Linköping University Medical Dissertation
No. 1395

Zopiclone degradation in biological samples

Characteristics and consequences in forensic toxicology

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Linköping 2014

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ISBN 978-91-7519-397-7
ISSN 0345-0082

Printed in Sweden by LiU-Tryck, Linköping, 2014

Cover illustration: Johanna Höglund

If you have knowledge,
let others light their candles with it.

Margaret Fuller (1810-1850)

❖

"In connection with a legal trial after a traffic accident, the suspected drug-impaired driver claimed that she/he had been drugged with zopiclone. Eight months had passed since traffic incident and blood sample collection. When the police requested zopiclone analysis and the sample was reanalyzed after eight months of storage at 4°C, no measurable zopiclone was found in the sample".

❖

"A woman 37 years old was found walking around the streets confused and absent minded, however not perceived as drunk. She was taken to the hospital and upon questioning she recalled being at a party but had a memory lapse of three hours. It was a suspected case of drug-facilitated sexual assault. A urine sample was obtained approximately 11 hours after the assault and stored at 4°C during two months during routine analysis and then stored at –20°C during about one month before analysis. Zopiclone and its metabolites were not detected, but instead high concentrations of 2-amino-5-chloropyridine were found in the urine sample with pH>8.2".

❖

"A woman 51 years old who reported a rape and a urine sample was obtained less than 24 hours after the assault. No other information about the case was available. In this case, the urine sample was stored one week at 4°C after arrival at the laboratory prior to analysis. 2-amino-5-chloropyridine was not detected, whereas zopiclone and its metabolites could be quantified in the urine sample with pH<6.5".

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CONTENTS

ABSTRACT

Bio-analytical results are influenced by *in vivo* factors such as genetics, pharmacological and physiological conditions and *in vitro* factors such as specimen composition, sample additives and storage conditions. Zopiclone (ZOP) is a short-acting hypnotic drug (Imovane®) used for treatment of insomnia. ZOP is metabolized by three major pathways; oxidation to the active zopiclone N-oxide (ZOPNO), demethylation to the inactive N-desmethylzopiclone (NDZOP) and oxidative decarboxylation to other inactive metabolites. ZOP is increasingly being encountered in forensic cases and is a common finding in samples from drug-impaired drivers, users of illicit recreational drugs, victims of drug facilitated sexual assaults and forensic autopsy cases. ZOP is a notoriously unstable analyte in biological matrices and analytical results depend on pre-analytical factors, such as storage time and temperature. The overall aim of this thesis was to investigate the stability of ZOP and the factors of importance for degradation during storage in biological samples and to identify consequences for interpretation of results in forensic toxicology.

In paper I, different stability tests in spiked samples were performed including short-term, long-term, freeze-thaw and processed stability. Analyses of ZOP were performed by gas chromatography with nitrogen phosphorous detection and ZOP concentrations were measured at selected time intervals. The degradation product 2-amino-5-chloropyridine (ACP) was identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The stability investigations showed a very poor short-term storage stability of ZOP.

Therefore, in paper II, the influence of pre-analytical conditions was further investigated in dosed subjects. Whole blood from volunteers was obtained before and after oral administration of Imovane®. In this study, the influence from physiological factors such as drug interactions, matrix composition and plasma protein levels were minimized. The results showed that ZOP was stable in whole blood for only one day at room temperature, one week in a refrigerator and at least three months frozen in authentic as well as in spiked whole blood. The rapid degradation of ZOP at ambient temperature can cause an underestimation of the true concentration and consequently flaw the interpretation. However, by also analyzing the degradation product ACP the original concentration of ZOP may be estimated.

In papers III and IV, two LC-MS-MS methods were validated for the quantitation of ACP, ZOP and NDZOP in blood and ACP, ZOP, NDZOP and ZOPNO in urine. These methods were used in a controlled pharmacokinetic study where whole blood and urine were obtained after oral administration of Imovane®. Samples of blood and urine were aliquoted, analyzed and stored under different conditions and the formation of ACP was monitored. Additionally, at each studied time point the pH of the blood and urine samples was measured using i-STAT® system. The results showed that ACP was formed in equimolar amounts to the degradation of ZOP and its metabolites.

In urine samples, the formation of ACP occurred at elevated pH or temperature and mirrored the degradation of ZOP, NDZOP and ZOPNO. The high concentrations of metabolites, which also degraded to ACP, made it impossible to estimate the original ZOP concentration.

The results from analysis of blood samples containing ACP were also used to develop mathematical models to estimate the original ZOP concentration. Both models showed strong correlation to the original ZOP concentration ($r=0.960$ and $r=0.955$) with $p<0.01$. This study showed that the equimolar degradation of ZOP and NDZOP to ACP could be used to estimate the original concentration of ZOP in blood samples.

Absence of ACP in the blood or urine samples analysed strongly suggests that degradation has not occurred and that the measured concentration of ZOP is reliable. For

proper interpretation in forensic cases, it is strongly recommended that ZOP and its metabolites as well as ACP are included in the analysis.

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. 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. Kunskap om stabiliteten hos kemiska föreningar i biologiska prover under förvaring är därför av väsentlig betydelse både analytiskt och tolkningsmässigt.

Zopiklon är den verksamma substansen i läkemedlet Imovane® som introducerades i Sverige 1991 för behandling av kortvariga sömnbesvär. Inom forensisk toxikologi undersöks förekomst av zopiklon när analys begärs. Zopiklon återfinns i såväl missbruksärenden, drograttfylleriärenden, våldsbrottärenden som i obduktionsfall. Zopiklon kan analyseras i olika biologiska material som till exempel blod, urin och hår. Beroende på förvaringsförhållanden, provmaterialets beskaffenhet och pH förändras mängden zopiklon i lösningar och i biologiskt material. Syftet med studierna i denna avhandling var att undersöka stabiliteten av zopiklon i biologiskt material och faktorer som har betydelse vid förvaring samt hur detta påverkar tolkningen av resultat inom rättstoxikologi.

Fyra olika studier genomfördes. I den första studien gjordes olika typer av stabilitetstester. Prover med tillsatt zopiklon (spikade prover) förvarades vid −20, 5 och 20°C och koncentrationerna av zopiklon följdes över tid. Studien visade att koncentrationerna av zopiklon i blod 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 bryts ner till 2-amino-5-klorpyridin (ACP). Denna nedbrytningsprodukt kunde identifieras vid ett enkelt försök på zopiklonspikade prover som inkuberats vid 37°C.

I den andra studien undersöktes hur provhantering innan analys kan inverka på koncentrationerna av zopiklon i autentiskt blod och påverka tolkningen av resultat. I studien deltog frivilliga individer och autentiska prover studerades tillsammans med spikade. Blodprov togs före (som spikades med zopiklon) och efter intag (autentiska prover) av läkemedlet Imovane®. Proven förvarades vid −20, 5 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 blod. Faktorer som materialets beskaffenhet, förekomst av andra droger och mängden av plasmaproteiner kontrollerades. 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 blod visade sig vara stabilt ungefär en dag vid förvaring i rumstemperatur, en vecka 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. Den snabba nedbrytningen vid högre temperaturer kan orsaka en underskattning av den verkliga koncentrationen och därmed tolkas felaktigt. Resultat från den första studien visade att mängden ACP ökade i proportion till minskningen av mängden zopiklon. ACP har rapporterats som en unik nedbrytningsprodukt till zopiklon och dess

metaboliter. Mätning av ACP kan därför komma till nytta vid utredningar av förekomst av zopiklon i fall där provmaterial har förvarats under lång tid.

I den tredje och fjärde studien validerades två analytiska metoder för att kunna mäta koncentrationer av zopiklon, metaboliterna till zopiklon och nedbrytningsprodukten ACP i både blodprover och urinprover. Metoderna användes därefter i en studie där friska frivilliga deltog och som lämnade blod- och urinprov efter intag av läkemedlet Imovane®. Provmaterialet förvarades vid −20, 5 och 20°C och nedbrytning av zopiklon och dess metaboliter följdes över tid tillsammans med bildningen av ACP. Dessutom mättes pH-värdet för urin- och blodproverna vid varje analystillfälle.

Resultaten visade att ACP bildades i samma mängd som mängden nedbrutit zopiklon och zopiklon-metaboliter. I förhållande till modersubstansen finns metaboliterna i blod endast i små mängder. I urin förekommer metaboliterna däremot i betydligt högre mängd. ACP-bildningen i urin inträffade när pH eller temperatur ökade. De höga halterna av metaboliter i urin, som också bryts ned till ACP, gjorde det omöjligt att uppskatta den ursprungliga zopiklonkoncentrationen. Mätning av ACP-koncentrationer i urin tillsammans med mätningar av zopiklon och zopiklon-metaboliter kom till användning i två rättsliga fall. Det var två våldtäktsfall där analys av ACP i urinprov kunde vara till hjälp vid tolkningen av huruvida koncentrationerna av zopiklon och zopiklon-metaboliter hade brutits ner eller ej.

Resultat från blodprover där ACP hade bildats användes för test av två matematiska modeller. Modellerna användes till att uppskatta ursprunglig zopiklonkoncentration. Via den enklare modellen beräknas den ursprungliga zopiklonkoncentrationen genom att addera den uppmätta zopiklonkoncentrationen med faktorn 3,02 gånger koncentrationen av ACP. Faktorn baseras på molära förhållanden mellan zopiklon och ACP. I denna modell har hänsyn inte tagits till metaboliternas bidrag till mängden ACP. Därför bör det uppskattade ursprungsvärdet av zopiklon tolkas som maximal zopiklonkoncentration i det analyserade provet.

Avsaknad av ACP-koncentration i blod- eller urinprov tyder på att det inte har skett någon nedbrytning och att den uppmätta koncentrationen av zopiklon eller av zopiklon-metabolit är tillförlitlig. För korrekt tolkning i rättsliga fall bör därför både zopiklon och dess metaboliter och ACP analyseras.

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: 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. 2011, 207: 35-39.

III. Quantitative analysis of zopiclone, N-desmethylzopiclone, zopiclone N-oxide and 2-amino-5-chloropyridine in urine using LC-MS/MS.
Nilsson GH, Kugelberg FC, Ahlner J, Kronstrand R.
J. Anal. Toxicol. 2014, accepted for publication.

IV. LC-MS/MS determination of 2-amino-5-chloropyridine to estimate the original zopiclone concentration in stored whole blood.
Nilsson GH, Kugelberg FC, Ahlner J, Kronstrand R.
Submitted manuscript.

ABBREVIATIONS

ACP	2-amino-5-chloropyridine
CE	Capillary electrophoresis
C_{max}	Maximal concentration
CV	Coefficient of variation
CYP	Cytochrome P450
DBS	Dried blood spots
DMS	Dried matrix spot
EDDP	2-ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolinium
GABA	γ-aminobutyric acid
GC	Gas chromatography
GHB	Gamma-hydroxybutyric acid
HPLC	High performance liquid chromatography
IS	Internal standard
LC	Liquid chromatography
LLOQ	Lower limit of quantification
LOD	Limit of detection
LSD	Lysergic acid diethylamide
ME	Matrix effects
MS	Mass spectrometry
NDZOP	N-desmethylzopiclone
NPD	Nitrogen-phosphorus detector
PE	Process efficiency
RE	Extraction recovery
RIA	Radioimmunoassay
SEM	Standard error of the mean
SD	Standard deviation
THC	Δ^9-tetrahydrocannabinol
THCCOOglu	11-nor-Δ^9-carboxy-tetra-hydrocannabinolic glucuronide
THCCOOH	11-nor-Δ^9-carboxy-tetra-hydrocannabinolic acid
TOF	Time of flight
ZOP	Zopiclone
ZOPNO	Zopiclone N-oxide or N-oxide zopiclone

INTRODUCTION

Forensic toxicology

Forensic toxicology is defined as *"the application of toxicology for the purpose of the law"* [1]. Forensic toxicology is made up of three different areas, which also are the main areas of work in forensic laboratories: Post-mortem toxicology, Human performance toxicology and Forensic urine drug testing.

Requests from forensic medicine, police, criminal justice system, health care and social service are dealt with at forensic laboratories. Medico-legal autopsy cases from forensic medicine belong to the post-mortem toxicology field. Request from the police about drug related violent crimes, driving under influence of alcohol and/or drugs and use of illicit drugs are in the human performance toxicology field. Cases of suspected drug abuse within prison populations together with suspected drug abuse, intoxication or compliance from health care or social service come to the forensic urine drug testing area. The Swedish Forensic Toxicology Laboratory is a governmental institution that serves a population of 9.5 million citizens. **Fig. 1** shows the total number of cases submitted for analysis in the period 2000 to 2013 and example of distribution of cases in different areas are shown as a pie diagram.

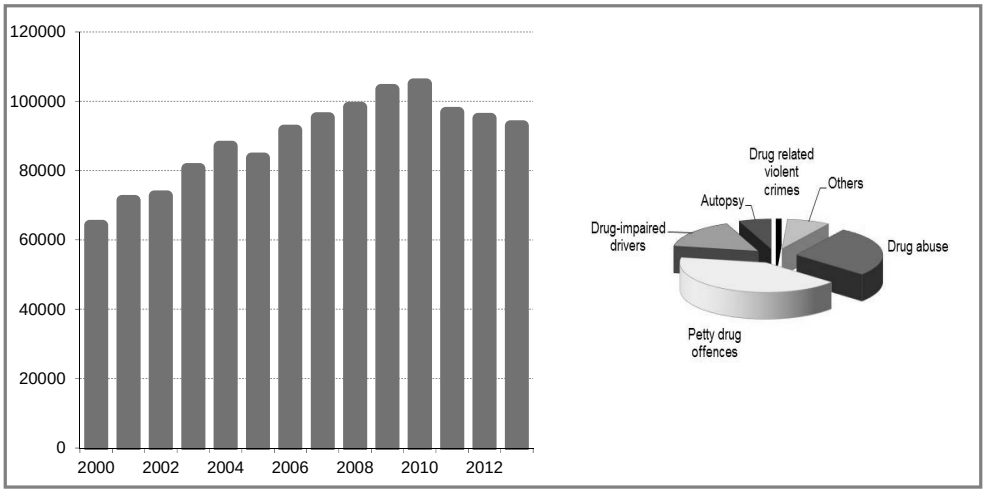

Fig. 1. Number of forensic toxicology cases in Sweden 2000 to 2013 and example of distribution of the different cases from the year 2013 (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

The aim of forensic toxicology is to establish the presence or absence of drugs and their metabolites, chemicals and other poisons in biological material. Compounds of interest are illicit drugs e.g. tetrahydrocannabinol (cannabis), amphetamines (ecstasy), cocaine, opiates/opioids (heroine, morphine), benzodiazepines, lysergic acid diethylamide (LSD), gamma-hydroxybutyric acid (GHB), therapeutic drugs e.g. analgesics, antidepressants, antiepileptic, hypnotics, sedatives, muscle relaxants, cardiovascular drugs or other compounds like alcohols, gases (carbon monoxide, cyanide, butane), steroids and designer drugs. The main issues in forensic interpretation are if drugs had been involved in the crime, if a drug or drugs had caused intoxication or altered a person's behaviour or if the drug was the cause of death.

Biological samples

The most common biological samples used for drug testing are blood, urine and hair. The identification and the quantification of drug and metabolite concentrations in blood are valuable for the assessment of drug intake in connection with various crime and sometimes for establishing the cause of death. The time of sampling is important, especially if there is any suspicion of drug influence at the time a crime was committed. 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 **Fig. 2**, the kinetic profile of drug presence in blood, urine and hair is illustrated.

