Charité University Hospital
Institute of Legal Medicine and Forensic Sciences
Department of Forensic Toxicology

Detection of psychoactive substances (antidepressants, antipsychotics, benzodiazepines, alcohol) in human hair and other matrices using liquid chromatography-mass spectrometry (LC-MS/MS)

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy

of Freie Universität Berlin

by

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born in Magdeburg

April 2020

Research of the present study was conducted from 2016 until 2020 under supervision of Prof. Dr. Michael Tsokos at the Institute of Legal Medicine and Forensic Sciences at the Charité University Hospital.

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Disputation day: 23.06.2020

Wer die Natur wirklich erforscht, wird dabei zum Mystiker. Und wer dabei kein Mystiker wird, ist kein echter Naturwissenschaftler (Albert Hoffmann, 1906 - 2008)

Acknowledgements

Many people must be thanked as fellow companions on the way to develop this thesis.

There is my supervisor Prof. Dr. Michael Tsokos whom I gratefully thank for the opportunity to work on this interesting and valuable topic and who gave me a place in the Institute of Legal Medicine to acquire the necessary skills and experience. My second supervisor Prof. Dr. Maria Kristina Parr from the Free University of Berlin always had an open mind, heart and enthusiasm when I asked for guidance and I am very thankful for that.

I humbly thank my colleagues in the Department of Forensic Toxicology PD. Dr. Sven Hartwig, Dr. Franziska Krumbiegel, Lena Westendorf, André Niebel, Denise Thurmann, Friederike Mörlein, Denise Sonntag, Isabell Mroske, Carmen Hoffmann, Dagmar Simmert, Sibylle Menzel, Andrea Handt and Sabine Borzekowski. Without you I would never have learned what I had to know as a scientist and as a person. Furthermore, I want to thank Prof. Dr. Dr. Fritz Pragst who always had an open door and constructive feedback when I struggled with a difficult problem. I also thank Janine Stramm, Carola Walter, Ayesha Alameri, Mahir Uzonovic, Jenny Liang for their help with the hair samples. In addition, I would like to thank all employees of the Institute of Legal Medicine who supported me and my work in direct and indirect ways and gave me the opportunity to profit from their work.

Franziska, thank you for being one of the nicest office mates I can imagine and for all the counsel and support you gave me throughout the years. Also, I would like to thank PD Dr. Hartwig for his advices and open door that gave me greater understanding in all aspects of forensic science.

I sincerely thank my family for bringing me into this life, raising me into the person I am today and always supporting me.

I would never have made it through those past years if it would not have been for you Denise, my fellow friend. You, Tobias, Robert and all the others carried me through my darkest moments and through the most joyous moments and I just thank you for being there with me.

Tabea, my love, I thank you for everything. I am excited for what life holds for us.

Fabiola, thank you for coming into my life, showing me this vibrant aliveness and waking me up to who I am now. I hope you are ok, wherever your soul is now.

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List of abbreviations

ATYP	Atypical neuroleptics
DDD	Defined daily doses
DFC	Drug-facilitated crimes
DIN ISO	German Institute for Standardization(DIN), International Organization for Standardization (ISO)
EDDP	2-(Dthylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
ESI	Electrospray ionization
EtG	Ethyl glucuronide
FAEE	Fatty acid ethyl esters
GC-FID	Gas chromatography flame ionization detection
GC-MS	Gas chromatography with mass spectrometry
GTFCh	German Society of Toxicological and Forensic Chemistry
HPLC-DAD	High-performance liquid chromatography with diode-array detection
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LC-QQQ-MS	Liquid chromatography with triple quadrupole mass spectrometry
LC-TOF/QTOF-MS	Liquid chromatography with quadrupole time-of-flight mass spectromety
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MRM	Multiple reaction monitoring
m/z	Mass-to-charge ratio
ng/mg	Nanogram per milligram
pg/mg	Picogram per milligram
Q1/2/3	Quadrupole 1/2/3
RSD	relative standard deviation
SoHT	Society of Hair Testing
SSRI	Selective serotonin reuptake inhibitors
TCA	Tricycliy antidepressants
TECA	Tetracyclic antidepressants
THC	11-nor-Δ9--tetrahydrocannabinol-11-carboxylic acid
TYP	Typical neuroleptics
UV	Ultraviolett

