stability differences under the conditions usually encountered in our laboratory. *Authentic* blood samples from nine cases were included, where venous blood specimens had been obtained by medical personnel and sent by the police to the Department of Forensic Genetics and Forensic Toxicology, Linköping, for analysis. Aliquots were reanalyzed after 1, 3, 5 and 8 months of storage at 5°C and the concentration of zopiclone was measured. The first measured concentration was used as starting value. *Spiked blood* samples were prepared to compare with the authentic samples. To 1 g aliquots of drug-free blood, zopiclone standard solution was added at ten different concentrations (0.02, 0.03, 0.06, 0.09, 0.10, 0.15, 0.20, 0.30, 0.40 and 0.50 μg/g) and after initial measurement the spiked samples were stored at 5°C. The initially measured concentration was used as starting value. The zopiclone concentration was measured in duplicate after 1, 3, 5 and 8 months of storage.

Freeze-thaw stability

Aliquots of drug-free blood were spiked in triplicates with zopiclone standard solution to one low concentration (0.02 μg/g) and one high concentration (0.2 μg/g). The samples were analyzed before and after three freeze (20 ± 2 h at –20°C) - thaw cycles (in total less than 2 h at room temperature). Also, authentic blood samples were used. Aliquots from eight samples were analyzed, then frozen at −20°C for 20 ± 2 h, thawed at room temperature for less than 2 h, and reanalyzed. Because of limited authentic material, the authentic samples could only go through one freeze-thaw cycle.

Processed stability

Six samples were reinjected to evaluate processed sample stability of zopiclone concentrations in extracts of butyl-acetate. The extracts were reinjected 21 h (one day) and 45 h (two days) after on-instrument storage at ambient temperature.

Degradation

In addition to the stability investigation, the formation of zopiclone degradation products was investigated in blood samples during storage for 24 h at 37°C. To 1 gram of drug-free blood 0.3 μg/g of zopiclone (0.77 nM) was added and the aliquots were incubated 0, 1, 3, 6, 18, and 24 h before extraction and analysis.

Influence of pre-analytical conditions

Nine healthy drug-free volunteers participated in the study approved by the regional ethics committee in Linköping, Sweden (#M164-08). Before oral administration of a single dose of 10 mg Imovane®, blood samples were drawn in tubes containing EDTA (2 x 3 mL) and tubes containing sodium fluoride and potassium oxalate (6 x 9 mL). After administration, at the estimated peak time of 1.5 h, blood samples were taken in tubes containing sodium fluoride and potassium oxalate (6 x 9 mL).

The EDTA blood samples were directly transported to a local clinical laboratory (at University Hospital, Linköping) for analysis of erythrocyte volume fraction, plasma albumin and plasma α-1-glycoprotein. The pre-dosed blood was pooled individually; then divided into glass tubes and spiked with stock standard solution of zopiclone to give target concentrations of 0.15 μg/g (n = 4) and 0.08 μg/g (n = 5), respectively. The levels were expected to reflect authentic blood levels. The post-dosed blood was pooled individually and aliquoted in glass tubes.

After the initial concentration was measured in triplicate, the remaining tubes were stored at 20°C, 5°C or –20°C. The initially measured mean concentration was used as starting value. Measuring started within 8 ± 1 h after the first sampling. To investigate the effect of time and temperature in authentic and spiked blood, the zopiclone concentration was measured in triplicate at selected times after storage (days at 20°C, weeks at 5°C and months at –20°C).

Ethical considerations

In the first study (Paper I), biological material from forensic cases was used. Data was collected from a laboratory database at the Department of Forensic Genetics and Forensic Toxicology. The samples were reanalyzed and evaluated unidentified with no connection to the cases. In the second study (Paper II), biological material from volunteers was used. Research procedures adhered to obligations to the study participants and the protocol was approved by the Regional Ethics committee, Faculty of Health Sciences, Linköping University, Sweden (# M164-08).

Equipment

Vacutainer tubes and/or VenoSafe tubes (Terumo Europe NV, Leuven, Belgium) containing 100 mg sodium fluoride and 25 mg potassium oxalate as preservatives and Vacutainer tubes (BD Vacutainer, Plymouth, United Kingdom) containing EDTA were used for blood sample collection. Aliquots of 1 g of whole blood were stored in glass tubes (DURAN®, Mainz, Germany). Eppendorf pipettes (accuracy and precision controlled each four months) were used. Blood samples were weighed on a Sartorius LC421 scale (calibrated once

a year and controlled in house each month). Hettich (Universal 30 RF) centrifuge was used for centrifugation (calibrated once a year).

Chemicals and solutions

Acetonitrile, methanol, n-butyl acetate, methyl-*tert*butylether of analytical grade came from Merck (Darmstadt, Germany). Hydrochloric acid, formic acid, sodium hydroxide, tris(hydroxymethyl)aminomethane, potassium dihydrogen phosphate and dipotassium hydrogen phosphate * 3 H_2O were supplied by Merck (Darmstadt, Germany) and ammonium formiate by Sigma Aldrich (Steinheim, Germany). The 0.5 M phosphate buffer pH 7.0 used in extraction was prepared by mixing a 0.5 M dipotassium hydrogen phosphate solution with 0.5 M potassium dihydrogen phosphate solution to pH 7.0. The 1 M trisbuffer was adjusted to pH 11. Reference solutions (stock standard solutions) were prepared in acetonitrile with certified zopiclone (Sigma-Aldrich, St. Louis, MI, USA) to concentrations of 0.1 mg/mL, 0.01 mg/mL, 1 μg/mL and 0.1 μg/mL. The reference solutions were stored at -20°C with a tested stability of at least 12 months. Prazepam (Sigma-Aldrich, St. Louis, MI, USA) was prepared in methanol and used as internal standard (IS) with a concentration of 0.01 mg/mL. The breakdown product 2-amino-5-chloropyridine was obtained from Sigma-Aldrich (Steinheim, Germany). Deionized water was prepared on a Direct Q laboratory plant (Millipore).