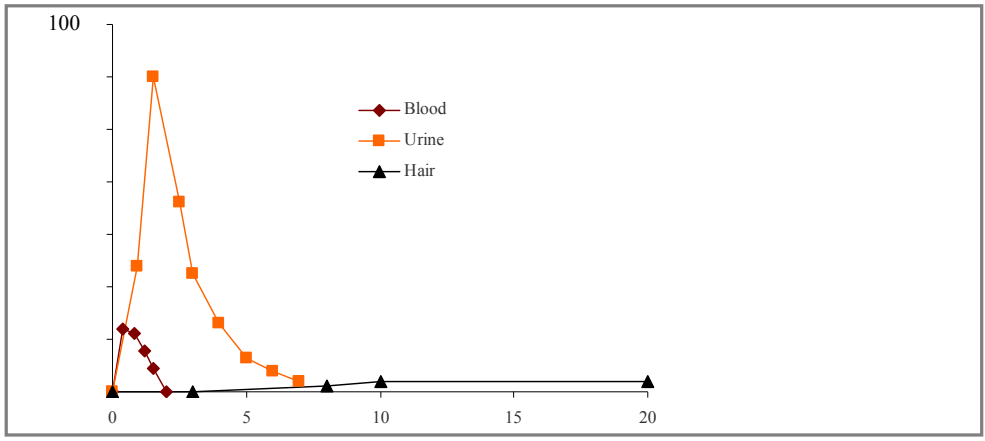

Fig. 2. Schematic kinetic illustration of drug presence in blood, urine and hair over time (days).

In Sweden specimens of venous whole blood are taken by a nurse or physician, urine samples are collected by the police officers and post-mortem samples (e.g. femoral blood, urine, vitreous humor, hair, liver, brain, kidney and lung) are taken by forensic pathologists. 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 [2]. 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 up to one year to enable reanalysis if necessary.

Pre-analytical conditions

Laboratory activities are commonly classified as pre-, intra- and post-analytical processes. The pre-analytical phase includes request form, sample collection, transport, registration, preparation and aliquoting, storage, freezing and thawing [3]. 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 [4,5] and

attention was directed to the pre-analytical process in laboratory medicine as well as in forensic toxicology [6-8]. Toxicological laboratory analysis results are influenced pre-analytically by *in vivo* factors such as 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 [9-14]. In post-mortem toxicology, additionally consideration of putrefaction, anaerobic metabolism and redistribution has to be taken [15,16].

Analytical strategies

As a generally rule in forensic toxicology, the identification of drugs and other substances in biosamples should be confirmed by two independent assays according to international guidelines (SOFT/AAFS Forensic Laboratory Guidelines – 2006). The usual procedure involves an initial screening for positive findings by analytical techniques such as immunoassays or more recently liquid chromatography time of flight mass spectrometry (LC-TOF/MS) is performed (**Fig. 3**). Second, for confirmation and quantification of positive results another analytical technique is used e.g. gas chromatography mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS). Photographs of a GC-MS and a LC-MS/MS instrument are shown in **Fig. 4**.

Fig. 3. Screening for positive findings by immunoassay shown to the left and by liquid chromatography time of flight mass spectrometry (LC-TOF/MS) shown to the right.

Fig. 4. Confirmation and quantification of positive findings by gas chromatography mass spectrometry (GC-MS) shown to the left and by liquid chromatography tandem mass spectrometry (LC-MS/MS) shown to the right.

For correct interpretation of toxicological analytical results the analysis must be reliable. Analytical methods should therefore be validated for drug identification or quantification, under the conditions of intended use. Method validation includes several analytical parameters; selectivity, calibration model, accuracy (bias) and precision, limit of detection (LOD), lower limit of quantification (LLOQ), recovery/extraction efficiency and parameters affected by specimen composition such as matrix effects and stability [17-22]. Analytical result showing presence or absence of a drug in the specimen yields information relevant to the time of analysis and the stability of the drug in the actual matrix has to be considered in connection with result interpretation [23].

Storage stability in biological samples

Stability has been defined as "*the chemical stability of an analyte in a given matrix under specific conditions for given time intervals*" [24]. In forensic toxicology, the analyte can be a drug, metabolite and/or a degradation product in the biological matrix such as whole blood, serum, plasma, urine, hair, oral fluids or tissues. The knowledge of stability of a drug and its major metabolites in biological specimens is very important in forensic cases for the interpretation of analytical results [8,23,25]. Overestimation or underestimation of unreliable results may lead to erroneous conclusions in the judicial inquiry. Many substances are unstable in biological samples and undergo degradation, whereas the concentrations of others might increase 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) [2,8,23]. The duration of storage starts from the time of sampling and proceeding 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 analysed. By contrast, a drug which is absent may be formed during storage and detected in the analysis. Hence, the stability of drugs in biological specimens has been extensively studied in the area of analytical toxicology.

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 [18,19,26], but generally accepted guidelines have not yet been established [23,27]. However, best practices for stability experiments and stability evaluation have been recommended [28].

Several different types of stability tests, including biological matrix and standard stock solutions are required for complete stability evaluation [18,19,21,22,26,28]. Long-term stability studies usually cover a storage period normally expected for a laboratory, when samples are stored under the same storage conditions as routinely used. 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 stored under auto sampler conditions.

Tests of drug stability is done by comparing samples at two concentration levels before (comparison samples/reference samples) and after (stability samples) exposing to test conditions has been suggested [18-20,26]. The reference samples can either be freshly prepared or stored below –130°C. After storage at selected temperature and for various time intervals, reference and stability samples are analysed together and the results are compared. For instance, after comparing reference and stability samples by *F*-test, the stability can be evaluated by interval hypothesis testing [19]. An acceptable stability has been recommended when mean concentration ratios between reference samples and stability samples are within 90 and 110%, or acceptance interval of 85–115% from the control samples mean for 90%-confidence interval of stability samples [26]. However, it has also been recommended to use the same criteria as for accuracy and precision. Thus, stability is acceptable when the results are within 15% of the nominal values [28]. This means, that analyte instability is defined as a deviation of >15% from the expected concentration (nominal/initial measured).

Various experimental designs and different procedures for data evaluation exist for investigation of drug stability. 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 trend regarding degradation [29-40]. Among reported investigations, also studies on authentic material from volunteers dosed with the drug or from laboratory cases have been performed [33,40-44].

Stability investigations have been evaluated in several different ways by statistical parametric tests like Student's *t*-test [34] paired *t*-tests [40,43], analysis of variance (ANOVA) [36,44] or by nonparametric tests like Kruskal-Wallis and Mann-Whitney [39]. 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 [38,42]. Stability has also been evaluated on a percentage base with regard to analyte decrease or increase during storage [30,31,37,41,44].

Stability investigations of drugs in biological specimens

Specific stability studies of several forensically important drugs in blood and other fluids or tissues have been published including *cocaine*, *its metabolites and its degradations product* in whole blood, post-mortem blood or plasma [32,37,45,46], *benzodiazepines, antidepressants, antipsychotics, analgesics and/or hypnotics* in whole blood, plasma or post-mortem blood [29,30,43,47-51], *morphine and/or its glucuronides and/or buprenorphine* in whole blood, plasma or post-mortem blood [31,33,52], *11-nor-Δ^9-carboxy-tetra-hydrocannabinol glucuronide (THCCOOglu)* in plasma [38,53], *toluene and acetone* in liver, brain and lungs [39], *3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA) and 3,4-methylene-dioxyamphetamine (MDA)* in whole blood [34], *carbon monoxide* in post-mortem blood [54], *GHB* in blood, serum or post-mortem blood [44,55,56] and *ethanol* in whole blood, plasma, post-mortem blood or vitreous humor [40,57,58].

Furthermore, stability of some drugs in urine have been investigated such as *11-nor-Δ^9-carboxy-tetra-hydrocannabinol acid (THCCOOH), THCCOOglu, amphetamine, methamphet-*

amine, ephedrine, morphine, codeine, cocaine, benzoylecgonine and/or *phencyclidine* [35,41,42,53], *LSD)* [59], the LSD metabolite *2-oxo-3-hydroxy lysergic acid diethylamide (O-H-LSD*) [36] and *GHB* [44].

Targeted testing for drug stability in hair strands is not common, but stability of *amphetamine* and *methamphetamine* in reference material has been tested [60].

Depending on storage conditions (e.g. time, temperature and/or pH) changes in drug concentrations occurred in these experimental studies. For example, *GHB* in post-mortem blood and urine (metabolic production) [44], *THCCOOH* in urine, *THCCOOglu* in urine and plasma (decarboxylation, enzymatic and chemical hydrolysis) [38,42], *acetone* in liver, brain and lungs (reduction) [39] and *cocaine* in whole blood and post-mortem blood (enzymatic and chemical hydrolysis) [32]. In post-mortem blood, degradation was noticed for e.g. the benzodiazepine metabolite *7-amino-nitrazepam* and for the hypnotic drug *zopiclone* (ZOP) [43]. Addition of fluoride preservatives have been shown to inhibit the formation of alcohol in whole blood [58] and GHB in whole blood and in post-mortem blood [55].

Chemical hydrolysis of drugs is a common reaction leading to degradation. To preserve hydrolytically labile drugs, dried blood spots (DBS) has been used. Drugs such as *benzodiazepines, cocaine, 6-acetylmorphine* and *ZOP* have proved less prone to degradation in dried matrix spot (DMS) samples than for corresponding blood samples [61-63].

During 2000s, oral fluid has received a lot of attention as an alternative matrix for drug testing and stability studies on drugs in the saliva have been done; *opioids, cocaine, amphetamines, benzodiazepines* and other *psychoactive drugs*, *methadone, 2-ethyl-1,5-dimethyl-3,3-diphenyl-pyrrolinium (EDDP), Δ^9-tetrahydrocannabinol (THC)* and *THCCOOH* [64-68]. To increase the stability of opiates, cocaine and amphetamines, the addition of a preservative (citrate buffer and sodium azide) was needed [64]. Other drugs such as THC, EDDP, tramadol and carisoprodol decreased in concentration during storage, but ZOP exhibited a minor concentration loss in oral fluid when stored frozen [65,67,68].

Zopiclone

ZOP 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 and was registered in Sweden in 1991. ZOP is increasingly being encountered in forensic cases and is a common finding in samples from drug-impaired drivers, users of illicit recreational drugs, victims of drug facilitated sexual assaults and forensic autopsy cases [25,69-76]. ZOP is considered a non-benzodiazepine from the cyclopyrrolone class. It contains a single asymmetric carbon atom and with 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. ZOP is a racemic mixture composed of the (+)-enantiomer with S-configuration and the (−)-enantiomer with R-configuration [77]. The chemical structure of ZOP is shown below (**Fig. 5)**.

The racemic mixture of ZOP 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 sold under the brand name Lunesta® (e.g. USA). ZOP are prescribed as therapy for short-term insomnia. Insomnia might entail difficulty in falling asleep, frequent nocturnal awaking and/or early morning awaking. The usual dose of Imovane® to treat insomnia is 5 mg or 7.5 mg taken before bedtime [78].

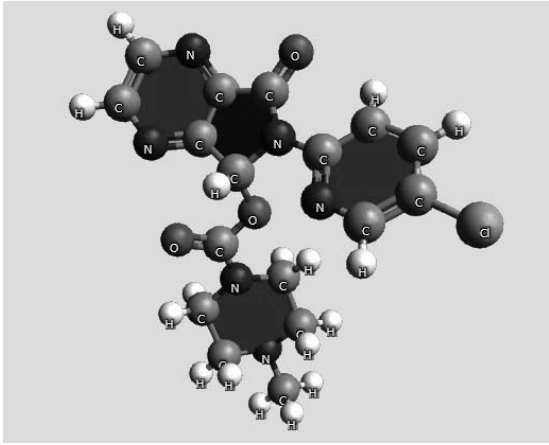

Fig. 5. Zopiclone, 6-(5-chloro-2-pyridyl)-7-(4-methyl-1-piperazinyl) carbonyloxy-6,7-dihydro (5H) pyrrolo-(3,4-b) pyrazin-5-one (*position of asymmetric carbon).
Empirical formula: $C_{17}H_{17}ClN_6O_3$ Molecular weight: 388.81 g/mole

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) ligand-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 [79]. ZOP 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 [80,81]. However, at maximal concentration (C_{max}) the functional effect has been calculated to 60% for α1β2γ2, 55% for α5β2γ2 and to 25% for α3β2γ2 and the affinity, potency and efficacy varies between the α1−α5 subtypes [82]. Additionally, it had been explained that α1 subtypes mediates sedative effects, α2 and α3 anxiolytic actions and that α5 regulate memory function [83].

The pharmacological profile of ZOP differs from the benzodiazepines either because of a differential affinity for different $GABA_A$ receptor subtypes or partial agonistic properties. Further, it has been found that ZOP behaves as a partial agonist at the $GABA_A$ receptor with a lower intrinsic activity relative to benzodiazepines. S(+)-ZOP has higher affinity for benzodiazepine sites than R(−)-ZOP, but both enantiomers are active at $GABA_A$ receptors [81,84-86]. At α1 subunit, ZOP shows agonist activity whereas S(+)-ZOP differs from its racemic mixture and has greater efficacy at α2 and α3 subunits. The R(−)-ZOP interacts allosteric to effect the activity of S(+)-ZOP [82,83,87]. Moreover, some studies suggest that the ZOP metabolite S(+)-desmethylzopiclone enantiomer elicits an anxiolytic effect without central nervous depression [88].

The hypnotic effects of ZOP for inducing sleep could be seen within 30 minutes of dosing with S(+)-ZOP and after an hour of dosing with the racemic ZOP [83]. Drugs that act on the central nervous system can have adverse effects on performance and behaviour of the

individual. Plasma concentrations are elevated up to ten hours after ZOP dosing generating residual effects on psychomotor and cognitive functions [82,89]. Additionally, dependence and abuse of ZOP has been recognized [75,90,91].

Pharmacokinetics

After oral administration of the racemic drug, ZOP is rapidly absorbed from the gastrointestinal tract, with a bioavailability of approximately 80% [92]. Plasma protein binding of ZOP was reported as 45% in one study [93] and 80% in another [94]. Both albumin and α-1-acid glycoprotein contribute to protein binding but also other plasma proteins might be involved (e.g. globulins, lipoproteins). It has been noticed that the protein binding is stereo selectivity. S(+)-ZOP showed higher binding to α-1-acid glycoprotein and albumin than R(−)-ZOP. However, a higher total protein binding was observed for R(−)-ZOP and an explanation for this was that R(−)-ZOP also binds to other proteins [94]. The distribution of ZOP into body tissues including the brain is rapid and widespread, and ZOP is excreted in urine, saliva and breast milk. ZOP is metabolized by decarboxylation, oxidation, and demethylation.