1 Introduction

1.1 The importance of hair and nail concentrations of psychoactive substances in forensic toxicology

Over the last thirty years, the retrospective detection of xenobiotics in hair has found its own valuable place in the work of forensic toxicologists. To date, numerous applications of hair analysis are performed in forensic laboratories all over the world. They can include aspects such as the human performance (workplace drug testing, abstinence control, doping control), the diagnosis of a recent drug abuse or chronic intoxication (drug abuse history, exposure to drugs during pregnancy, toxic environmental pollution), criminal liability and drug addiction, or the wide field of post mortem toxicology (health impairment or tolerance effects from chronic drug abuse, body identification, pattern of drug abuse, proof or exclusion of a drug administration, repeated criminal poisoning) [1]. On the other hand, hair analysis can be used in criminal assaults (single or repeated drug administration, control of regular intake by the offender), therapy adherence control (segmental hair analysis) or in the detection of excessive alcohol abuse [1].

Considerable research attention has been devoted to gain insight in the possibilities of the analysis of human hair. Several scientific reviews have described the useful advantages of hair as matrix for the detection of xenobiotics [2-7]. Compared to body fluids (e.g. blood, urine) it allows for a wider detection window after drug exposure while sampling itself is non-invasive and discrete [2]. Preferably, the hair is sampled from the scalp, as most of the research is conducted with scalp hair. If scalp hair is not available (e.g. from reduced hair growth or haircut), body hair from different body sites (e.g. beard, arm, leg, breast, pubic area) can be used under certain limitations for interpretation.

Today, toxicologists are not only able to detect and quantify a high number of substances in human hair, they are also in the position to make valid interpretations of these results. Tools like the segmental analysis or the detection of metabolites have demonstrated their value to achieve even better interpretations of the analytical results.

Until now, the vast majority of research in hair analysis has focused on the detection of drugs of abuse and markers of alcohol consumption. Based on this profound knowledge scientific communities like the Society of Hair Testing (SoHT) have been able to recommend cut-off values for a high number of illegal drugs [8]. In addition, metabolite-parent drug ratios have been proposed to discriminate between an actual drug use or a contamination [8, 9].

Due to their sedative properties [10, 11], their relatively high toxicity [12] and their broad prescription frequency [13], psychotropic drugs (antidepressants, antipsychotics) can often be part of certain forensic case constellations. Only few studies reported the examination of the detection of drugs like antidepressants, antipsychotics or sedatives. Approaches for a qualitive library-based identification of xenobiotics in different matrices have shown to be applicable for the detection of these substances [14]. Yet, the identification alone does not allow to make assumptions about the frequency of intake. Quantitative data, established from the analysis of a population with known intake, can be used for comparison and better interpretation of the frequency of use. Therefore, the examination of quantitative data on this group of substances is an important area of enquiry. A broad base of quantitative data of these substances is crucial to properly investigate certain case constellations. An example is given to illustrate the complexity of such constellations.

A young child was found dead at home. Autopsy revealed unambiguous signs of an intoxication while the toxicologic examinations proved an intoxication with tricyclic antidepressants. The question arises if the antidepressants were stored close to the child and the child accidentally took them. On the other, the antidepressants may have been intentionally used to sedate the child. Hair analysis may give a rough guidance to the frequency of exposure over a longer time before death. This may point to a systematic sedation over time and provide valuable information for the investigation.

The reality for most of forensic toxicologist is different. First, very few laboratories are able to quantify these substances in hair. Second, the available data is scarce since the scientific literature in this field is often limited to the presentation of case reports or investigations on small case numbers. A comparison with reference data or data from systematic studies is hardly possible and therefore interpretation of such results is difficult.