Analytical methods

Gas chromatography

The measurement principle GC-NPD was used for zopiclone quantification.

To 1.0 g of the sample 30 μL internal standard (prazepam 0.01 mg/mL) and 0.5 mL 0.5 M phosphate buffer pH 7.0 were added. Extraction was made with 0.3 mL butyl-acetate for 10 minutes and after phase separation by centrifugation (5000 rpm), the organic extract was transferred to a sampler vial and analyzed by GC-NPD.

GC-NPD analyses were performed on a Hewlett Packard (HP) 5890 GC (Waldbronn, Germany). 5 μL aliquots of the extract were injected with

automatic injector HP 7634A at 250°C into an analytical column, DB-5 capillary column 15 m by 0.25 mm ID and 0.25 μm thickness (J&W, Folsom, CA, USA). The initial oven temperature was 200°C and was then programmed to 300°C at a rate of 25°C /min, held at 300°C for 4 min before an increase to 320°C. The carrier gas was helium with a constant pressure. The detector temperature was 320°C. Results were evaluated using Chromeleon version 6.7 (Dionex Corparation, Sunnyvale, CA, USA). Example of a typical chromatogram is shown in **Fig. 3**.

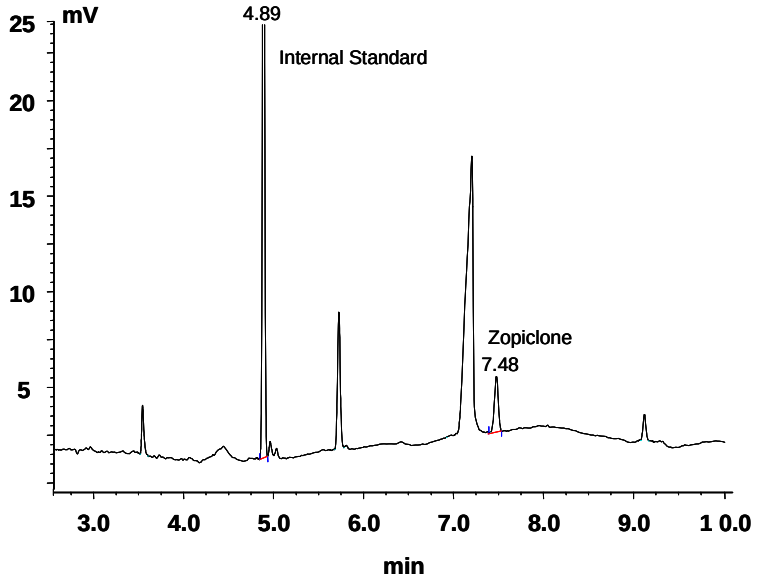


Fig. 3. Example of chromatogram from blood extract of a control sample at low level 0.02 μg/g. Internal standard is prazepam (4.89) and the peak before zopiclone (7.48) is cholesterol.

This GC-NPD assay is routinely used and method validation was performed before the stability investigations. Calibration curves were made by adding zopiclone reference solution at five different concentrations (0.01, 0.05, 0.2, 0.5 and 1 μg/g) to drug-free fresh whole blood and analyzed in duplicate. Linear calibration curves were obtained in the range of 0.01 to 2 μg/g. Precision was expressed as the percentage coefficient of variation (%CV) of measurements. Inter- and intraday variability were determined at two levels (0.02 and 0.50 μg/g). Intra-day variability (n = 5) was 6.1%CV and 4.4%CV and inter-day variability (n = 45) was 26.6%CV and 24.4%CV for the low and high level, respectively. The limit of detection (LOD) was 0.007 μg/g and the limit of

quantification (LOQ) was 0.02 μg/g. Recovery in the assay was 51%. Zopiclone metabolites and degradation products were not included in this method.

Liquid chromatography

To identify and quantify zopiclone breakdown products a brief experiment was performed. After the incubation at 37°C, 0.5 mL 1 M trisbuffer was added to the samples. Extraction was made with methyl-*tert*butylether for 10 minutes and after phase separation by centrifugation (5000 rpm), the organic extract was removed and evaporated with nitrogen. The residue was reconstituted in 0.2 mL of a 50% solvent mixture of A and B (see below) and analyzed by LC-MS-MS.

The LC/MS/MS system consisted of a Waters ACQUITY UPLC® (Ultra Performance LC) with a Binary Solvent Manager, Sample Manager, and Column Manager (Waters Co., Milford, MA, USA) connected to an API 4000™ triple quadrupole instrument (Applied Biosystems/MDS Sciex, Stockholm, Sweden) equipped with an electrospray interface (TURBO V™ source, TurboIonSpray® probe) operating in the MRM mode.