In the liver ZOP is partly metabolized to an inactive N-demethylated (13-20% of dose) and an active N-oxide metabolite (9–18% of dose) [92]. The cytochrome P-450 (CYP) enzymes CYP3A4 and CYP2C8 are involved in the metabolism of ZOP. Both metabolites depend on CYP3A4 activity but the N-desmethylzopiclone (NDZOP) formation also has a correlation to CYP2C8 activity [95]. From ester hydrolysis involving oxidative decarboxylation (50% of the administered dose) inactive metabolites are formed. Some of these metabolites are excreted as carbon dioxide via the lungs. The three main pathways of ZOP metabolism are shown in **Fig. 6** below. In urine, the NDZOP and N-oxide metabolites (ZOPNO) account for 30% of the initial dose whereas less than 7% of the administrated dose is excreted as unchanged ZOP. Elimination half-life ($t_{1/2}$) of ZOP falls within the range of 3.5 to 6.5 hours. No gender difference in pharmacokinetics of ZOP has been observed, although in patients with liver or renal dysfunction small differences exist and the plasma half-life of ZOP increases with age [92,93].

All the pharmacokinetic processes, absorption, distribution, metabolism and excretion, are influenced by chirality. The plasma concentration of S(+)-ZOP is higher than that of its antipode R(−)-ZOP after oral administration of the racemic mixture and the urine concentration of R(–)-NDZOP and R(−)-ZOPNO are also higher than the corresponding S(+)-enantiomers [96].

Adverse metabolic drug interactions can occur when the efficacy or toxicity of a medication is changed by concomitant administration of another substance. Pharmacokinetic interactions generate a change in the metabolism of drugs [97]. Inhibitors of CYP3A4 increase plasma ZOP concentrations [98,99] whereas CYP3A4 inducers decrease the plasma concentration of ZOP [100].

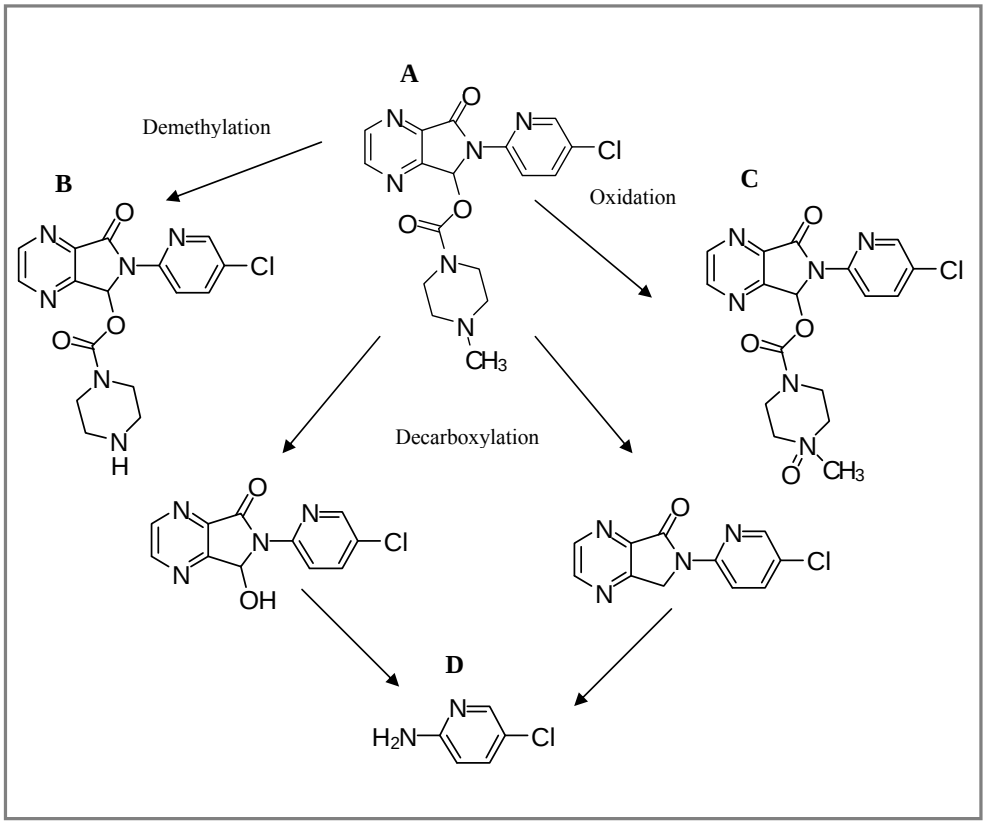

Fig. 6. The three main pathways of zopiclone (racemic) metabolism in humans. (**A**) Zopiclone, (**B**) N-desmethylzopiclone, (**C**) N-oxide-zopiclone and (**D**) 2-amino-5-chloropyridine (ACP).

Occurrence of zopiclone in forensic cases

At the clinically recommended dose of 7.5 mg, the peak concentration of ZOP in plasma of 0.06 mg/L is reached within 1–2 h [78]. Blood concentrations of about 0.1 mg/L are possible after therapeutic use and toxicity might occur at serum levels above 0.15 mg/L [101,102]. During the past few years there have been an increasing number of reports about the abuse and misuse of ZOP [90]. One study showed a prevalent misuse of ZOP when its degradation product 2-amino-5-chloropyridine (ACP) was detected in urine [69]. In Sweden, the first petty drug offences involving ZOP appeared 1994. The distribution of number of cases and the concentration in blood of those arrested for petty drug offences 2000 to 2013 is shown in **Table 1**.

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

Year	Blood samples				Urine samples
	Number of ZOP positives	Mean (µg/g)	Median (µg/g)	Highest (µg/g)	Number of ZOP positives
2000	3	0.03	0.03	0.03	62
2001	7	0.06	0.03	0.19	51
2002	12	0.18	0.16	0.50	45
2003	10	0.08	0.07	0.15	71
2004	6	0.05	0.05	0.06	75
2005	15	0.10	0.04	0.50	144
2006	22	0.12	0.06	0.90	164
2007	26	0.09	0.06	0.28	234
2008	28	0.10	0.05	0.70	332
2009	37	0.08	0.05	0.30	268
2010	42	0.14	0.06	1.0	262
2011	33	0.07	0.06	0.20	260
2012	54	0.07	0.05	0.62	301
2013	66	0.08	0.04	0.38	279

ZOP and other sedative hypnotic drugs are frequently detected in blood and urine from people suspected of driving under the influence of drugs [25,71,72,76,103,104]. Because of its therapeutic use as a sleep-aid, use of ZOP is contra-indicated when skilled task such as driving is performance [105-107].

ZOP concentrations in blood from drivers over a period of about six years showed that 80% had higher blood concentrations than expected from normal therapeutic doses [76]. A connection between road-traffic accidents and ZOP use has been reported and users of ZOP were advised not to drive the next day [105]. Only one year after the drug was introduced in Sweden, ZOP was detected in a case of drug impaired driver. The number of ZOP cases and the concentrations in blood of people arrested for drug-impaired driving 2000 to 2013 are shown in **Table 2**.

Table 2. Number of cases positive for zopiclone (ZOP) and the concentrations in whole blood from cases of drug-impaired drivers arrested in Sweden (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

Year	Number of ZOP positives	Mean (µg/g)	Median (µg/g)	Highest (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
2010	128	0.14	0.08	1.2
2011	110	0.09	0.06	0.94
2012	113	0.08	0.04	0.71
2013	100	0.12	0.05	0.92

Several studies have implicated sedative hypnotic drugs as a means to cause incapacitation and thus facilitate criminal actions [108-110]. In samples from female victims of alleged sexual assault in Sweden, ZOP has been identified as a commonly used drug [74,111]. The number of ZOP cases and the concentrations in blood in cases of drug-related violent crimes in Sweden 2008 to 2013 are shown in **Table 3**.

Table 3. Number of cases positive for zopiclone (ZOP) and the concentrations in whole blood from cases of drug related violent crimes in Sweden (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

	Blood samples				Urine samples
Year	Number of ZOP positives	Mean (µg/g)	Median (µg/g)	Highest (µg/g)	Number of ZOP positives
2008	10	0.06	0.04	0.15	10
2009	13	0.08	0.06	0.40	16
2010	19	0.07	0.05	0.23	24
2011	14	0.06	0.05	0.17	21
2012	25	0.06	0.03	0.29	25
2013	23	0.06	0.03	0.49	23

Fatalities resulting from poisoning with ZOP combined with alcohol or other drugs have also been described [73,112-115] and cases of fatal ZOP overdose with ZOP concentrations of 1.4-3.9 mg/L in the blood have been reported [116]. An overdose death after ingesting 90 mg of ZOP suggested that this amount could be a minimum lethal dose (in femoral blood the ZOP concentration was 0.254 mg/L) [114].

Median concentration from drug intoxication deaths was reported as 0.70 mg/L for ZOP but this median concentration decreased when the number of co-ingestion drugs increased [73]. In Sweden, ZOP is one of the most frequently identified prescription drugs in post-mortem femoral blood [70,73]. Only one year after this hypnotic was introduced in Sweden, ZOP was detected in an autopsy cases. The number of positive findings has increased dramatically and in 2012 and 2013, ZOP was identified as the most common prescript drug in forensic autopsy cases. The number of cases of ZOP and the concentrations in autopsy femoral blood for the years 2000 to 2013 are shown in **Table 4.**

Table 4. Number of zopiclone (ZOP) cases and the concentrations in femoral blood samples from forensic autopsies in Sweden (data collected from the laboratory database at the Department of Forensic Genetics and Forensic Toxicology).

Year	Number of ZOP findings	Mean (µg/g)	Median (µg/g)	Highest (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
2010	286	0.27	0.09	3.8
2011	333	0.19	0.07	3.1
2012	421	0.19	0.07	2.7
2013	456	0.25	0.07	8.1

Analytical methodology

ZOP is a white to light yellow crystalline solid, which is only slightly soluble in water, but very soluble in most organic solvents (e.g. ethanol, acetonitrile, dichloromethane) [92,102]. Analysis of ZOP in biological specimens is complicated by its instability in certain solvents such as methanol, and under acid or basic pH conditions [101,117,118]. Standard solution should be prepared in acetonitrile and extraction must be done at neutral pH to improve stability [101,119].

Several analytical methods, including 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 identification and quantification of ZOP in whole blood, serum and/or plasma [118,120-136]. Some assays (HPLC, LC-MS, LC-MS/MS) have also been developed to separate and determine ZOP [127,137-139] and/or its metabolites (NDZOP and ZOPNO) in urine [140-142]. However, because of analytical detection or validation problems when ZOP was analysed in urine, the ZOP analyte was excluded from two of these methods [139,141]. In another method the metabolites were identified but not quantitated [142]. A couple of methods have been developed to include also the analysis of ZOP metabolites NDZOP and/or ZOPNO in plasma. One method (HPLC) has been reported to detect ZOP and its major metabolite NDZOP [129], and the other both of metabolites together with the parent drug [131]. Stereo specific methods [LC, capillary electrophoresis (CE), radioimmunoassay (RIA), HPLC, LC-MS/MS] have been developed to separate the enantiomers in urine [143-145] and in plasma [119,146-148]. Three methods (HPLC, GC-MS, LC-MS/MS) are suitable for measuring ZOP's degradation product ACP in urine, whole blood, DMS and in post-mortem urine, blood and stomach content [63,149,150]. Additionally, methods such as HPLC and CE were used to determine ACP impurity in ZOP tablets [151,152].

In vitro stability studies

It has been confirmed that physical processes e.g. temperature have an effect on ZOP stability and it has been shown that ZOP undergoes degradation by chemical hydrolysis at basic pH by ring opening and conversion to ACP [63,117,149,150].

Specific long-term stability investigations showed a 21% decrease of the ZOP concentration in post-mortem femoral blood after storage for twelve months at –20°C [43]. Studies of the stability of ZOP have also been carried out from stability experiments in connection with method development and validation. *Long-term stability* tests with spiked human plasma showed that ZOP was stable for one month [118,129], for 74 days [131] and for six months [119] when stored at –20°C or lower. *Freeze-thaw stability* tests verified that ZOP was stable in spiked human plasma for three freeze-thaw cycles [118,129] but stability for five freeze-thaw cycles has also been reported [131]. *Short-term* or *in process stability* tests give no evidence of degradation in plasma when quality control samples were stored at room temperature for 12 h [131] or 24 h [129]. No loss of ZOP was detected when blood samples (plasma) was stored at 4–8°C for less than 6 h. The concentration of ZOP in blood was reduced by 25% and 29% for the (–)- and (+)-enantiomers, respectively, after 20 h storage at ambient temperature [119]. *Processed sample stability* determined by reanalysis of an extract (under ordinary instrument conditions) has shown that ZOP is stable for up to 24 h in water-methanol extract [129], stable in 0.05% formic acid-acetonitrile-methanol extract for 52 h, but

unstable in ethanol extracts [118,131]. *In vitro* half-life of ZOP in spiked whole blood samples was 3 days at 20°C and 28.4 days at 4°C compared with 90 days at –20°C [63].

Both albumin and α-1-acid glycoprotein are involved in ZOP protein binding [94]. Protein binding might protect drugs from *in vitro* breakdown, although no relationship between concentration decrease in connection during storage and plasma protein binding has been confirmed [29]. However, large individual differences exist in plasma protein concentrations and competition between different drugs for binding sites occur *in vitro*, stability studies in blood on authentic samples might be important. Blood and urine are the commonest matrices for analysis of ZOP in forensic toxicology [87]. Stability tests of ZOP in stored urine samples was not investigated or reported in recently validated methods for forensic applications [137-142]. Because of the short half-life, ZOP is rapidly elimination from blood so urine may be a good choice of matrix, for example, in drug facilitated crimes when the victim might delay reporting the crime [153]. Since interpretation of ZOP concentrations in whole blood and urine are important in forensic toxicology, detailed knowledge of the ZOP stability in these matrices is essential for reliable analysis and interpretation of results.

AIMS OF THESIS

The overall aim of this thesis was to investigate the stability of the hypnotic drug ZOP and factors of importance for degradation during storage in biological samples and to identify consequences for interpretation of results in forensic toxicology.

Specific aims

Paper I

To investigate the stability of ZOP in human blood during storage under different conditions, stability differences between authentic and spiked blood samples, 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.

Paper III

To develop and validate a method for the quantitative analysis of ZOP, NDZOP, ZOPNO and ACP in urine in order to study their rates of degradation or formation under conditions of time, temperature and pH.

Paper IV

To investigate how ACP could be used to estimate the original concentration of ZOP in authentic samples. For that purpose, an analytical LC-MS/MS method for the quantitation of ACP, ZOP and NDZOP in blood was developed and validated. ACP formation, ZOP and NDZOP degradation were investigated and mathematical models were derived to calculate the original concentration of ZOP.

INVESTIGATIONS

Stability tests of zopiclone in whole blood (Paper I)

Initiation

This investigation of storage stability in whole blood samples, started with a forensic case. It was in connection with a legal trial of a traffic accident, when the suspected drug-impaired driver claimed that he (or she) had been drugged with ZOP. Eight months had passed since the traffic incident and blood sample collection. When the police requested ZOP analysis and the sample was reanalysed after eight months of storage at 5±4°C, no measurable ZOP was found in the sample. At that time, stability tests of ZOP in whole blood had not been investigated or reported. The aim of this study was to investigate the stability of ZOP in human blood during storage under different conditions, stability differences between authentic and spiked blood, freeze-thaw and processed sample stability.

Study design

Short- and long-term stability: For short- and long term stability investigation aliquots of pooled drug-free whole blood was spiked to one low ZOP level (0.2 μg/g) and one high level (0.5 μg/g). The studied levels used, were chosen on the basis of concentrations found in authentic blood samples and being high enough to follow decrease over time. Samples were measured initially (reference samples) and repeated measured after being exposed to different storage conditions (–20, 4, and 20˚C) during a study period up to 12 months (stability samples). Long-term stability was evaluated by comparing ZOP concentrations between reference samples and stability samples.