For the purpose of further illustration, this scenario is extended by an additional aspect:

An older brother of the child died 3 years ago without suspicion raised by the authorities. It was assumed to be a natural death by that time. An exhumation of the body may enable a toxicological investigation of organ tissue which reveals high levels of the same tricyclic antidepressant that was found in the younger sibling. Due to prolonged process of decay no hair is available for the investigation of a repeated administration before death. Only fingernails were still available for toxicological investigations.

The analysis of nails is even less well-investigated concerning psychotropic drugs and the toxicologist ends up with very difficult interpretation of the case.

Another example is given to illustrate further constellations of forensic value:

A woman went to the police with symptoms of dizziness and sedation and files a report of sexual abuse against her boyfriend. She states that she was sedated by him. A blood sample is taken at the hospital and screened for pharmaceutical drugs and drugs of abuse. The analysis reveals an acute influence of benzodiazepines. Asked by the police, the boyfriend states that he never sedated his girlfriend, nor did he know about benzodiazepines. A prescription for the benzodiazepine bromazepam is found in the home of the woman. The polices orders the analysis of a hair sample of both persons. In the hair sample of the man, no benzodiazepines were found. The woman was tested with high levels of bromazepam which was interpreted as frequent use in the months before the incident.

Again, the hair analysis is crucial for the outcome of the investigation of the case but data on bromazepam concentrations in hair is scarce.

In all constructed cases it is difficult to do well-founded interpretation without quantitative data. On the other hand, the cases may be interpreted differently and maybe not in the favor of the victims if a hair analysis is not done.

Out of this conflict and difficulties in daily forensic work the main ideas and objectives for this inaugural dissertation have been shaped.

1.2 Aims and objectives

Today's routine forensic work in hair analysis is mainly concerned with the investigation of alcohol markers and narcotics, for which a high level of experience has been gained over the last 30 years. However, the number of requests for the investigation of active pharmaceutical ingredients and psychotropic drugs is increasing rapidly. Case constellations such as those described above can occur at any time and confront the forensic scientist with challenging interpretations. The main objective of this thesis is therefore to meaningfully expand the current data situation regarding the detection of psychotropic drugs (antidepressants and antipsychotics) in hair in order to enable a comprehensive interpretation of such hair findings in daily forensic work.

Controlled dose studies in which volunteers take a defined amount of substances and then have their hair examined would be the best approach to obtain meaningful data. From an ethical point of view, however, such studies must be weighed very carefully. This calls for a search for alternate ways to enable research on this topic. In postmortem investigations, hair is routinely preserved and is available for research purposes. However, the significance of such investigations is somewhat limited, as it is not possible to estimate with reasonable certainty which dose was taken in the period before death. Nevertheless, the data can be assessed in retrospective studies, offers a good orientation and is ethically safer to obtain. In postmortem case investigation there is hardly valid information on the prescribed medication of the deceased. On the other hand, preparation and extraction of hair samples is a time consuming and intensive effort. In order not to waste time and money on negative hair samples an effective selection of suitable cases is crucial. Therefore, an observational study should be conducted to identify postmortem cases from the Institute of Legal Medicine and Forensic Sciences with a possible intake of antidepressants or antipsychotics prior to death. It was assumed that these deceased could have taken these substances of a longer period of time. These cases were to be included in a second study where the available hair samples were analyzed for antidepressants, antipsychotics and benzodiazepines in hair.

Therefore, another aim of this work was the development of a fast and robust liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the detection and quantification of antidepressants, antipsychotics and benzodiazepines in human hair. This method was then validated according to guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh) [15] to ensure the quality of the obtained results. For an adequate statistical representation of the missing dose information, the data should be presented in percentiles, as also applied by Musshoff et al. [16] and Tsanaclis et al. [17] as effective. The presentation in

percentiles should be able to represent the variations within the investigated purposes, hair lengths and populations well.