The acquisition method included two transitions for zopiclone (389.1/217.0 and 389.1/245.1), N-desmethyl-zopiclone (375.1/217.1 and 375.1/245.0), N-oxide-zopiclone (405.2/143.1 and 405.2/245.0), and 2-amino-5-chloropyridine (128.9/112.1 and 128.9/76.1). Ultra Performance Liquid Chromatography (UPLC®) was performed using a 1.7 μm, 50 × 2.1 mm ACQUITY UPLC® ethylene bridged hybrid (BEH) C_{18} column (Waters Co.), operated in gradient mode at 0.6 mL/min with a total run time of 3 min. Solvent A consisted of 0.05% formic acid in 10 mM ammonium formiate and Solvent B of 0.05% formic acid in acetonitrile. External calibration curves were used for quantitation.

Quality controls

Quality controls at two levels, low (0.02 μg/g) and high (0.50 μg/g) were extracted and measured together with each batch of samples in the long- and short-term stability investigation (GC-NPD). Quality control samples at three levels, negative, low (0.02 μg/g) and high (0.20 μg/g), were used in the same way in the freeze-thaw and in the processed stability investigations as well as in the study of the influence of pre-analytical conditions. The quality control samples were freshly prepared before each assay by spiking drug-free blood

matrix with the reference standard solution at each concentration. Method precision (random error, variation) and accuracy (systematic error, mean bias) were evaluated for these quality control samples.

Clinical chemical analysis

The erythrocyte volume fraction, plasma albumin and plasma α-1-glycoprotein were analyzed at the clinical laboratory at University Hospital, Linköping. Erythrocyte volume fraction (EVF) was measured by impedance technique (Cell-Dyn Sapphire, Abbot Laboratories, Saint-Laurent, Québec, Canada), plasma albumin and plasma α-1-glycoprotein were analyzed by immuno chemical technique (ADVIA 1800, Siemens, Deerfield, IL, USA and BN ProSpec, Siemens, Deerfield, IL, USA).

Statistical analysis

The descriptive statistics used were means and standard deviation. Wilcoxon Signed Ranks Test was used, when comparison between the different storage times was evaluated. When differences between authentic and spiked samples were evaluated, Mann-Whitney *U*-test was used. A probability of less than 5% ($p < 0.05$) was regarded as significant. The statistical analysis was performed using SPSS 16.0 for Windows in Paper I and SPSS Statistics 17.0 in Paper II. In paper II the stability was also evaluated by using a lower acceptance limit (initial value – 2 x SD of method). The ± 2 standard deviations (SD) are a common limit for accepting batch controls in a series of samples with a 95% confidence.

RESULTS

Paper I

Long-term and short-term stability

Effect of time and temperature

Long- and short-term stability was investigated at two concentrations of zopiclone and at three different storage conditions. A significant difference between samples stored at –20°C, 5°C or 20°C was found ($p < 0.05$). The results from the higher concentration (0.59 μg/g) and the lower concentration (0.23 μg/g) showed the same degradation profile with no significant differences. Data from samples at the high concentration (0.59 μg/g) are depicted in **Fig. 4** showing that zopiclone is most stable at –20°C. After five months 74-88% of the initial zopiclone concentration was left of the low and high levels, respectively. However, after six months of storage some decrease in zopiclone concentration was noticed which reached a 36-52% decrease in the end of studied period. In samples stored at 5°C degradation of zopiclone was seen already after a few weeks. After four weeks, the residue was 42-61%, after three months 12-13%, and after five months there was no measurable concentration left. At a storage temperature of 20°C, the concentration decreased more than 50% after the first three days of storage.

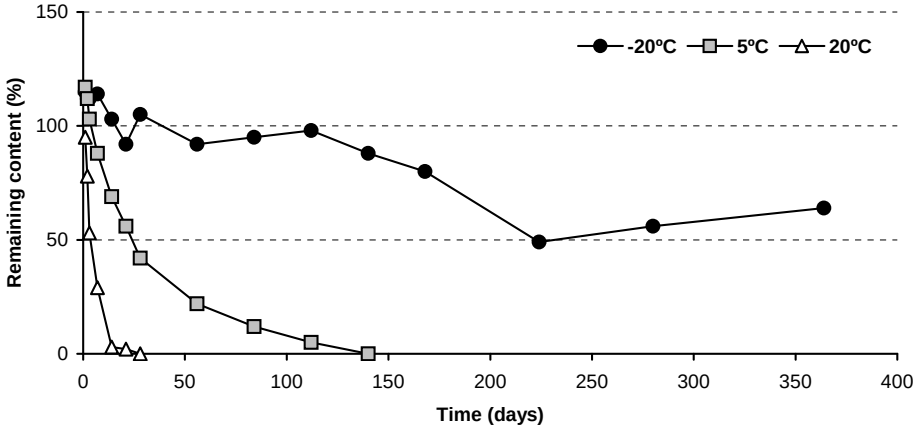


Fig. 4. Effect of time and storage conditions in whole blood containing 0.59 μg/g of zopiclone (high level).

Authentic and spiked stability samples

As regards long-term stability, authentic and spiked samples were compared during storage at 5°C. Zopiclone degradation over time was seen in both authentic and spiked blood samples stored at 5°C (**Fig. 5**).