Tested hypothesis: H_0 = There is no significant difference in ZOP concentration during storage depending on started concentration level, storage time or temperature. H_1 = There is a difference in ZOP concentration during storage.

Authentic and spiked stability differences: Long-term stability differences between authentic and spiked blood samples were compared under the conditions usually encountered in our laboratory (4˚C). Aliquots of spiked and authentic whole blood were measured before (reference samples) and were monthly repeated after storage up to eight months (stability samples). For spiked blood, the initially measured concentration was used as starting value (reference samples) whereas for authentic samples the first measured concentration was used as starting value (reference sample). The studied levels used in spiked samples (0.02–0.50 μg/g) were chosen to reflect the concentration levels of the authentic blood samples.

Tested hypothesis: H_0 = There is no significant difference in the stability of ZOP between authentic and spiked blood samples during storage. H_1 = There is a difference in stability of ZOP between authentic and spiked blood samples during storage.

Freeze-thaw stability: Spiked blood sample at one low concentration (0.02 μg/g) and one high concentration (0.2 μg/g) were analysed before (reference samples) and after three freeze-thaw cycles (stability samples). Authentic blood samples were tested through one freeze-thaw cycle. ZOP concentration was compared between reference samples and stability samples.

Processed stability: Extracted samples were reinjected to evaluate processed stability of ZOP concentrations in extracts of butyl-acetate. The extracts were reinjected one and two days after on-instrument storage at ambient temperature and the results were compared with the results from the first injection.

Degradation experiment: In addition to the stability investigation, the formation of ZOP degradation products was investigated. ZOP and ACP were measured from aliquots of ZOP spiked whole blood (0.3 µg/g) before and after storage up to 24 h at 37°C. The concentration of ZOP was plotted against the concentration of ACP.

Influence of pre-analytical conditions on the interpretation of zopiclone (Paper II)

Initiation

In the previous study (Paper I), the initially measured concentration was used as starting value for spiked blood, whereas for authentic samples the first measured concentration was used as starting value. Data of pre-analytical stability for ZOP in authentic whole blood was therefore missing. Additionally, pooled drug-free blood from different donors was used as matrix for the spiked blood. Such matrix pool is rarely homogeneous because of clots and the source of matrix might influence drug stability. As mentioned in the introduction, individual differences in plasma protein concentrations might also influence pre-analytical drug stability. For supplying information of that influences, concentrations of albumin and α-1-acid glycoprotein might be individually controlled. The specific aim of this study was 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.

Study design

For the study purpose a controlled study was performed. Pre-dosed whole blood individually pooled was used to get a homogenous matrix for the spiked study samples. The pre-dosed blood was aliquoted and ZOP spiked to give target concentration of 0.15 µg/g and 0.08 µg/g respectively. The levels were expected to reflect authentic blood levels. The post-dosed blood after Imovane® intake was pooled individually and aliquoted. The measuring started within 8±1 h after sampling, and was daily repeated after storage up to five days at 20˚C, weekly up to twelve weeks at 4˚C and monthly up to three months at −20˚C. For comparing ZOP stability between authentic and spiked whole blood, post-dosed authentic samples were measured initially together with spiked samples (reference samples), and after being exposed to different storage conditions (stability samples). For GC-NPD evaluation, historical calibrations curves were used and all samples were run in triplicates. Samples were also obtained for external plasma albumin and plasma α-1-acid glycoprotein albumin measuring.

Hypothesis a) H_0 = There is no significant difference in the stability of ZOP between authentic and spiked blood samples during storage. H_1 = There is a difference in stability of ZOP between authentic and spiked blood samples during storage.

b) H_0 = There is no significant difference in ZOP concentration during storage at any of the studied conditions. H_1 = There is a difference in ZOP concentration during the storage.

Quantitative analysis of zopiclone, N-desmethylzopiclone, zopiclone N-oxide and 2-amino-5-chloropyridine in urine using LC-MS/MS (Paper III)

Initiation

Stability of ZOP or its metabolites in urine samples has not yet been investigated or reported. Temperature and pH are factors that influence both enzymatic and chemical hydrolysis and by extension impact on stability of drugs in urine samples. Since interpretation of ZOP concentrations or its metabolites presences in urine are made, stability investigation of these analytes in urine is needed. The aim of this study was to develop and validate a method (LC-MS/MS) for the quantitative analysis of ZOP, NDZOP, ZOPNO and ACP in urine with the intention of studying their degradation or formation under conditions of time, temperature and pH.

Study designs

LC-MS/MS validation: The LC-MS/MS method was validated for selectivity, matrix effects (ME), process efficiency (PE), LLOQ, calibration model, precision and accuracy and stability according to published guidance for methods used in research projects [22].

Degradation and formation study: A controlled study was performed and authentic urine samples were obtained after Imovane® intake. For investigation of the effect of time, temperature and pH, the concentration of ZOP, NDZOP, ZOPNO and ACP of the authentic study samples were quantified by LC-MS/MS and the pH was measured. The measuring started within 2 h after sampling, and was daily repeated after storage up to five days at 20˚C, weekly up to twelve weeks at 4˚C and monthly up to three months at −20˚C. For LC-MS/MS evaluation, daily calibrations curves were used.

Forensic cases: The developed method was also used in two suspected cases of drug-facilitated sexual assault. The first case was a woman who was found walking around the streets confused and absent minded, however not perceived as drunk. She was taken to the hospital and upon questioning she recalled being at a party but had a memory lapse of three hours. A urine sample was obtained approximately 11 h after the assault and stored at 4°C during two months during routine analysis and then stored at –20°C during about one month before analysed using the developed method. The second case was a woman who reported a rape and a urine sample was obtained less than 24 h after the assault. No other information about the case was available. In this case, the urine sample was stored one week at 4°C after arrival at the laboratory prior to analysis.

LC-MS/MS determination of 2-amino-5-chloropyridine to estimate the original zopiclone concentration in stored whole blood (Paper IV)

Initiation

In the first study (Paper I), the degradation of ZOP and the formation of ACP were studied in a brief experiment. ACP (molecular weight of 128.6 g/mol) was formed in equimolar amounts to ZOP (molecular weight of the 388,8 g/mol) degradation. The aim of this study was to investigate how ACP could be used to estimate the original concentration of ZOP in authentic

samples. For that purpose, an analytical LC-MS/MS method for the quantitation of ACP, ZOP and NDZOP in blood was validated. ACP formation, ZOP and NDZOP degradation were investigated and mathematical models to calculate the original concentration of ZOP were formulated and tested.

Study designs

LC-MS/MS validation: The LC-MS/MS method was validated for selectivity, ME, PE, extraction recovery (RE), LLOQ, calibration model, precision and accuracy and stability according to published guidance for methods used in research projects [22].

Formation and degradation study: A controlled study was performed and authentic whole blood samples were obtained after Imovane® intake. For investigation initial measurement began within 4 h after sampling, and was subsequently daily repeated after storage up to five days at 20˚C, weekly up to twelve weeks at 4˚C and monthly up to three months at −20˚C. All samples were run in duplicates and daily calibrations curves were used.

Mathematical models: The original ZOP concentration (t0) was calculated according to the two following formulas:

1. [ZOP]ti + k([ACP]ti *(1–x))
2. [ZOP]ti + k[ACP]ti

ti = current concentration, 1–x = 1–[NDZOP]/[ZOP]
k = 1 when molar concentrations are used and k = 3.02 (389 (molecular weight of ZOP)/129 (molecular weight of ACP) when concentrations are expressed as ng/g.

Evaluation: Pearson Correlation was used to analyse bivariate correlation between t0 and the results from each formula. A simple linear regression was performed to investigate how the respective formula (independent variable) influenced the t0 (dependent variable).

Tested hypothesis: H_0 = There is a significant correlation between calculated concentration and original concentration. H_1 = There is no significant correlation.

Biological specimens

Paper I: Drug-free whole blood (containing sodium fluoride and potassium oxalate) used as matrix for spiked study samples was obtained from the local blood bank at the University Hospital in Linköping. Authentic whole blood were obtained by medical personnel and sent by the police to the Department of Forensic Genetics and Forensic Toxicology, Linköping, for analysis. The authentic blood samples were collected in tubes with 100 mg sodium fluoride and 25 mg potassium oxalate. The spiked samples were analyzed initially, aliquoted, stored at –20, 4, and 20˚C, and repeatedly analyzed during the study of 12 months. Aliquots of spiked and authentic whole blood were stored together at 4˚C, and repeatedly analyzed during the eight months study.

Paper II: Drug free blood (containing sodium fluoride and potassium oxalate) used for matrix in spiked study samples was purchased from the local blood bank at the University Hospital in Linköping. Authentic blood was obtained from drug-free volunteers (n=9) before and 1.5 h after administration of a single dose of 10 mg Imovane®. The blood samples were collected in tubes with 100 mg sodium fluoride and 25 mg potassium oxalate. Pre-dosed blood was

pooled individually, aliquoted, spiked to target ZOP concentrations, analyzed initially then stored at –20, 4, and 20˚C, and repeatedly analyzed during the study. The post-dosed blood was pooled individually, aliquoted, stored and analyzed with the spiked samples. Prior to initial analysis, the samples were stored at refrigerator temperature.

EDTA blood samples were collected before Imovane® intake, transported to a local clinical laboratory (at University Hospital, Linköping) for analysis of erythrocyte volume fraction, plasma albumin and plasma α-1-acid glycoprotein.

Paper III: Pooled drug-free urine (pH≈7.0) for development, validation, calibration, and quality control standards was obtained from volunteers in our laboratory (Department of Forensic Genetics and Forensic Toxicology, Sweden). Authentic urine samples were collected 10 h after oral administration of a single dose of 5 mg (n=4) or 10 mg (n=5) Imovane®. The samples were immediately analyzed and then distributed to storage (at –20, 4, and 20˚C) for investigation of time, temperature and/or pH dependent degradation.

Paper IV: Drug free blood (containing sodium citrate 26.3 g/L, sodium dihydrogen phosphate 2.51 g/L, glucose 25.5 g/L and citric acid 3.27 g/L) for development, calibration and quality controls was purchased from a local blood bank. For validation, drug free blood from donors was collected in tubes containing sodium fluoride (100 mg) and potassium oxalate (25 mg). Drug positive blood samples were collected as part of a human pharmacokinetic study. These blood samples were collected in the same source of tubes (containing sodium fluoride and potassium oxalate) 2 h after oral administration of a single dose of 5 mg (n=4) or 10 mg (n=5) Imovane®. Samples were analyzed initially, aliquoted, stored at –20, 4, and 20˚C, and repeatedly analyzed during the study. Prior to initial analysis, the samples were stored at refrigerator temperature.

Ethical considerations (Papers I–IV)

In the first study (Paper I) and in supplementary measurements, 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 de-identified with no connection to the cases. In the second, third and fourth study (Papers II–IV), biological material from volunteers was used. Prior to the study, all participants had given written informed consent. The studies were conducted according the code of ethics of the World Medical Association (Declaration of Helsinki). The protocols were approved by the Regional Ethics committee, Faculty of Health Sciences, Linköping University, Sweden (Paper II: # M164-08, Papers III–IV: #2010/41-31).

Equipment (Papers I–IV)

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 whole blood were stored in glass tubes (DURAN®, Mainz, Germany). Urine samples were stored in polystyrene tubes (Nunc™, Roskilde, Denmark). Eppendorf and/or Gilson 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) and TurboVap® LV Evaporator was used for evaporation.

Chemicals and solutions (Papers I–IV)

Papers I–IV: Sodium hydroxide, potassium dihydrogen phosphate and dipotassium hydrogen phosphate * 3 H_2O, sodium acetate trihydrate, Tris (hydroxymethyl)-aminomethan, ammonium acetate and acetic acid, hydrochloric acid, formic acid (analytical reagent grade), n-butyl acetate (analytical grade) acetonitrile, methanol and tert-butyl methyl ether (gradient grade for liquid chromatography) were purchased by Merck (Darmstadt, Germany). Ammonium formiate came from Fluka® Sigma-Aldrich (Steinheim, Germany). The water used for solutions was purified on a Milli-Q® Water Purification System from Millipore (Molsheim, France).

Papers I–II: The reference material ZOP was obtained from (Sigma-Aldrich, St. Louis, MI, USA), ACP from Fluka® Sigma-Aldrich (Steinheim, Germany) and prazepam from (Sigma-Aldrich, St. Louis, MI, USA). Two sets of standard solutions of ZOP were separately prepared at concentrations of 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL and 0.0001 mg/mL. One of the set was used for calibration samples and the other for quality control (QC) samples to one low (0.02 µg/g) and one high level (0.5 µg/g). The standard solution of ZOP were dissolved in acetonitrile whereas prazepam was prepared in methanol and used as internal standard (IS) with a concentration of 0.01 mg/mL.

Papers III–IV: The reference material ACP was obtained from Fluka® Sigma-Aldrich (Steinheim, Germany), ZOP and zopiclone-D_4 (ZOP-D_4) from Cerilliant® Sigma-Aldrich (Texas, USA), NDZOP was obtained from TRC (North York, Canada) and ZOPNO from LGC® (Luckenwalde, Germany). Two sets of standard solutions of ZOP, NDZOP, ZOPNO and ACP were separately prepared at concentrations of 0.1 mg/mL, 0.01 mg/mL and 0.001 mg/mL. The standard solutions of NDZOP, ZOPNO and ACP were dissolved in methanol while ZOP was dissolved in acetonitrile (due to ZOP instability in methanol). From one set of standard solutions, five combined working solutions containing all analytes were made in acetonitrile to be used for calibration samples. From the other set, two (blood method) or three (urine method) combined working solutions were made for QC samples. A stock solution of ZOP-D_4 (internal standard) was prepared at 0.02 mg/mL in acetonitrile and a working solution was prepared at 0.001 mg/mL. All solutions were stored at –20˚C.

Analytical methods

Sample extraction/sample preparation (Papers I–IV)

Papers I–II: 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) and the organic extract was transferred to a sampler vial. QC samples at three levels (zero, low, and high) were extracted along with each batch of samples. An aliquot of 5 µL was injected into the GC-NPD system.

Paper I (degradation experiment): To identify and quantify ZOP 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-tertbutylether 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 solvent A and B. An aliquot of 2 µL was injected and analyzed by LC-MS/MS system.

Paper III: The studied samples were prepared by adding 40 µL internal standard (IS), 0.05 M sodium acetate buffer pH 6.0 (760 µL) and 200 µL urine were directly into an auto sampler vial (1.5 mL). Calibration samples at five levels and QC samples at four levels (zero, low, medium and high) were prepared by spiking to appropriate concentrations and to appropriate buffer volume. An aliquot of 5 µL was injected into the LC-MS/MS system.

Paper IV: To 0.5 g of blood 25 µL zopiclone-D4 and 0.5 mL 0.5 M Tris buffer pH 8.5 were added before liquid-liquid extraction with 3 mL tert-butyl-methyl-ether for 10 min. After centrifugation (5000 rpm) the samples were frozen for 20 min at −80°C. The organic phase was transferred to a new tube containing 20 µL 1% hydrochloric acid in methanol, mixed and evaporated at ~30°C under a stream of nitrogen. The residue was reconstituted in 100 µL mobile phase. Calibration samples at five levels and QC samples at three levels (zero, low, and high) were extracted along with each batch of samples. An aliquot of 5 µL was injected into the LC-MS/MS system.