A work by Krumbiegel et al. [18] revealed that nails and hair showed comparable results in screening tests and thus nails became the focus of attention as an alternative matrix to hair. Thus, it was aimed to develop and validate [15] LC-MS/MS methods for the determination of alcohol markers, narcotics and psychotropic drugs in nails and hair and use them for an investigation on incorporation mechanisms and the comparability of the two matrices. Therefore, hair and nail samples from postmortem case of the Institute of Legal Medicine and Forensic Sciences were to be investigated. A segmental analysis of both, hair and nails were to be conducted for the study of further insights on the incorporation pathways into the nail. The comparability of quantitative data obtained from both matrices were to be investigated as well.

2 Background

2.1 Hair analysis between expectations and limitations

2.1.1 Life cycle and structure of human hair

The complex structure of human hair is reflected in its anatomy, physiology and its chemical properties which will be discussed in the following chapters. The visible part above the skin is the hair shaft, a heterogenous fiber formed of tightly glued keratinized cells [19]. Three concentric structures with different functions build the fiber from the outside to the inside: the cuticle, cortex and medulla (Figure 1a, [1]). The cuticle is a protective layer that anchors the hair shaft in the follicle and consists of a single layer of overlapping elongated cells [19]. Chemicals, heat, ultraviolet (UV) light, or mechanical stress can damage the protective layer which often results in a loss of cuticle cells towards the distal end of the shaft [19] (also known as “split ends”). The next inner layer, the cortex, represents the major content of the hair shaft and contains long keratinized cortical cells which are formed into fibers [19]. A special effect supports the strong tightness of this layer. During growth, small spaces between the cells are filled with a fluid that dries out when the hair grows out [19]. These small spaces, called fusi, are filled with air which works as kind of chemical cement and glues the cells together [19]. Melanin granules, pigments that give the hair color, are mainly located in the cortical cells [19] and partly in the cuticle cells [5]. Finally, the innermost layer, the medulla, consists of loosely packed medullar cells that dehydrate, shrivel and then leave small vacuoles along the shaft. Only a small percentage of medullar cells is found in human hair while they may also be completely absent or discontinuous along the center of the hair shaft [19].