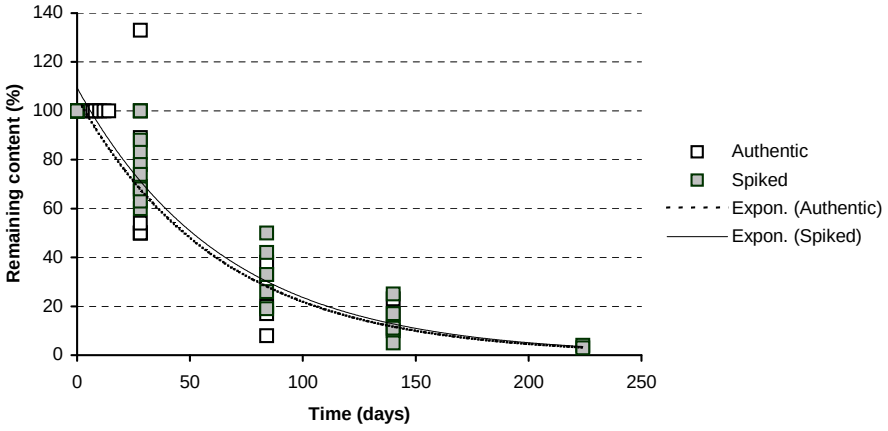


Fig. 5. Authentic and spiked samples (at levels between 0.01-0.49 μg/g) during eight months of storage at 5°C. Within the authentic group, storage before first measurement varied between 2-14 days. For all the spiked samples first measurement was at day zero.

A significant difference between the initial content and the residue was noticed already after one month ($p < 0.05$) in the spiked group as well as in the authentic group (**Table 4**). Mann-Whitney *U*-test showed no significant differences between the two groups, but within both of the groups the remaining content varied. Additionally, in the authentic group the first measured concentration was used as starting value while the initially measured concentration was used as starting value in the spiked group (**Fig. 5**).

Table 4. A compilation of zopiclone remaining content in authentic and spiked human blood samples during storage for 8 months at 5°C.

Material	Initial content (%)	Mean remaining content (%) and (range) after storage in months			
		1	3	5	8
Authentic (n = 9)*	100	75** (50-133)	13** (0-38)	6** (0-23)	0** 0
Spiked (n = 10)	100	77** (55-100)	19** (0-50)	8** (0-25)	0.9** (0-4)

*Other drugs, e.g. alprazolam, carisoprodol, citalopram, codeine, diazepam, dihydropropiomazine, ephedrine, ethanol, lamotrigine, levomepromazine, meprobamate, nordazepam, oxazepam and/or tramadol were present in eight of the nine authentic samples. Within the authentic group, storage before first measurement varied between 2-14 days.
**$p < 0.05$

Freeze-thaw stability

Freeze-thaw stability of zopiclone in whole blood was evaluated in both authentic and spiked samples. There were no differences in zopiclone concentration before and after the freeze-thaw cycle for any of the eight authentic samples with a zopiclone concentration ranging from 0.03 to 0.15 μg/g. In the spiked samples, there were no differences at the low level (0.02 μg/g) after three freeze-thaw cycles, but some decrease (8%) at the high level (0.20 μg/g) was noticed after three cycles.

Processed stability

After storage on the analytical instrument during two days at ambient temperature, no evidence of degradation was seen in the processed sample stability test of the sample extract reanalysis.

Degradation products

The zopiclone degradation was investigated in spiked samples during 24 h incubation at 37°C. In these samples the zopiclone metabolites N-desmethyl-zopiclone and N-oxide-zopiclone were not detected. However, 2-amino-5-chloropyridine was formed in approximately equimolar amounts to zopiclone decay (**Fig. 6**).

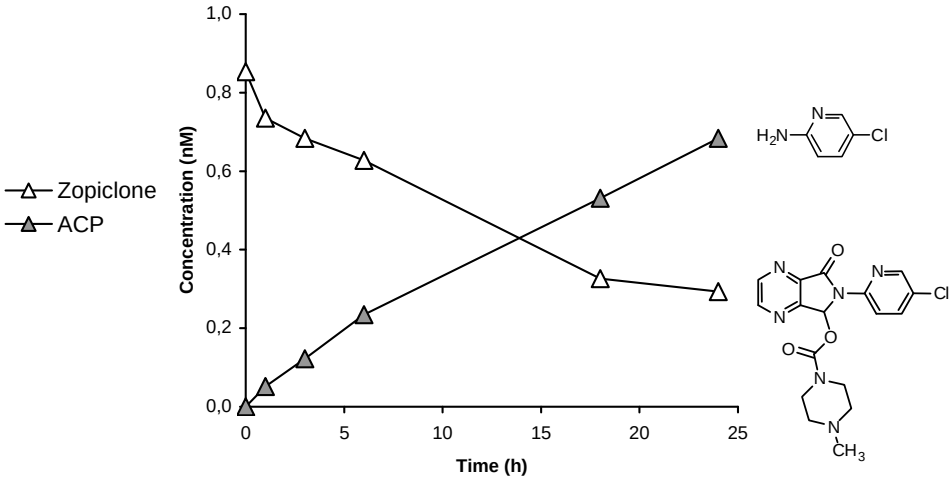


Fig. 6. Degradation of zopiclone and formation of 2-amino-5-chloropyridine (ACP) in spiked whole blood at 37°C.

Quality control samples

Results from the quality control samples during short- and long-term stability investigation in Paper I are shown in **Table 5**.