Gas chromatography (Papers I–II)

GC-NPD analysis was performed on a Hewlett Packard (HP) 5890 GC (Waldbronn, Germany). 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. 7**.

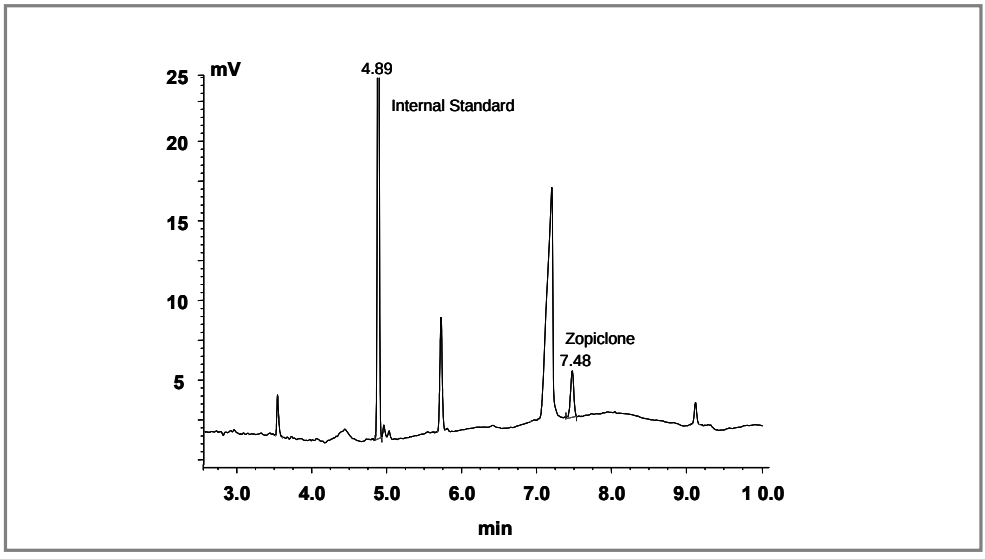

Fig. 7. Example of chromatogram from blood extract of a control sample at low level 0.02 µg/g. Internal standard is prazepam (4.89) and the peak before zopiclone (7.48) is cholesterol.

This GC-NPD assay is routinely used and method validation was performed before the stability investigations (Papers I–II). Linear calibration curves were obtained in the range of 0.01 to 2 µg/g. Inter- and intraday variability were determined at two levels (0.02 and 0.50 µg/g) and expressed as percentage coefficient of variation (CV%). 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. LOD was 0.007 µg/g and LLOQ was 0.02 µg/g. Recovery in the assay was 51%. ZOP metabolites and degradation products were not included in this method.

Liquid chromatography (Papers I, III–IV)

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.

Paper I: The acquisition method included two transitions for ZOP (389.1/217.0 and 389.1/245.1) and ACP (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.

Papers III–IV: The acquisition method included two transitions for ZOP (389.1/245.0 and 389.1/217.0), NDZOP (375.0/245.0 and 375.0/217.2), ZOPNO (405.2/245.2 and 405.2/143.2), ACP (129.1/76.1 and 129.1/93.1) and one transition for ZOP-D4 (393.1/245.0). The first specified transitions were used for quantification. Ultra Performance Liquid Chromatography (UPLC®) was performed using a 1.7 µm, 50 × 2.1 mm ACQUITY UPLC® ethylene bridged hybrid (BEH) C18 column (Waters Co.), operated in gradient mode at 0.6 mL/min with a total run time of 3.5 min. Solvent A consisted of 5 mM ammonium acetate buffer pH 5.0 and Solvent B of 0.05% acetic acid in methanol. Analysed data were processed using Sciex Analyst software (version 1.4.2).

Clinical chemical analysis (Paper II)

The erythrocyte volume fraction, plasma albumin and plasma α-1-glycoprotein were analysed 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 analysed by immunochemical technique (ADVIA 1800, Siemens, Deerfield, IL, USA and BN ProSpec, Siemens, Deerfield, IL, USA).

pH measurement (Paper III and supplementary analysis)

The pH of urine and blood samples was measured on an i-STAT® Analyser (Abbott Laboratories, Abbott Park, IL USA) in the range of 6.50−8.20. A few drops of urine or blood were applied into single use disposable i-STAT® G3+ Cartridges (Abbott Laboratories, Abbott Park, IL USA) with integrated calibration and the pH was calculated at 20˚C. The i-STAT® system is mainly used for whole blood analysis, but other fluids were accepted and

the pH of some urine samples was checked against a standard pH meter PHM210 from Radiometer (Copenhagen, Denmark).

Statistical analysis

The descriptive statistics used were mean, median, standard deviation and analysis of variance (ANOVA) were performed in Excel (version 2003). Wilcoxon Signed Ranks Test was used, when comparison between the different storage conditions 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. Pearson Correlation was used to analyse bivariate correlation between t0 and the results from each formula. A simple linear regression was performed to investigate how the respective formula (independent variable) influenced the t0 (dependent variable). The statistical analysis was performed using SPSS 16.0 for Windows (Paper I), SPSS Statistics 17.0 (Paper II) and IBM SPSS Statistics 20 (Paper IV).

RESULTS

Stability tests of zopiclone in whole blood (Paper I)

Storage stability

Short- and long-term stability: Long- and short-term stability was investigated at two concentrations of ZOP and at three different storage conditions in spiked whole blood duplicates. 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.

ZOP was most stable at –20°C. After five months 74–88% of the initial ZOP concentration was left of the low and high levels, respectively. However, after six months of storage some decrease in ZOP concentration was noticed which reached a 36–52% decrease in the end of studied period. In samples stored at 5°C degradation of ZOP 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.

Authentic and spiked stability differences: As regards long-term stability, authentic and spiked samples were compared during storage at 5°C. ZOP degradation over time was seen in both authentic and spiked blood samples stored at 5°C (at levels between 0.01–0.49 µg/g). 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 5**). 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 (between 2–14 days storage after sampling) was used as starting value while the initially measured concentration (day zero) was used as starting value in the spiked group.

Table 5. 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

Supplementary stability tests

Freeze-thaw stability: Freeze-thaw stability of ZOP in whole blood was evaluated in both authentic and spiked samples. There were no differences in ZOP concentration before and after the freeze-thaw cycle for any of the eight authentic samples with a ZOP concentration ranging from 0.03 to 0.15 µg/g. In the spiked triplicates 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 ZOP degradation was seen in the processed sample stability test (n=6) of the sample extract (butyl-acetate) reanalysis.

Identification of degradation product

Degradation experiment: The ZOP degradation was investigated in spiked samples (n=1) during 24 h incubation at 37°C. In these samples the ZOP metabolites NDZOP and ZOPNO were not detected. However, ACP was formed in approximately equimolar amounts to ZOP decay (**Fig. 8**).

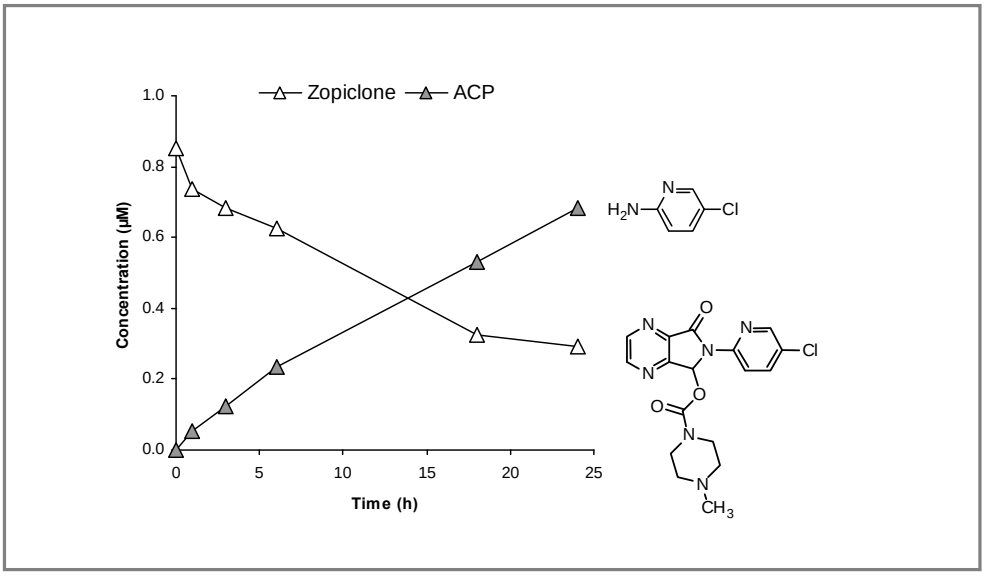

Fig. 8. Degradation of zopiclone and formation of 2-amino-5-chloropyridine (ACP) in spiked whole blood at 37°C.

Quality control

The precision (CV%) of the method during the 12 months investigation was 27% at low level (n=75) and 21% at high level (n=75) and the accuracy was in the range of 70–89%. During the eight months stability investigation in authentic and spiked blood the CV was 20% at low level (n=48) and 17% at high level (n=48) and the accuracy was in the range of 75–88%.

Influence of pre-analytical conditions on the interpretation of zopiclone (Paper II)

Stability in authentic blood compared with spiked blood

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 ZOP blood levels lower than expected and therefore four of the spiked samples were excluded (spiked at 0.15 µg/g). ZOP 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 ZOP in authentic and spiked blood showed the same decreasing trends at 20°C and 5°C as depicted in **Fig. 9.**

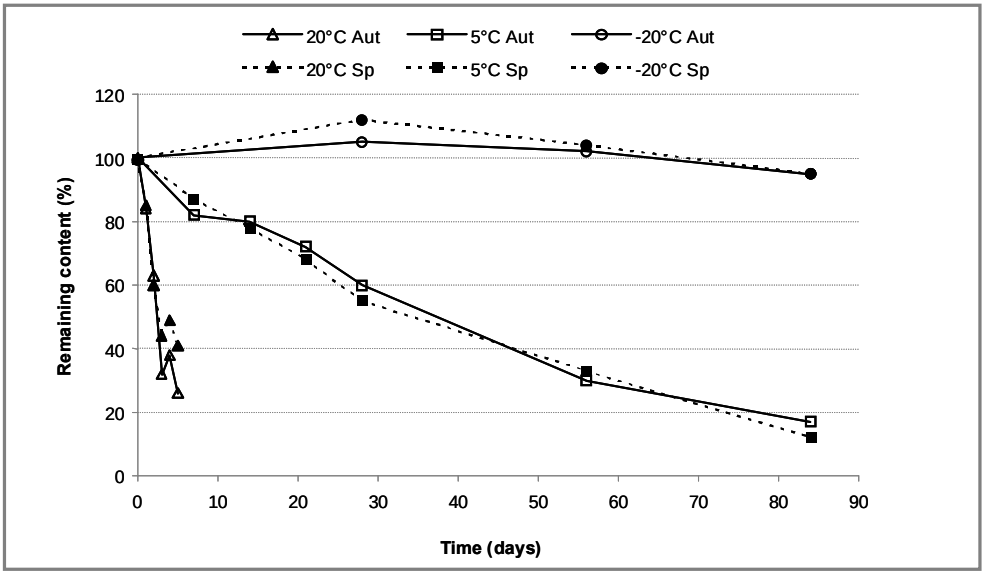

Fig. 9. Influence of storage conditions on zopiclone in authentic whole blood samples for nine subjects and in spiked whole blood samples for five subjects (mean values) during a period of 5 days at 20°C, 84 days at 5°C and at –20°C.

Stability evaluating

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. α-1-acid-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 ZOP content was seen already after one day of storage at 20°C in the authentic blood and after two days of storage in the spiked blood. At 5°C there was a significant difference in remaining content after two 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

The precision (CV) of the method during the three months investigation was 9% at low level (n=36) and 11% at high level (n=36) and the accuracy was in the range of 105–117%.

Quantitative analysis of zopiclone, N-desmethylzopiclone, zopiclone N-oxide and 2-amino-5-chloropyridine in urine using LC-MS/MS (Paper III)

LC-MS/MS validation

Selectivity tests showed the absence of endogenous as well as exogenous interferences when different sources of drug-free urine matrix without additives, drug-free urine spiked with IS and drug-free urine spiked with hypnotics and sedatives type were analyzed. PE was more than 92% for all of the analytes at the tested levels. Ion suppression or ion enhancement (ME) was evaluated both qualitatively by post-column infusion as well as quantitatively at two concentrations with accepted results. The calibration model elected was for ZOP and ZOPNO quadratic (weighing 1/x) whereas linear for NDZOP and ACP (weighing 1/x), in the calibration range between 5–3000 ng/mL for ZOP, 15–3000 ng/mL for NDZOP, 10–3000 ng/mL for ZOPNO and 20–3000 ng/mL for ACP. Intraday precision, total precision and accuracy were within acceptance limit (<15% for precision and within 80–120% for accuracy). The method could be used for quantitative analysis of ZOP, NDZOP, ZOPNO and ACP within a chromatographic time of 3.5 min.

Stability tests were performed under conditions expected for the study with stability requirements <15% of the nominal values. The analytes were all stable in the urine matrix through three freeze-thaw cycles and in prepared samples for 24 h in the autosampler at 10˚C. In case of storage prior to analysis, prepared samples also could be kept under this condition. The differences between peak areas from stored standard solutions versus freshly prepared were all less than 10% except from concentrations of NDZOP that differed 13% and 25% for the solutions of 0.01 mg/mL and 0.001 mg/mL respectively. Storage stability of the analytes in urine was determined at three different temperatures and showed stability for ACP during all studied conditions. ZOP was stable two days at 20˚C, three weeks at 4˚C and one month at −20˚C. NDZOP was stable one day at 20˚C, three weeks at 4˚C and one month at −20˚C. ZOPNO was stable less than one day at 20˚C, one week at 4˚C but one month at −20˚C.

Evaluation of room temperature storage showed that ACP was formed in equimolar amounts of ZOP, NDZOP as of ZOPNO.

Degradation or formation study

The degradation and formation study investigation showed that time, temperature and pH influenced the stability of analytes and showed that the ACP formation was dependent upon these factors. Because of refrigerator temperature problem, four subjects' samples were excluded from the study. The degradation of ZOP, NDZOP and ZOPNO over time at the three different storage conditions, and the very close equimolar ACP formation were reported in Paper III. In **Fig. 10** degradation of ZOP, NDZOP, ZOPNO are shown separately for each analyte together with the ACP formation.