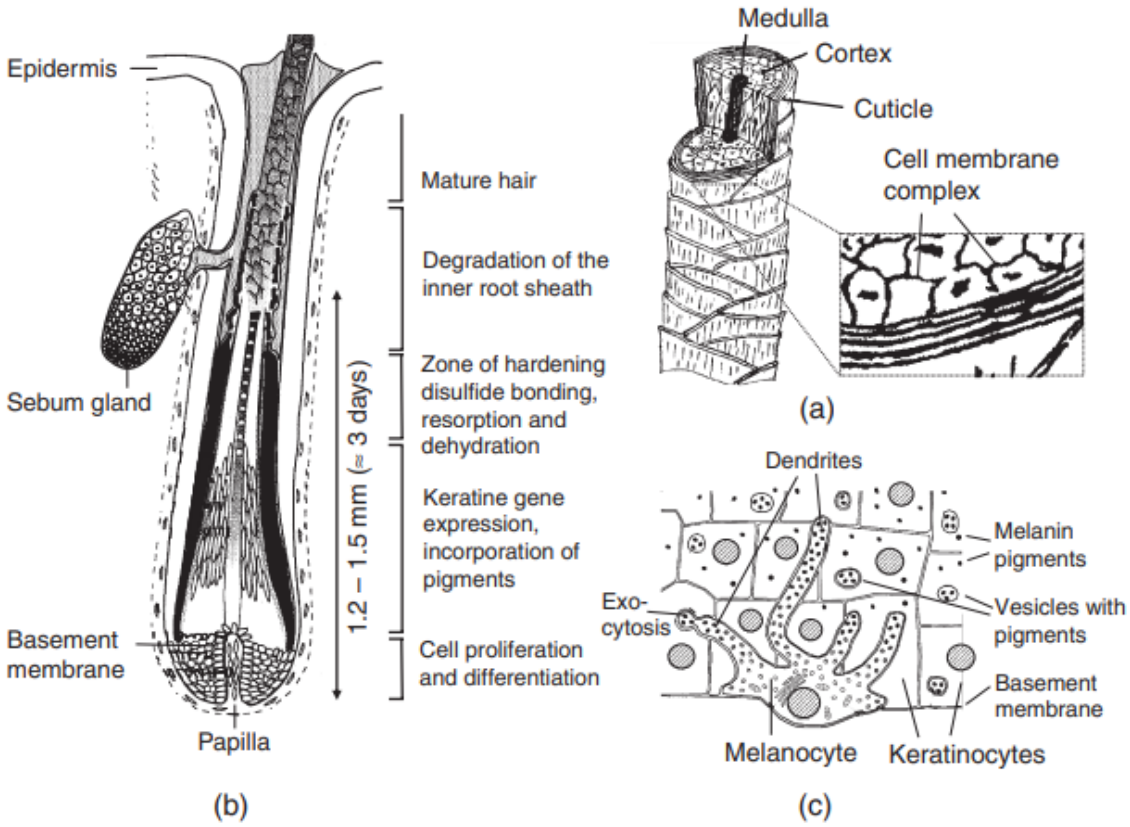

Figure 1 - overview of a human hair shaft (a), overview of a hair follice (b), overview of the cell types in the basement membrane (c), taken from Pragst et al. [1]

Hair follicles are sac-like organs at the root of the hair and place of growth of a hair [19]. Located in the epidermis of human skin they are embedded approximately 3-4 mm below the surface [19]. Figure 1b gives an overview of the structure of the follicle. Harkey describes three functional zones along the axis of the hair [19]: an innermost zone, containing the basement membrane in and around the bulb on the basement of the follicle. The basement membrane contains keratinocytes and melanocytes [1] that divide, increase in volume, and elongate [19]. From this germination center, which is located around the hair bulb papilla, the rapid mitosis [1] forces the cells to move up along the axis into the keratogenous zone [19]. Here, genes for the formation of keratine are expressed and cell differentiation for the cortex and cuticle occurs [1]. While cortex cells change into a spindle-like form, cuticle cells change into a shingle-like structure [1]. Further up the shaft, the zone of hardening, disulfide bonding, resorption and dehydration is characterized by a loss of all cytoplasmatic content (cell nucleus and water release) [1, 19]. The cell membrane complex [1], a dense “horny” mass of coalesced cells is formed [19]. It consists of proteins and protein-lipid

complexes from previous cell membranes and is assumed to be site of deposition for lipophilic drugs [1]. In addition, it serves as the main diffusion point for the incorporation and elimination of drugs into hair [1]. The next zone is characterized by fully mature hair. Overall, the follicle is embedded in a rich capillary system that ensures supply with nourishment during the hair growth [19]. This connection to the blood stream is also mainly important for the incorporation of basic drugs [19] into hair.

The sebaceous and apocrine glands are not only closely located to the follicle but are also functionally associated [19]. In most parts of the body, these glands and the follicles build functional fusions known as the pilosebaceous unit. In this unit, glands and follicles are so close to another that the ducts of both glands empty into the follicle. A third type of gland, the sweat gland, is located near the follicle [19]. All glands serve a different purpose: the sebaceous gland produces sebum, a waxy lipophilic substance, that covers the hair shaft before it emerges out of the skin [19]. Apocrine glands also cover the hair shaft with an oily, colorless and odorless substance yet its functions are still not fully understood. The apocrine glands are only expressed in the armpits and intimate sites [1, 19]. Sweat produced in the sweat gland moistures the hair shaft and can be a vehicle for the incorporation of hydrophilic drugs [1].

The basement membrane contains melanocytes and keratinocytes closely together (Figure 1c) in a ratio of about 1:5 [1]. Melanin pigments are produced in melanosomes within the melanocytes. Melanin granules or hair pigments were already mentioned as being responsible for the hair color. Trough long dendrites the melanocytes transport these melanosomes to neighboring keratinocytes where the melanin pigments are phagocytized and then imbedded in the keratinocytes [1]. The composition (amount, distribution, type) of these melanin pigments finally defines the color the hair [19].