Table 5. The descriptive statistics for the quality controls in the long- and short-term zopiclone stability investigation: a) spiked blood (during 12 months) and b) authentic and spiked blood (during 8 months). L = low level and H = high level.

Quality Control	Theoretical value (μg/g)	Mean value (μg/g)	SEM	SD	Precision CV%	Mean Bias	Accuracy %
a) L (n = 75)	0.02	0.014	0.0005	0.004	27	-0.006	70
a) H (n = 75)	0.50	0.444	0.011	0.092	21	-0.056	89
b) L (n = 48)	0.02	0.015	0.0004	0.003	20	-0.005	75
b) H (n = 48)	0.05	0.440	0.011	0.076	17	-0.060	88

Paper II

Authentic and spiked stability samples

Stability differences between authentic and spiked blood samples from the same donor were compared.

The spiked levels were intended to reflect those in the authentic samples. However, the dosed subjects presented with zopiclone blood levels lower than expected and therefore four of the spiked samples were excluded (spiked at 0.15 μg/g). Zopiclone concentrations in the authentic samples 90 minutes after oral administration varied between 0.017–0.060 μg/g (n = 9) and the target concentration in the spiked samples was 0.08 μg/g (n = 5). The measured concentration at the time of spiking ranged between 0.064-0.083 μg/g.

The remaining content of zopiclone in authentic and spiked blood showed the same decreasing trends at 20°C and 5°C as depicted in **Fig. 7**.

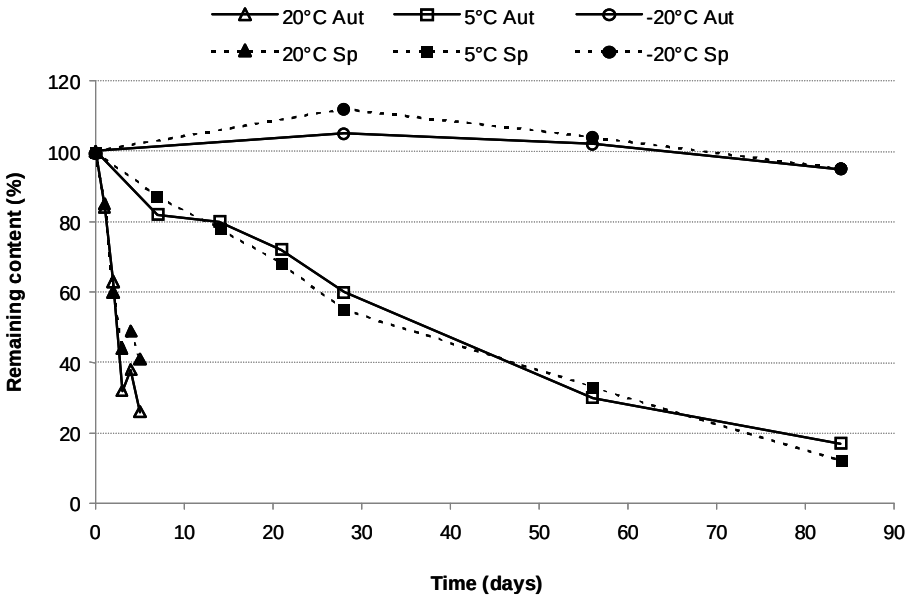


Fig. 7. Influence of storage conditions on zopiclone in authentic whole blood samples for nine subjects and in spiked whole blood samples for five subjects (mean values) during a period of 5 days at 20°C, 84 days at 5°C and at -20°C.

Mann-Whitney *U*-Test showed no significant differences for any of the five individually compared subjects when authentic and spiked differences were evaluated. This corresponded to all the storage conditions. Erythrocyte volume fraction was normal and plasma protein concentrations were normal for all subjects except for two. P-α-1-glycoprotein was in these cases slightly below the low reference level at 0.52 g/L, but no influence from this discrepancy was noted. However, differences in initial concentration as well as in the rate of concentration decrease over time were observed between the subjects.

A rapid concentration decrease and a significant difference in zopiclone content was seen already after 1 day of storage at 20°C in the authentic blood and after 2 days of storage in the spiked blood. At 5°C there was a significant difference in remaining content after 2 weeks in the authentic blood and in the spiked blood ($p < 0.05$). After storage at low temperature (–20°C) there were no differences in the authentic blood or in the spiked blood during the observed period. **Table 6** shows the rapid decrease in concentration at 20°C and at 5°C, respectively.

Table 6. Summary of remaining content of zopiclone after short- and long-term storage at 20°C and at 5°C.

Material	Initial content (%)	Mean remaining content (%) at 20°C After days of storage					Mean remaining content (%) at 5°C After weeks of storage					
		1	2	3	4	5	1	2	3	4	8	12
Authentic (n = 9)	100	84*	63*	32*	38*	25*	82	80*	72*	60*	30*	17*
Spiked (n = 5)	100	85	60*	44*	49*	40*	87	78*	68*	55*	33*	12*

*$p < 0.05$

Quality control samples

Results from the quality control samples during stability investigation in Paper II are shown in **Table 7**.

Table 7. The descriptive statistics for the quality controls in the authentic and spiked zopiclone stability investigation during 3 months of storage.