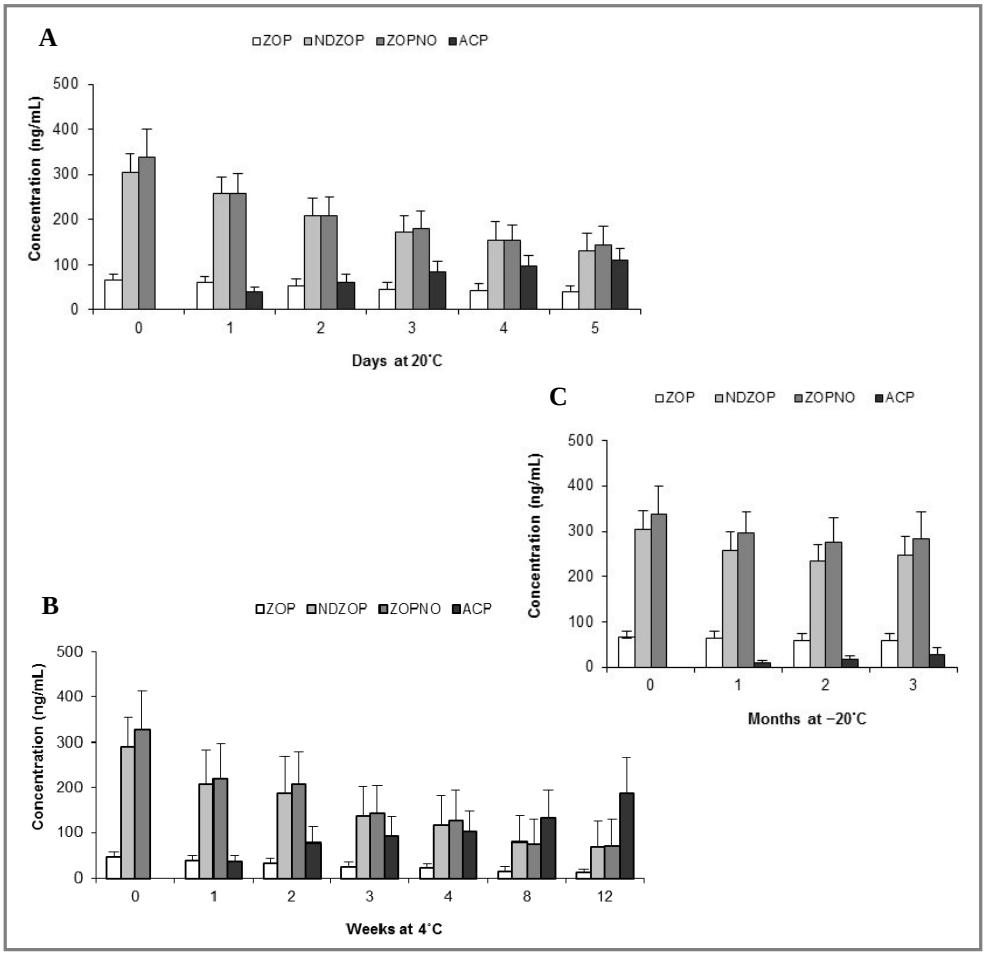

Fig. 10. Degradation of zopiclone (ZOP), N-desmethylzopiclone (NDZOP) and zopiclone N-oxide (ZOPNO) and the 2-amino-5-chloropyridine (ACP) formation over time in stored urine samples at three different storage conditions (**A**) at 20˚C (n=9) (**B**) at 4˚C (n=5) and (**C**) at −20˚C (n=9). The concentrations in the bars are based on mean ± s.e.m. values. Values <LLOQ has been calculated as zero.

However, pH had the greatest impact on ACP formation. More than 20% of ACP (of total amount ZOP, NDZOP and ZOPNO) was formed within one day at 20˚C, one week at 4˚C and

two or three months at −20˚C in urine samples with initial pH of 7.7. Corresponding ACP formation in urine samples with initial pH of 6.8 and 6.9 was seen first after five days at 20˚C, 12 weeks at 4˚C and was not seen after three months at −20˚C. The pH dependent ACP formation is exemplified in **Fig. 11** and shows the formation from each subject.

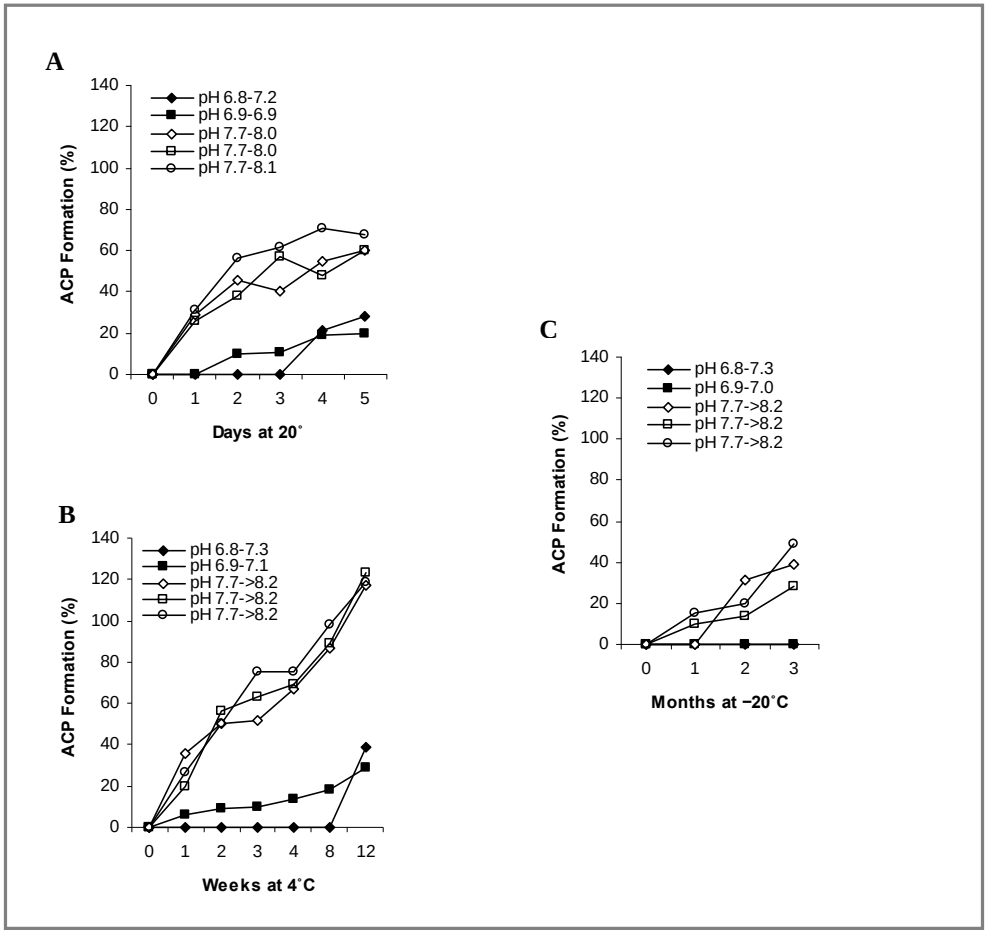

Fig. 11. Formation of 2-amino-5-chloropyridine (ACP) in urine samples (n=5) with different pH values and during different storage conditions (**A**) at 20˚C (**B**) at 4˚C and (**C**) at −20˚C.The ACP formation is expressed as a percentage of total amount zopiclone (ZOP), N-desmethylzopiclone (NDZOP) and ZOPNO. The pH (mean values) is specified in the range between initial (day zero) and ended pH value.

In addition, storage stability in authentic samples could be evaluated from the study. ZOP was stable one day at 20˚C, less than one week at 4˚C and three months at –20°C. NDZOP was stable one day at 20˚C, less than one week at 4˚C and one month at –20°C. ZOPNO was stable less than one day at 20˚C, less than one week at 4˚C and one month –20°C.

Forensic cases

In the first case, ZOP, NDZOP and ZOPNO were not detected but instead high concentrations of ACP were found in the urine sample with pH>8.2. In the second case, ACP was not detected, whereas ZOP, NDZOP and ZOPNO could be quantified in the urine sample with pH<6.5 (**Table 7**).

Table 7. 2-amino-5-chloropyridine (ACP), zopiclone (ZOP), N-desmethylzopiclone (NDZOP), zopiclone N-oxide (ZOPNO) and pH in urine samples from two forensic cases. ND: Not detected.

	ACP (ng/mL)	ZOP (ng/mL)	NDZOP (ng/mL)	ZOPNO (ng/mL)	pH
Case 1	3600	ND (<5)	ND (<15)	ND (<10)	>8.2
Case 2	ND (<20)	8	107	20	<6.5

Quality control

The correlation coefficients were >0.999 for all calibration curves (n=38) during the study with control CV% less than 10% for all analytes at three measured concentrations low, medium and high (n=38).

LC-MS/MS determination of 2-amino-5-chloropyridine to estimate the original zopiclone concentration in stored whole blood (Paper IV)

LC-MS/MS validation

The selectivity tests showed no interfering peaks present at the retention times of the analytes or internal standard when drug-free samples from different donors, drug-free blood spiked with internal standard and drug-free urine spiked with possible interfering substances were analysed. PE was >82% for ACP, ZOP and ZOP-D4 and 40% for NDZOP. RE was >85% for ACP, >82% for ZOP, 96% for ZOP-D4 and 45% for NDZOP. Ion suppression or ion enhancement (ME) was evaluated both qualitatively by post-column infusion as well as quantitatively at two concentrations with evaluated accepted results. The calibration model elected was for ACP and ZOP linear (weighing 1/x) whereas quadratic for NDZOP (weighing 1/x), in the determined calibration range between 1–150 ng/g for ACP, 2–150 ng/g for ZOP and 1–30 ng/g for NDZOP. Intraday precision, total precision and accuracy were within acceptance limit (<15% for precision, <20% for precision at LLOQ and within 80–120% for accuracy). The method could be used for quantitative analysis of ACP, ZOP and NDZOP within a chromatographic time of 3.5 min. An example of chromatogram is shown in **Fig. 12.**

Stability tests were performed under conditions expected for the study with stability requirements <15% of the nominal values. ACP was stable through all stability tests except processed stability at low level. ZOP was stable one day at 20°C, one week at 4°C, and one month at –20°C. ZOP was stable during 24 h as processed sample and stable through three freeze-thaw cycles. NDZOP was stable less than one day at 20°C, less than one week at 4°C, and two weeks at –20°C. NDZOP was stable during 24 h as processed sample and stable through one freeze-thaw cycle. The stability experiments at 4°C showed that ACP was formed in equimolar concentrations independently from both ZOP and from NDZOP. In addition, storage stability for ZOP and NDZOP in authentic samples could be evaluated from the formation and degradation study (see below) where ZOP was stable one day at 20˚C, one week at 4˚C and three months at –20°C. NDZOP was stable less than one day at 20˚C, one week at 4˚C and two months at –20°C.

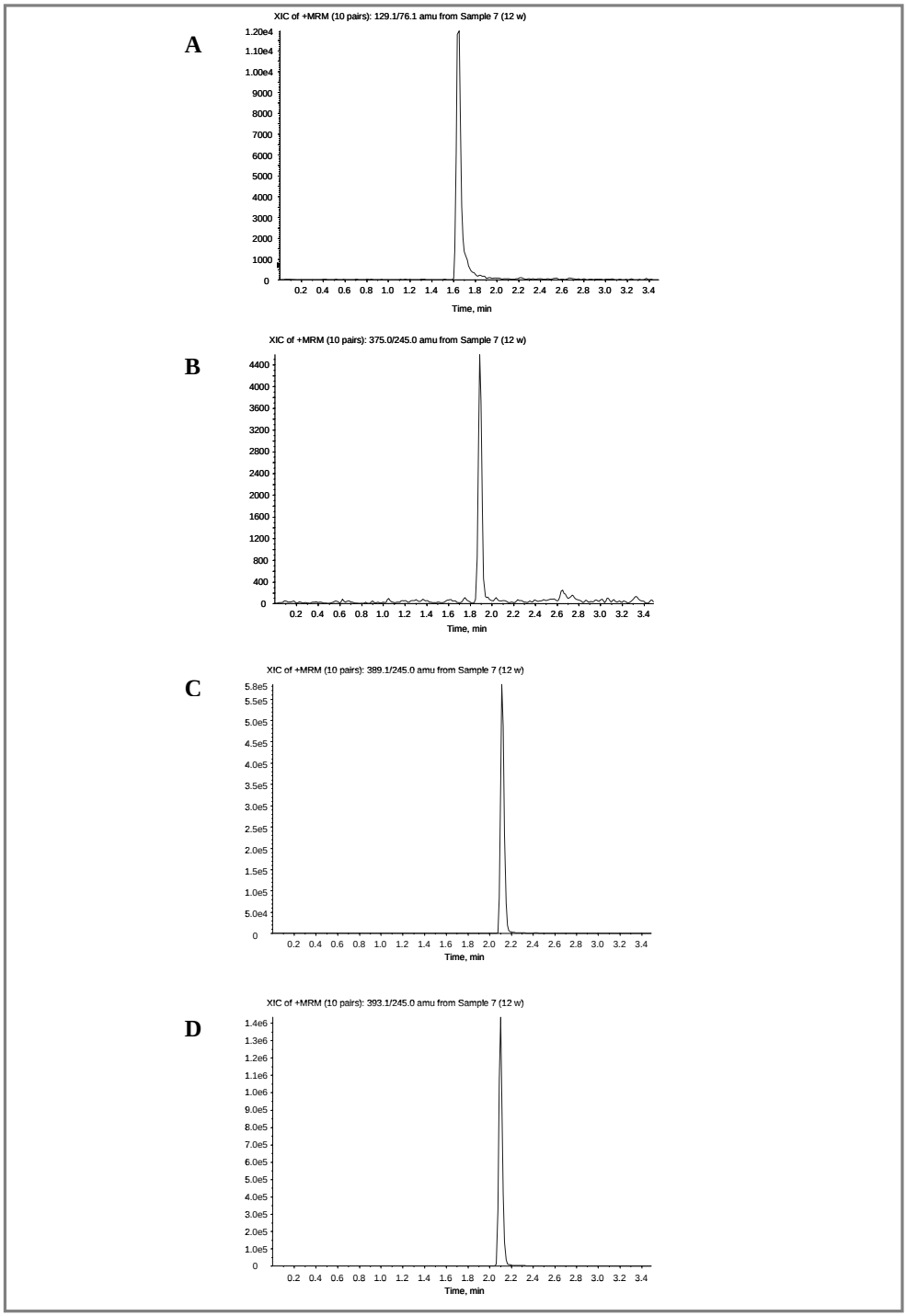

Fig. 12. LC-MS/MS chromatogram from a subject stored 12 weeks at 4˚C containing **A** 2-amino-5-chloropyridine (13 ng/g), **B** N-desmethylzopiclone (1 ng/g), **C** zopiclone (29 ng/g) and **D** zopiclone-D4 (internal standard).

Formation and degradation study

The results from the study showed that ACP increased from 1.3 to 7.8 ng/g during five days at 20˚C. At 4˚C, ACP increased from 1.1 to 8.2 ng/g during 12 weeks of storage. ACP in authentic blood was formed in amounts equimolar to the ZOP and NDZOP degradation after storage at 4°C for 12 weeks, and after storage at 20°C for four days. At –20°C, no ACP formation was observed (**Fig. 13**). Because of refrigerator temperature problem, four subjects' samples were actually stored at 10˚C and were therefore excluded from this figure.