It is widely accepted that hair grows in a cyclical process, which is regulated by the activity of cytokines (hormones) [20]. The cycle is characterized by three stages: the anagen phase (growth phase), the catagen phase (transitional phase) and the telogen phase (resting phase) [20]. During the anagen phase, the phase of active growth, cells in the bulb (root) are rapidly divided. A thin filament is built out of the elongation of these cells [3] followed by an upward movement of the cells into the follicular canal. There, the cells differentiate into different cell types of the cuticle, cortex or the medulla and begin to keratinize. This phase can last for several years [1, 5, 21]. After the anagen phase, the hair enters a transitional phase, the catagen phase, which lasts about 2-3 weeks [3, 1]. The hair shaft becomes fully keratinized, the cell division stops and the bulb begins to degenerate during this phase [5]. The third phase or resting phase (telogen phase) is

characterized by a complete stop of hair growth while the hair shaft retains in the upper follicular canal [19]. It is then easily removed from the scalp by pulling. For scalp hair this resting phase is up to 10 weeks long [19]. Hair from other body sites can remain in the telogen phase for 2-6 years [19]. The existing literature emphasizes that hair follicles grow individually and not synchronous [19]. The amount of hair in the growing stage or in the resting stage differs for hair from different body sites [19]. Previous research has supported the hypothesis that a proportion of approximately 85 % of the adult scalp hair is in the anagen phase while the remaining 15 % are in the resting phase [19, 21].

A number of scientists have turned their attention to the growth rates of hair from different body sites [1, 5]. The review of this literature showed a general growth rate of 0.6 cm - 1.4 cm per month for scalp hair [1, 5]. A recommendation by the SoHT proposes the estimation of an average growth rate of 1 cm per month [8]. However, Kintz et al. [5] pointed out that this might be an oversimplification since there was also a publication that found a maximum growth rate of 3.36 cm per month [22]. In addition, the growth rate depends on the type of hair, the body location and age of the person [6]. A lack of published information on growth rates for children and teenagers was identified [5]. The hair growth of infants is driven by complex changes during pregnancy and is therefore even more difficult to interpret. Finally, it is estimated that the growing hair needs approximately 7-10 days to reach the head surface [5]. Therefore, the proximal end of a cut hair strand (cut close to the scalp) does not represent the most recent period of hair growth [5]. Hair from the scalp is the hair of choice with the fastest growth rate and the highest proportion of hair in the anagen phase [5]. Significantly different growth rates and proportions of anagen/telogen phase have been reported for different body sites [8] which is considerable due to the mentioned variations. For example, leg hair grows about 0.6 cm per month [23]. Pubic hair and beard hair grow slower and have a higher proportion of telogen hair which leads to a longer detection window. However, pubic hair is prone to be contaminated by urine [5].

Regarding the cyclic growth and the different growth rate, the window of detection needs careful interpretation with respect to the type of hair the body location of the hair sample. If a hair strand of 6 cm proximal scalp hair is analyzed, it should represent the last six months before sampling (assuming an average growth rate of 1 cm/month). In this hair strand there is about 15 % hair in the telogen phase. Thus, this hair has stopped growth sometime before the last six months. This hair then reflects a different time window than the 85 % anagen hair in this 6 cm hair strand. Therefore, the analyzed hair strand of 6 cm reflects the last six up to twelve months before sampling due to the telogen hair. That effect can be reduced if the head is fully shaven and only

the newly grown hair is used for an analysis after a defined growth period. For interpretation, it is advised to apply the respective growth rates for scalp and body to achieve a more precise time window of the analyzed hair length.