Quality control	Theoretical value (μg/g)	Mean value (μg/g)	SEM	SD	Precision CV%	Mean Bias	Accuracy %
Low level (n = 36)	0.020	0.021	0.0003	0.002	9	0.001	105
High level (n = 36)	0.200	0.233	0.004	0.026	11	0.033	117

GENERAL DISCUSSION

Stability investigations

These investigations have shown the importance of knowledge about drug stability in biological samples. The aim of the studies was to investigate and to compare stability of zopiclone in spiked and authentic whole blood.

In connection with sampling, transport, registration and aliquoting bio-samples are kept at ambient temperature. Stability investigations of analytes in samples stored under realistic conditions are therefore of great value. Short-term stability investigation of zopiclone in spiked whole blood showed a very poor stability during storage at 20°C. A decrease of more than 50% was noticed after three days of storage; first (Paper I) for spiked samples at high zopiclone concentrations (0.23 and 0.59 μg/g), second (Paper II) for spiked samples (0.06-0.08 μg/g) and for authentic samples (0.02-0.06 μg/g) at low concentrations. Stability of zopiclone in spiked and authentic whole blood according to the second investigation (Paper II) was approximately one day. This short stability at 20°C means that sampling, transport, registration and preparation steps have to be done within 24 h or at a controlled lower temperature. Many samples are kept overnight at ambient temperature in connection with transport and at least a few hours at room temperature in connection with registration. This will affect the measured concentration. Handling and storage conditions of bio-samples must be considered when analytical results are interpreted [15] and since interpretations of zopiclone concentrations in whole blood are used in forensic toxicology (**Table 1** and **Table 2**) the analytical results have to be carefully interpreted.

Before and after analysis, bio-samples usually are stored at refrigerator temperature (5°C) and long-term stability investigation at this temperature was needed. In spiked samples (Paper I) at two different zopiclone concentrations (0.23 and 0.59 μg/g) stored at 5°C, degradation of zopiclone was seen already after a few weeks. After four weeks, the residue was 42-61% of the starting concentration, after three months 12-13% and after five months there was no measurable concentration left (**Fig. 4**). In spiked samples and in authentic samples (Paper I) at different zopiclone concentrations (0.01-0.49 μg/g) a significant difference between the initial content and the residue was

noticed after one month of storage (**Table 4**). The mean remaining content after this time was 75% and 77% for the authentic and spiked samples, respectively. However, among the samples individual differences existed and for some samples the residue was only 50-55% at this time. After three months 13-19% of the first measured zopiclone concentration remained. After five months 6-8% was left, but samples with no measureable concentration were also seen. By weekly testing (Paper II) of spiked (0.06-0.08 μg/g) and authentic blood samples (0.02-0.06 μg/g) the stability was estimated to less than two weeks. After four weeks the mean remaining content was 55% and 60% for the spiked and authentic samples, respectively. After 12 weeks 12-17% remained (**Table 6**). The one-week stability at 5°C means that analysis should be carried out within this time. Laboratory routines should be reviewed if samples are kept at this temperature for a long time, because after five to eight months of storage it might not be possible to show if zopiclone was present in the first place or not.

Zopiclone results in forensic cases have to be interpreted with caution if the sample has been stored at 5°C for a long time *and/or* if the sample has been stored at room temperature 24 hours or longer in the pre-analytical phase.

Long-term stability was also investigated at freezer temperature at –20°C. It is a common temperature used for long-term storage of bio-samples after the analysis is made. Stability investigation at this condition is important if a reanalysis is made. Results from the investigations (Paper I-II) showed that zopiclone is most stable at –20°C. After five months 74-88% was left of the low and high spiked levels, respectively (Paper I) and in authentic and spiked samples at different concentrations (Paper II) no evidence of degradation was noticed during the studied period of three months.

Knowledge of freeze-thaw stability applies in cases of reanalysis of frozen and thawed bio-samples. Freeze-thaw stability was therefore tested for zopiclone in spiked and in authentic whole blood (Paper I). Results from the freeze-thaw cycles showed good stability through the tested cycles. Considering this, it is possible to keep samples at –20°C even before analysis. If the analysis cannot be done within a week, the samples should be frozen and zopiclone stability can be extended to at least three months.

In routine laboratory work, sometimes problems originate during analysis. Knowledge of processed stability (extracted samples) is therefore useful in cases of delayed injection owing to unforeseen circumstances e.g. instrument problems or technical errors. The stability investigation (Paper I) included this type of test and showed that zopiclone in sample extracts of butyl acetate were

stable for two days when stored at ambient temperature, which should be sufficient to enable reinjections of processed samples.

The results from the various investigations of zopiclone in whole blood during different storage conditions agreed well. These results also agree with earlier stability tests of zopiclone in plasma [69,70,78]. However, zopiclone spiked-samples decreased more in concentration after 12 months of storage at –20°C compared to authentic post-mortem femoral blood [35]. This is very interesting, because pH of post-mortem blood tends to be acidic, whereas alkaline shift takes place in fresh blood after storage at 4°C and at 20°C [25]. For zopiclone degradation, pH has been reported as one important factor [68]. It has also been shown that pH values in whole blood do not change within a storage period of six months at –20°C [25]. This might correspond to the best zopiclone stability in whole blood. The results from the freeze-thaw stability tests were in agreement with previous findings in plasma [69,78]. Processed stability tests of zopiclone in butyl-acetate could not be compared with prior tests, but stability tests have shown that zopiclone is unstable in ethanol extracts [69] but stable in water-methanol extracts [78]. This shows the importance of this study type and additionally, this test can be useful for choosing extract solvent, when zopiclone analysis is transferred from one measurement principle to another.