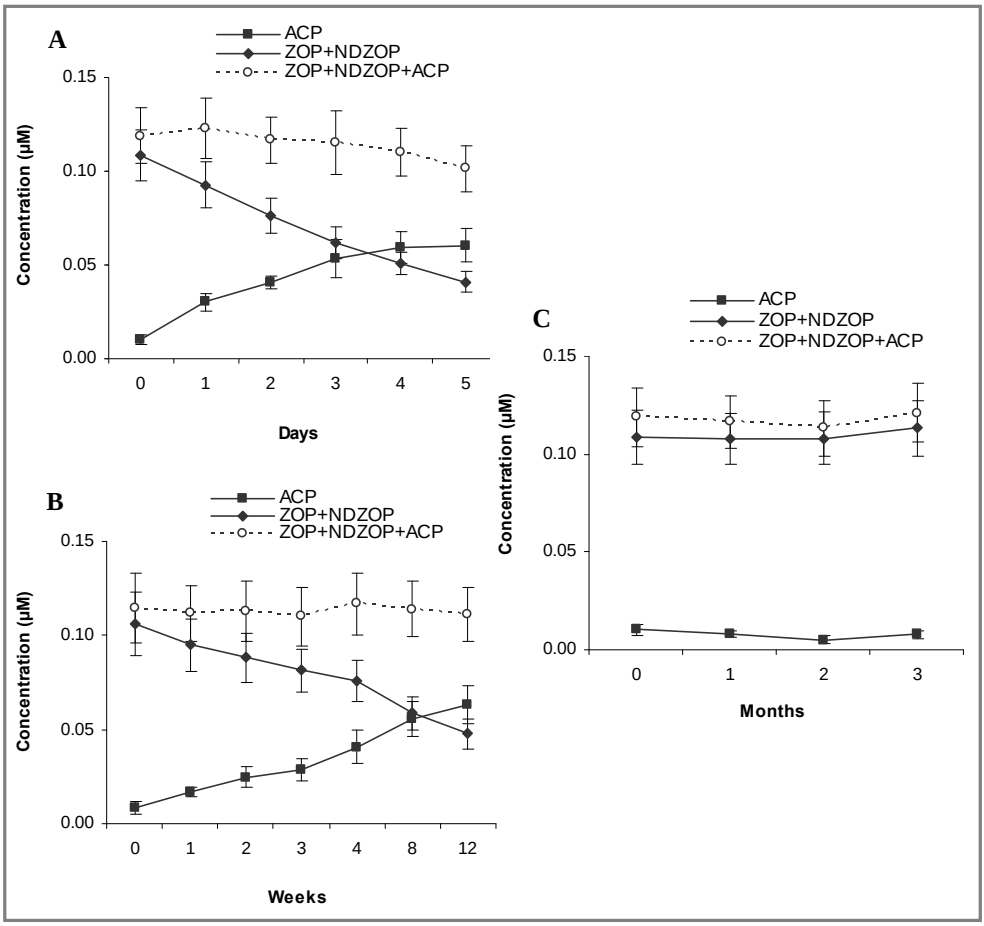

Fig. 13. 2-amino-5-chloropyridine (ACP) formation in relation to the zopiclone (ZOP), and N-desmethylzopiclone (NDZOP) degradation over time in authentic whole blood samples stored at three different storage conditions **A** at 20˚C (n=9) **B** at 4˚C (n=5) and **C** at −20˚C (n=9). Each point represents the mean ± s.e.m. Values <LLOQ have been calculated as zero.

Estimation

Results from samples in which ACP had been formed (n=99) were used to test two models to estimate the original ZOP concentration. The correlation tests for both formulas showed strong correlations to the original ZOP concentration (r=0.960 and r=0.955) with p<0.01. From simple linear regression, adjusted R Square (R2) was 0.921 and 0.913 and the low p-value (p<0.001) indicated significant models for the estimation formulas. The regression based linear fit line and the 95% confidence intervals are demonstrated in **Fig. 14.**

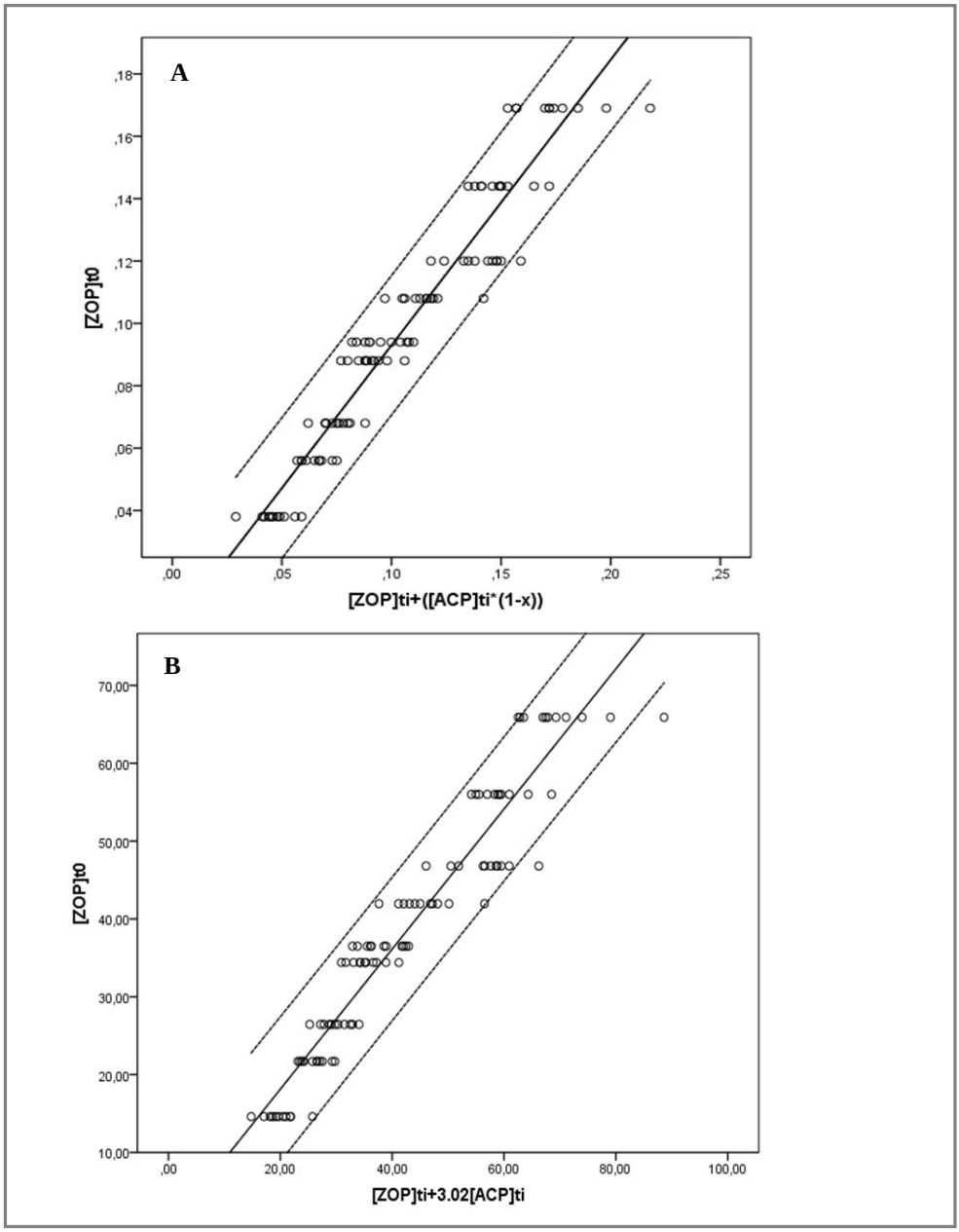

Fig. 14. Zopiclone (ZOP) original concentration [t0] (n=99) explained by **A** [ZOP] ti + ([ACP]ti * (1–x)) with calculations in μM and **B** [ZOP]ti + k[ACP]ti with calculations in ng/g. The linear regression fit lines is shown with 95% confidence of individual. Abbreviations in the formulas: ti is actual concentration, ACP is 2-amino-5-chloropyridine, NDZOP is N-desmethylzopiclone, x= [NDZOP]/[ZOP], k=389 (molecular weight of ZOP)/129 (molecular weight of ACP) = 3.02.

Quality control

The correlation coefficients were >0.999 for all calibration (n=39) curves during the study with control CV% less than 15% for all analytes at low level as well as at high level (n=52).

Supplementary data

In connection with the formation and degradation study in Paper IV, also the pH in the studied samples was measured. Additives might affect stability and explain any differences between stability investigations. Therefore, pH in whole blood samples containing fluoride was compared with whole blood samples containing heparin. In addition, pH in post-mortem femoral blood samples from forensic cases kept different period of time at 4–6 ˚C was investigated. Also, ZOP, NDZOP and ACP concentrations were measured using the LC-MS/MS method (Paper IV).

pH in whole blood samples

In the formation and degradation study, initial pH of the blood samples in the formation and degradation study varied between 7.8 and >8.2 and with no noticeable changes during the studied period.

Comparison of pH at 20˚C and at 37˚C between additives in blood with sodium fluoride and potassium oxalate and in blood with sodium-heparin as additive showed a mean value of 7.9 and 7.7 respectively (n=7). The results are shown in **Table 8.**

Table 8. Comparison of pH at 20˚C and at 37˚C in blood containing sodium fluoride and potassium oxalate and in blood containing sodium-heparin.

Sample number	pH 20˚C		pH 37˚C	
	NaF	Na-Heparin	NaF	Na-Heparin
1	7.983	7.696	7.700	7.442
2	8.004	7.705	7.719	7.449
3	7.791	7.871	7.527	7.599
4	7.787	7.741	7.523	7.482
5	8.021	7.691	7.734	7.437
6	7.934	7.712	7.656	7.456
7	8.008	7.744	7.723	7.485
Mean value	7.933	7.737	7.655	7.479

LC-MS/MS analysis and pH measurement in post-mortem samples

In post-mortem femoral blood, the pH was acidic at least between two and ten weeks of storage and ACP concentrations were observed in all analysed samples.

The results of pH in post-mortem femoral blood containing potassium fluoride and the results from LC-MS/MS analysis after different storage times at 4–6 ˚C are shown in **Table 9** below.

Table 9. Post-mortem femoral blood containing potassium fluoride stored at 4–6 ˚C for different periods of time.

Sample number	Storage time (weeks)	pH (20˚C)	ZOP (ng/g)	NDZOP (ng/g)	ACP (ng/g)
1	2	<6.5	324	96	53
2	2	<6.5	27	25	7
3	2	<6.5	513	155	102
4	3	<6.5	326	21	32
5	5	<6.5	258	21	38
6	5	<6.5	148	84	48
7	5	<6.5	77	17	22
8	5	<6.5	90	12	12
9	10	6.5	115	66	46
10	10	6.5	39	7	13
11	10	6.6	151	23	85
12	11	6.7	189	40	136

DISCUSSION

The investigations described in this thesis have shown the importance of considering the stability of drugs in biological samples. The overall aim was to investigate the stability of the hypnotic drug ZOP and factors of importance for degradation during storage in biological samples and to identify consequences for interpretation of results in forensic toxicology.

Methodological aspects

The studies were based both on existing stability study designs (Papers I–II) and on guidelines for methods used in research projects [22] (Papers III–IV). Stability data in a particular matrix should not be extrapolated to other matrices [26]. These stability investigations of ZOP 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.

In stability tests during method validation it is common to use a drug-free pooled matrix but both blood and urine are complex matrices and therefore pooled matrix might influence the results (Paper I). To exclude 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). In Paper IV, the matrix used in stability tests during method validation was the same as that used in the formation and degradation study.

Whole blood collected from a local blood bank was found to contain a pH stabilizer and was therefore not used for the method validation tests. The pH of the urine matrix used for stability tests might influence drug stability. Initial pH of study samples (Paper III) was in the range between <6.5 to 7.7 while the pH of the urine matrix used for stability tests in method validation was 7.0. For a comprehensive stability study, pooled drug-free urine should cover both acidic and basic conditions.

Stability investigations using blood samples from dosed subjects are recommended as most appropriate [26] but this usually involves only low concentrations. Usually one low and one high concentration within the calibration range of the analytical method should be tested [19,26]. In these studies both low and high levels in the range between 10–590 ng/g were obtained, which included both therapeutic (Papers II and IV) and toxic levels from forensic cases (Paper I) as well as low and high levels according to the analytical methods.

By increasing the number of replicates and sample size random error will be decreased. In the method validation six replicates were performed for stability tests (Papers III–IV), but because of sample volume in the studies only duplicates (Papers I, III–IV) or triplicates (Paper II) were applied. Also, the sample size was increased by the repeated studies. As a whole, the stability studies in whole blood have been both internal validated by repeating studies (Papers I, II and IV) as well as by an external validation [63] increasing the reliability.

Stability calculations based on statistical tests are sometimes used, but defined acceptance criteria for stability is preferred if concentration differences are small or if the precision of the analytical method is poor [23]. Intra- and inter analytical processing errors (variability errors and bias errors) have to be considered in the calculations [26]. In these studies the stability was evaluated both statistically, by acceptance criteria connected to analytical variations and by the stability criteria of <15% loss from the nominal value. The nominal value or the reference value in these studies was either the initially measured concentration or the measured concentration from freshly prepared samples. Additionally, by stability test performance in the method validation (Papers III–IV), when fresh (reference) samples and stored stability samples were analysed simultaneously the between-run variation was eliminated. The nonparametric Wilcoxon Signed Ranks Test

and Mann-Whitney U-test wereused because of outliers and not normally distributed results. The different evaluation procedures gave the same result for ZOP storage stability.

In some analytical methods for ZOP the procedure included an enzymatic hydrolyzing step with β-glucuronidase [127,138], mainly because the methods also included benzodiazepines. Another study showed that ZOP metabolites are excreted mainly unconjugated [142]. Urine samples have also been processed without hydrolysis because of analyte instability during incubation [137]. No hydrolyzing step was used in the method in Paper III.

In Paper IV, there were some limitations. The analytical method did not include ZOPNO, which also degrades to ACP. Therefore, the formulas will overestimate the original concentration of ZOP if ZOPNO was present in the sample and had degraded. ACP could also be an impurity from Imovane®, but should be less than 0.5% [151]. In addition, ACP may be an *in vivo* metabolite from the oxidative decarboxylation pathway of ZOP. Both these additional origins for ACP may result in overestimation of the ZOP concentration. Of the three, ZOPNO is the one that might significantly influence the estimation. The reasons for not including ZOPNO were both analytical and practical. It has proven difficult to analyse ZOPNO in blood [63] or plasma [119] and in the method development it showed very poor recovery using liquid/liquid extraction. A more expensive and complicated solid phase extraction is needed to determine ZOPNO [131]. That would be the most optimal state and should give a better estimated original ZOP concentration (Paper IV).

Stability investigations

Bio-samples are often kept at ambient temperature during sampling, transport, registration and aliquoting prior to analysis. Stability investigations of analytes in samples stored under these conditions are therefore of great value when results are interpreted. The short-term stability investigation of ZOP in both spiked and authentic whole blood showed a very poor stability during storage at 20°C. The concentrations of ZOP decreased by more than 50% after three days of storage (Paper I) and the stability of ZOP was approximately one day (Paper II and IV). Many samples are kept overnight at ambient temperature when transported from the sampling site to the laboratory and are kept for at least a few hours at room temperature during registration. The poor stability at 20°C warrants that sampling, transport, registration and preparation should be done within 24 h or at a controlled lower temperature.

Before and after analysis, bio-samples are stored refrigerated and knowledge of long-term stability at various temperatures is therefore needed. At 4°C, degradation of ZOP occurred already after a few weeks and after five months of storage, there was no measurable concentration of ZOP in blood samples (Paper I). By repeated testing of both spiked and authentic samples, a stability of one week was established (Papers II and IV). After storage for four weeks the degradation of ZOP was approximately 50%, which is in agreement with the 28.4 days reported as *in vitro* half-life of ZOP [63]. This means that analysis should be carried out within this time period. Laboratory routines should be reviewed if samples are kept at this temperature for a longer time.