In forensic cases it is not uncommon that more than one drug is present in the blood samples. Because of differences in plasma protein concentrations or possible plasma protein binding rivalry between drugs *in vitro*, stability studies on authentic samples were included in the studies (Paper I-II). Zopiclone stability was compared between authentic and spiked samples in whole blood. In the first study (Paper I) no conclusion could be drawn, because of unknown initial concentration and differences in storage condition for the authentic samples **(Fig. 5)**. Additionally, plasma protein concentrations were unknown for these samples and other drugs were present in eight of nine cases **(Table 4)**. The second study was performed to test the stability of zopiclone in regard to individual protein binding effects and to compare differences in stability between spiked and authentic human blood from the same donor. The subjects in this study were healthy young people between 22-32 years old with no other medications prescribed, with normal erythrocyte volume fraction and normal plasma protein concentrations. The results showed no stability differences between authentic and spiked whole blood from the same donor (Paper II) and the stability at 5°C from the first study (Paper I) was confirmed. No connection between plasma protein concentrations and stability was noticed.

Paper I verified that zopiclone in blood undergoes degradation to 2-amino-5-chloropyridine during storage [85]. The *in vivo* metabolites N-desmethyl-zopiclone and N-oxide-zopiclone were not found in the spiked whole blood. The measurement of 2-amino-5-chloropyridine may be an additional help to the toxicologist when interpreting results.

Pre-analytical aspects

These studies have demonstrated the importance of controlled pre-analytical conditions after sampling and before analysis. In the light of the short stability of zopiclone at ambient temperature, the laboratory should ensure stability by control of temperature during transportation to the laboratory. These results may very well be extrapolated to other drugs and medications that are known to be unstable. Therefore laboratories should perform short-term stability studies to mimic transportation, registration and aliquoting prior to analysis. If not, the concentrations measured will not reflect those present in the blood at the time of sampling and the interpretation will be misleading.

Storage stability is only one pre-analytical factor to be considered when interpreting analytical results. The same dose of Imovane® was given and blood drawn at the same time for all the subjects, but zopiclone concentrations ranged between from 0.017-0.060 μg/g (Paper II). This was most likely explained by differences in zopiclone uptake and distribution between the subjects. The subjects in this study were young people with no diagnose of diseases (e.g. reduced liver or kidney function). However, this study was not a pharmacokinetic study and only parameters that might influence zopiclone *in vitro* were controlled. Anyway, physiological factors as well as genetic and pharmacological factors also need to be considered e.g. the pharmacokinetic drug profile, polymorphism of drug metabolizing enzymes or drug chirality.

Methodological aspects

The stratification in these studies is based on prior knowledge about stability study designs. The stability experiments have been designed for forensic requirements. These stability investigations of zopiclone were evaluated in the same matrix and under storage conditions that are routinely encountered in forensic laboratories. This means that the results from these studies can be applied in practice.

During theses experiment new knowledge about designed stability test was established. Stability studies of an analyte have to be done in each matrix and stability data should not be extrapolated between different matrices [19]. Previous data about the stability of zopiclone in plasma [69,70,78] and post-mortem femoral blood exist [35]. The studies in this thesis expand on earlier work for fresh whole blood. However, confounding factors might occur in pooled matrix and the pooled blood matrix used to spiked blood might affect the results (Paper I). To exclude all differences arising from use of the pooled matrix, experiments should be performed using an identical biological matrix from a single person. This was done in the second study (Paper II).

Stability investigations using samples from dosed subjects are recommended as most appropriate [19], but in that way only one low concentration level will be evaluated. Usually one low and one high level should be tested [19] or levels depending on quantitation range of the analytical method [13]. In these studies both low and high levels in the range between 0.01-0.59 μg/g were obtained, which included both therapeutic (Paper II) and toxic levels from forensic cases (Paper I) as well as low and high levels according to the analytical method.

Duplicates were measured at each selected time interval in the first study (spiked blood samples) and differences were sometimes observed between the results (Paper I). Triplicates were therefore measured in the second study (Paper II). With several replicated measurements and with randomization confounders are minimized. All aliquots should be coded so they have the same probability of being included in a certain order. In these studies the randomization was not done optimally. The differences between initial value and first day value in the stability investigation might be caused by poor randomization (**Fig. 4**) (Paper I). Another possible factor when evaluating differences between the duplicates and/or triplicates in spiked samples; might be random error originating from adding zopiclone standard solution to the aliquots of 1 g blood matrix. The optimal way would have been to add the solution to the whole blood pool, but it might cause other problems, such as lack of homogeneity and deposit from acetonitrile. By increasing replicates and sample size random error will be decreased.

Because of thermo-instability, GC analysis is not the most optimal measurement principle for drugs like zopiclone [59]. However the quality controls indicate when something is going wrong during the analysis and at each time in these studies one control was measured before and one after the samples. Between-study difference in method precision (%CV) for zopiclone was observed (**Table 5** and **Table 7**). During the 12 months stability

investigation (Paper I) the %CV of the low-level quality control sample was 27%. This is fairly high and may account for some of the variation seen between measurements e.g. during storage at –20°C (**Fig. 4**). However, the results for three of the studied months were repeated and results were confirmed (Paper II). Repeated calibrations influence both precision and accuracy. The bias from calibration could not be excluded in theses stability tests and the agreement between measured mean concentrations to theoretical concentration (accuracy) varied between 70-117% (**Table 5** and **Table 7**). By analyzing freshly prepared or frozen control samples (reference samples) and testing stability at the same time, the measured concentrations will be compared from the same calibration curve [12,13,19].