Long-term stability was also investigated at freezer temperature of –20°C, which is commonly used for long-term storage of bio-samples after the initial analysis. Stability investigation under these conditions is important if a reanalysis is required. Results from the investigations (Papers I, II and IV) showed that stability of ZOP was best when samples were stored at –20°C. In both authentic and spiked samples (Papers II and IV) no evidence of degradation was found during the periods of storage tested. Stability of ZOP in frozen samples was at least three months.

Knowledge of freeze-thaw stability is also needed in connection with reanalysis. Freeze-thaw stability of ZOP was therefore tested in spiked (Papers I and IV) and in authentic whole blood (Paper I). Results from the freeze-thaw cycles showed good stability through the tested cycles. If the analysis of ZOP cannot be done within a week, the samples should be frozen at –20°C to ensure accurate results.

In routine laboratory work, instrument problems may appear during analysis, which leads to a delayed analysis. Knowledge of processed stability (extracted samples) is therefore useful in cases of delayed injection of extracts. The stability investigation in this thesis included this type of test and showed that ZOP reconstituted in butyl acetate (Paper I) was stable for two days. However, in samples reconstituted in ammonium acetate/methanol (Paper IV) ZOP was only stable for one day.

The stability data from the studies of ZOP in whole blood during different storage conditions agreed well. These results also agree with earlier stability tests of ZOP in plasma [118,119,129]. However, the concentration of ZOP in spiked samples decreased more after 12 months of storage at –20°C compared to authentic post-mortem femoral blood [43]. This is an interesting finding that might be explained by pH shift in the matrices where a low pH is favorable for ZOP stability [117]. The pH of post-mortem blood tends to be acidic, whereas an alkaline shift takes place in fresh heparinized blood after storage at 4°C and at 20°C but not at –20°C [33]. In whole blood preserved with sodium fluoride an alkaline shift was observed initially but without any noticeable change during the remaining storage period (Paper IV). In this study, whole blood samples containing sodium fluoride and potassium oxalate had a higher pH compared with blood samples preserved with heparin. The addition of sodium fluoride might protect from enzymatic degradation [154] but not from chemical hydrolysis. Stability studies should therefore be performed in the same matrix as normally received in forensic toxicology casework (Papers I, II and IV).

In whole blood, the stability of NDZOP was also investigated. Storage stability tests in Paper IV could be evaluated both from method validation and from the formation and degradation study. A previous study showed that NDZOP in human plasma was stable for at least 12 h at room temperature, during 5 freeze-thaw cycles, and during 74 days at –20°C [131]. The results from this study showed that NDZOP was stable for less than one day at room temperature both in method validation and in the formation and degradation study (Paper IV). Also the stability was poor during the freeze-thaw cycles where NDZOP was stable for one cycle only. The long-term stability results differed between method validation and the formation and degradation study. During method validation NDZOP was stable less than one week at 4°C and only two weeks at –20°C, whereas in the study it was one week at 4°C and two months at –20°C. That discrepancy might have two possible explanations. Firstly, low therapeutic levels were observed during the study compared to the high QC-level in the method validation stability test. Secondly, NDZOP might be more stable in authentic blood compared to spiked blood.

Stability tests of ZOP in stored urine samples have not been reported before [127,137-139,142]. Storage stability tests in Paper III could be evaluated both from method validation and from the degradation and formation study. Stability results from method validation indicated slightly better stability compared to results from the actual study. For instance at 20˚C, method validation showed that ZOP and NDZOP were stable for two days whereas in the study the corresponding stability was only one day. After storage at 4˚C the stability was less than one week for ZOP, NDZOP and ZOPNO in the study compared to three weeks for ZOP and NDZOP and one week for ZOPNO in the method validation. At −20˚C there was no difference in stability. In the method validation, stability samples were compared with freshly prepared control samples and calculated from the same calibration, but in the study, stored samples were compared with initial values and quantitated with different calibrations. This

may have influenced the results. The initial pH of the authentic urine samples used in the study varied from <6.5 to 7.7, while the pH of the spiked urine used for method validation was 7.0. Presumably, pH was the most important factor impacting on the difference in stability.

In the first study (Paper I) it was verified that ZOP in spiked blood undergoes degradation to ACP during storage [150] and this confirmation as well as the ZOP instability in whole blood samples has recently been repeated [63]. ACP showed good stability in both whole blood and urine samples during all stability tests in the method validations, except at the low level in whole blood processed samples. Considering this, the measurement of ACP may be an additional help to the toxicologist when interpreting results. Therefore, the degradation and formation studies were performed (Papers III–IV).

Degradation and formation of 2-amino-5-chloropyridine

The relationship between the formation of ACP and the degradation of ZOP, NDZOP or ZOPNO in whole blood and urine was a very important finding. Especially since ACP has been considered as an unique decomposition product to ZOP, NDZOP and ZOPNO, and no other drugs have been found decomposing to ACP [149].

However, ACP has also been explained as one among other inactive metabolites from a ZOP decarboxylation pathway [93] or as an impurity in ZOP tablets [151]. Urine samples in Paper III were collected 10 h after ZOP intake and then concentrations of ACP were below LLOQ (20 ng/mL). The ACP concentrations could therefore only be explained by formation from ZOP, NDZOP or ZOPNO degradation. Blood samples in Paper IV were collected 2 h after ZOP intake and then concentrations of ACP were between <1 and 1.9 ng/g. Hence, the minor detected amount of ACP in some blood samples might be explained either by the metabolism, tablets or by rapid ZOP degradation.

The investigations in Paper III showed that ACP is formed in equimolar amounts to ZOP, NDZOP and ZOPNO degradation in both spiked and authentic urine. Because high concentration of metabolites were present in urine and ACP is formed from both parent drug and its metabolites, no relationship between ACP concentrations and the original concentrations of ZOP, NDZOP or ZOPNO in urine could be established.

The investigations in Paper IV showed that ACP is formed in equimolar amounts to ZOP and NDZOP degradation in both spiked and authentic blood. In blood, the metabolites are present in much lower concentrations compared to the concentrations of ZOP. Therefore, ACP concentration was used in Paper IV to estimate the original ZOP concentration in a blood sample by mathematical formulas. From the data obtained (Paper IV) two formulas were tested, one that only includes the measured concentrations of ZOP and ACP, and one that also includes the concentration of NDZOP and where the portion of ACP that originates from the breakdown of NDZOP was subtracted. Both formulas showed significant correlations and explained more than 90% of the initial ZOP concentration. This can be explained by the fact that the concentrations of NDZOP in the samples collected two hours post dose were less than 29% of the ZOP concentration thus affecting the formula outcome little. NDZOP becomes more important later in the elimination when it is in similar concentrations as ZOP [131]. The impact of different ratios between ZOP and NDZOP in a blood sample on the accuracy of the formula should be further investigated to verify its applicability in case work. When ACP is detected together with ZOP, or when only ACP is detected the concentration estimated will be a maximum value. When both ZOP and NDZOP is present, it is possible to get closer to the original ZOP value by using the formula [ZOP]ti + k([ACP]ti *(1–x)) (**Fig. 14**).

In urine, the ACP formation occurred at elevated pH or temperature (Paper III). Previous studies have shown that urinary pH increases with increasing temperature [155]. In this study (Paper III) the measured pH lasted up to twelve weeks at 4˚C and at –20˚C and with different initial pH values. Over time, the pH increased under these conditions, but this had minor influence on the ZOP, NDZOP and ZOPNO degradation and the ACP formation at –20˚C. The effects of storage time, temperature and pH were clearly shown in Paper III and provide further information about *in vitro* ACP formation.

In the supplementary LC-MS/MS analysis of ZOP, NDZOP and ACP in post-mortem acidic femoral blood, measurable amounts of ACP were identified (**Table 9**). This was unexpected since ACP was not formed in acidic urine samples. The formation of ACP in post-mortem blood should therefore be further investigated.

Interpretation aspects

These studies have demonstrated the importance of well-controlled pre-analytical conditions after sampling and before analysis. The integrity of the analytical results can only be ensured if supporting stability data are available to confirm that degradation after sample collection has not occurred. The concentrations of ZOP in forensic cases have to be interpreted with caution if the sample has been stored at refrigerator temperature for a long time *and*/*or* if the sample has been stored at room temperature for 24 hours or longer in the pre-analytical phase. Otherwise, the concentrations measured will not reflect that present in the blood at the time of sampling and the interpretation will be misleading. ACP has been identified as a specific degradation product of ZOP, NDZOP and ZOPNO [149]. ACP analysis can therefore help to interpret concentrations of ZOP and/or its metabolites in stored whole blood or urine samples. Hence, for correct interpretation in forensic cases ZOP and its metabolites as well as ACP should be analysed. Absence of ACP strongly suggests that degradation has not occurred and the measured concentrations of ZOP, NDZOP and ZOPNO are correct. When ACP is found in a blood sample, the original concentration of ZOP can be estimated using a simple mathematical formula (Paper IV). When ACP is found in a urine sample the concentrations of ZOP, NDZOP and ZOPNO should be interpreted with great caution. In Paper III, two forensic cases are described and interpreted. In the case of a urine sample (pH>8.2) after long-term storage the involvement of ZOP was confirmed because of a high concentration of ACP indicating complete degradation of ZOP, NDZOP and ZOPNO. In the second short-termed urine sample (pH<6.5), the absence of ACP showed that the measured concentrations of ZOP, NDZOP and ZOPNO were reliable. These two examples show the benefits of analysing ACP in routine forensic toxicology. In addition, more complicated pre-analytical factors such as putrefaction, anaerobic metabolism and redistribution also complicate interpretation in post-mortem toxicology.

CONCLUDING REMARKS

Paper I

- This stability investigation showed that ZOP degrades in human blood depending on time and temperature and after long-term storage the drug might not be detectable.
- The best storage temperature was –20°C even for short storage times, because freeze-thaw cycles had no influence on the results.
- The rapid degradation of ZOP at ambient temperature can cause an underestimation of the true concentration and consequently flaw the analytical interpretation.
- Identification of ACP verified that ZOP undergoes degradation by chemical hydrolysis in spiked whole blood during storage.

Paper II

- Degradation of ZOP in authentic blood was about the same as that in spiked blood at the temperatures and times studied.
- ZOP is stable one day at 20°C, one week at 4°C and three months at –20°C in whole blood (containing sodium fluoride preservative).
- Considering the poor stability of ZOP at ambient temperature, the laboratory should carefully control temperature of storage during the pre-analytical phase to minimize degradation.

Paper III

- ACP is formed from ZOP and its metabolites NDZOP and ZOPNO in spiked and authentic urine samples. The formation is equimolar but the rate is dependent on the pH of the urine, time of storage and the temperature.
- Stability experiments during method validation should not only include different storage temperatures but also different urinary pH.
- For proper interpretation in forensic cases ZOP and its metabolites as well as ACP should be analyzed.
- When ACP is identified in a urine sample the concentrations of ZOP, NDZOP and ZOPNO should be interpreted with great caution.

Paper IV

- The equimolar degradation of ZOP and NDZOP to ACP can help to estimate the original concentration of ZOP in whole blood.
- Absence of ACP in the sample analysed strongly suggests that there has been no degradation and that the measured concentration of ZOP is reliable.

FUTURE PERSPECTIVES

The major focus of this thesis, was to study the stability of ZOP and its degradation in whole blood and urine during storage under different conditions.

The formation of the degradation product ACP was investigated and its absence or presence in blood and urine samples can be used to estimate the original concentrations of ZOP in blood. The absence of ACP in urine samples indicates that the original concentration of ZOP, NDZOP or ZOPNO are reflected, whereas the presence of ACP indicates degradation of the same. For proper interpretation in forensic cases, ZOP and its metabolites as well as ACP should be included in analysis.

- Measurements of ACP should be implemented in analytical methods routinely used in forensic toxicology laboratories.

- Estimation to the original ZOP concentration is feasible by use of mathematic models. However, before implementation in forensic practice the models need to be further investigated.

- In recent year, ZOP has been identified as the most common prescription drug in Swedish forensic autopsy cases representing all causes of death. The finding of ACP in post-mortem femoral blood at acidic pH was unexpected and further studies of the post-mortem relationship between ZOP and ACP in post-mortem blood specimens are necessary.

ACKNOWLEDGEMENTS

This thesis work was performed at the National Board of Forensic Medicine, Department of Forensic Genetics and Forensic Toxicology.

My sincere thanks to:

Robert Kronstrand, my supervisor, mentor and colleague. Thank you for accepting me as a graduate student and for sharing your deep knowledge in forensic toxicology, in forensic science and research, for your constant encouragement, never-ending optimism and for your professional guidance to complete this thesis.

Fredrik C. Kugelberg, my assistant supervisor and colleague. Thank you for sharing your extensive knowledge in forensic toxicology and for your endless support, guidance, advice and encouragement in science and research.

Johan Ahlner, my assistant supervisor and Head of the Department. Thank you for giving me the chance to make this thesis possible, for supporting the postgraduate studies and for your great enthusiasm in guiding me during the course of this work.

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

Ingrid Nyström, Liselotte Berglund and Malin Forsman; for your help with the human studies, for your great knowledge about planning and carrying out research trials, for collaboration and technical assistance. Maria Dolores Chermá Yeste, Eva Tärning, Karin Björnström Karlsson and Johan Ahlner; for your medical expertise and taking care of the subjects in the human subject study and Ingela Jakobsson for all your help.

Gunilla Thelander, Markus Roman and Maria Dahlgren; for sharing your technical knowledge and for your analytical expertise and assistance.

The “San-Francisco scientific group” at the SOFT-TIAFT meeting in San Francisco, California, USA (Robert K., Fredrik C. K., Wayne J., Martin J., Tor S., Anita H., Anna-Lena Z., Yvonne L. and Louise K.); for nice company and for support with my poster presentation.

The “Iceland scientific group” at the Nordic Forensic Toxicology Seminar in Iceland (Johan A., Fredrik C.K., Maria D. C. Y., Martin J., Gunilla T. and Nina R.); for nice company and for support during my oral presentation.

All the staff at the Institute of Legal and Traffic Medicine in Heidelberg, Germany, for welcoming me into your Institute and for taking good care of me during my practical training. Special thanks to Prof. Dr. Gisela Skopp for your great knowledge of pre-analytical issues in forensic toxicology, which has inspired me and taught me a lot. Thanks for letting me visit your department. Dr. Richarda Jantos, thank you for your excellent supervising in forensic science and research during my time in Heidelberg.

Anita Holmgren; for your fast and efficient help with retrieving data about forensic cases from the laboratory database.

Maria Edvardsson, my student friend from the Biomedical Science degree project; for the ideas about pH measurements.

Andreas Tillmar, Anna-Lena Zackrisson and Anna Jönsson, my PhD-colleagues; for your great encouragement, for valuable discussions in scientific methodology, statistics and practical issues.

Pernilla Haage, my PhD-student friend; for nice company and fun-times during our subject-specific postgraduate courses.

Gerd Jakobsson, my workroom companion; for helpful interventions, fruitful discussions, support and encouragement.

The entire staff at the Department of Forensic Genetics and Forensic Toxicology; for your contributions to create such a pleasant and amicable atmosphere to work in.

To my family:

Gördis och Ulla-Carin, my sisters; for care, support and friendship.

Fredrik, Johanna, Emelie, Emanuel and Ida, my sons and daughters; for your continuous love and support. Robert and Andreas my sons-in-law; for all valuable support. My granddaughter Siri for giving such great pleasure.

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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APPENDIX (PAPERS I–IV)

Papers