Stability calculations based on statistical tests are sometimes used, but defined acceptance criteria for stability is to preferred if concentration differences are small or if the precision of the analytical method is poor [15]. Intra-analytical processing errors (variability errors and bias errors) have to be considered in the calculations [19]. In this study the stability was evaluated both statistically and by acceptance criteria connected to intra-analytical variations. The nonparametric Wilcoxon Signed Ranks Test and Mann-Whitney *U*-test were used because of outliers and not normally distributed results. Evaluation by acceptance criteria gave the same results and confirmed the stability results.

Considering stability, these studies have not indicated any differences between authentic and spiked whole blood (Paper II). This finding indicated that spiked blood is a good alternative to authentic samples in stability studies. By using spiked samples it is possible to increase replicates, freeze the control/reference samples (if the analyte is stable through freeze-thaw) and perform analysis under the same analytical conditions. This should minimize variability and bias errors.

CONCLUDING REMARKS

Paper I

- This stability investigation showed that zopiclone degrades in human blood depending on time and temperature and might not be detectable after long-term storage.

- The best storage temperature was –20°C even at short storage times, because freeze-thaw had no influence on the results.

- The rapid degradation of zopiclone at ambient temperature can cause an underestimation of the true concentration and consequently flaw the analytical interpretation.

- Identification of 2-amino-5-chloropyridine verified that zopiclone undergoes degradation by chemical hydrolysis in whole blood during storage.

Paper II

- Degradation of zopiclone in authentic blood was about the same as that in spiked blood at the temperatures and times studied. Stability investigation by using spiked blood is preferable to authentic blood, since it is easier to control specimen composition and to increase the number of replicates for reliable stability evaluations.

- In the light of the poor stability of zopiclone at ambient temperature, the laboratory should ensure stability by careful control of temperature during storage in the pre-analytical phase.

FUTURE PERSPECTIVES

- In this thesis, the focus has been on zopiclone stability in whole blood. The identification of the product 2-amino-5-chloropyridine verified the degradation of zopiclone during storage. The measurement of 2-amino-5-chloropyridine may be an additional help to the toxicologist when interpreting results. Therefore the connection between zopiclone degradation and 2-amino-5-chloropyridine formation should be further investigated.

- 2-amino-5-chloro-pyridine can probably be formed also from the *in vivo* metabolites, N-desmethylzopiclone and N-oxide-zopiclone. Therefore, studies on their stability would also prove useful.

- Zopiclone is now marketed as a single enantiomer, which raises interpretive issues when using an achiral method of analysis. An enantiospecific method for quantitation of zopiclone in whole blood should be developed. In connection with method validation tests should be included that will allow comparing the stability of the enantiomers.

- The chemical breakdown of drugs might be influenced by the pH of the biological matrix, which changes during storage. The influence of pH needs to be investigated in future studies.

ACKNOWLEDGEMENTS

This thesis work was performed at National Board of Forensic Medicine, Department of Forensic Genetics and Forensic Toxicology with encouragement and support from many colleagues. Especially I would like to thank:

Professor Johan Ahlner, Head of the Department of Forensic Genetics and Forensic Toxicology, my first main supervisor and then my co-supervisor. Thank you for making this thesis possible, for supporting the postgraduate studies and for your great enthusiasm guiding me during this work.

Associate Professor Robert Kronstrand, my main supervisor and mentor. Thank you for sharing your great knowledge in forensic toxicology, in forensic science and research, for your constant encouragement and for your professional guidance to complete this thesis.

Associate Professor Fredrik C. Kugelberg, my co-supervisor. Thank you for your great knowledge in forensic toxicology and for your endless support, advice and encouragement in science and research.

Professor Wayne Jones, thank you for your great interest in this thesis, for advice, valuable comments and for proof reading the text.

Andreas Tillmar, Anna-Lena Zackrisson and Tor Seldén, my colleagues and PhD-student friends, thanks for valuable discussions in scientific methodology, statistics and practical issues.

Gunilla Thelander and Markus Roman, thanks for sharing your technical knowledge and expertise in forensic measurement principles and for your technical assistance.

Ingrid Nyström, thanks for your great knowledge about planning and carrying out research trials, for collaboration and technical assistance and for many interesting discussions.

Maria Dolores Chermá Yeste, thanks for your medical expertise and care of the subjects in the human subject study and Ingela Jakobsson for all your help.

The staff at the Department of Forensic Genetics and Forensic Toxicology; Anita H. for data collection, Malin F. for taking time with the subjects in the human study, the GC-team, my colleagues in the quality group and my team members for various help and support.

My mentor Simin Mohseni, my student friends Monica S., Marie D. and Maria E., for an instructive time in the Biomedical Laboratory Science degree project.

I will also thank my sisters Gördis and Ulla-Carin and most of all my sons and daughters Fredrik, Johanna, Emelie, Emanuel and Ida, for continuous love and support.

This work was supported by The National Board of Forensic Medicine and by The Centre of Forensic Science (CFV) Linköping University.

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