2.1.2 Incorporation of xenobiotics in hair

Over the last years an enormous amount of research has been conducted to study the mechanism of incorporation of xenobiotics into hair. Different models of incorporation have been described but still there is uncertainty about the precise mechanisms [1]. One of the basic assumptions, that substances follow a passive diffusion from the blood capillaries into the growing cells of the keratinization zone, failed to withstand experimental data [24-26] that indicated a more complex mechanism of incorporation [1]. Henderson et al. [27] proposed a multi-compartment model that is widely used in several books and scientific reviews [1, 3, 5, 28]. According to their model, incorporation occurs from blood during formation of hair, from sweat and sebum after formation and from the external environment after formation and the appearance of hair out of the skin [27]. Alternatively, an intradermal transfer of highly lipophilic drugs (e.g. tetrahydrocannabinol) [29] through several skin layers or binding of drugs to melanin-rich compartments is discussed [27]. It is still unclear which route of incorporation contributes to which extent, but it varies from drug to drug [5]. Pragst et al. [1] outlined that the chemistry of the substances itself is of great influence on the incorporation. On one hand there is the basicity and lipophilicity (lipophilic uncharged organic molecules are diffusion driven) of the substance itself and on the other hand the melanin content of the hair sample [1]. In addition to the general melanin-affinity of basic drugs [30, 31], the intracellular pH in keratinocytes [32] and melanocytes [30, 33] is in the acidic range. This leads to an accumulation of lipophilic and basic drugs in matrix cells [1]. Several studies investigating human and animal hair have found a correlation between the melanin concentration, which is correlated with the pigmentation, and incorporation of basic drugs into hair [33-37]. The incorporation of neutral compounds on the other hand has been found to be unaffected by pigmentation [35]. Acidic drugs however, like the metabolites of valproic acid or THC (11-nor-Δ9-tetrahydrocannabinol-11-carboxylic acid) are only incorporated in low concentration into the acidic matrix cells [1, 38]. Due to the gain of hydrophilicity during drug metabolism, metabolites are found in lower concentrations than their respective precursors [1, 39]. Once incorporated, most drugs are quite stable. Their stability depends on the chemical structure and increases with increasing polarity [1]. That leads to slower elimination of polar metabolites compared to their parent drugs [1]. In summary, basic drugs are consequently found in higher concentrations compared to neutral or acidic and lipophilic drugs [5].

2.1.3 Influence of cosmetic treatment on hair analysis

A widely accepted hypothesis is that drug concentration in hair can be altered after incorporation by different cosmetic treatments [1]. Aggressive oxidative bleaching or permanent wave can lead to an extensive decrease of the original drug concentration [1]. A report from Cirimele et al. [40] presented results from a person with separately treated hair (bleaching with hydrogen peroxide) and found up to 80 % higher drug concentrations of opiates in the unbleached hair compared to the bleached hair. This decrease of drug concentration by bleaching was demonstrated for cannabinoids as well [41]. In general, these aggressive treatments use strong basic solutions that damage the hair structure on different levels [3]. Bleaching as an oxidative-alkaline treatment with hydrogen-peroxide leads to a degradation of melanin granule by cleavage of the disulfide bonds and finally the decoloration of solubilized pigments [42]. The destruction of melanin results in a loss of binding sites for drug molecules in bleached hair and consequently the incorporation rate and drug concentration decreases [41, 42]. Hydrogen-peroxide is also capable of directly degrading the small drug molecules [42]. Perming or permanent wave disrupts keratinous disulfide bonds that are reconstructed in the hair matrix [3, 42]. Besides the mentioned reaction of hydrogen peroxide reactions to melanin pigments the hair cuticle is opened and the cortex of the hair swells when using a permanent dye [42]. Colorless precursor molecules in the dyeing preparation react with hydrogen peroxide in order to establish a new color [42].

Also, “natural” factors like sunlight and weather conditions have been found to impact the drug concentration in hair [43]. Individual drug stability as well as the intensity of UV light and contact to water were identified as influencing factors [43, 44].

Further empirical work confirmed the degrading effect on drug concentrations under cosmetic treatment for several analytes although some analytes were assumed more stable to cosmetic treatment than others [45, 46]. Alcohol consumption biomarkers in hair however are especially prone to concentration alteration by cosmetic treatments [5]. A comprehensive study by Suesse et al. [47] investigated the effects of cosmetic treatment on the markers for excessive alcohol abuse ethylglucuronide (EtG) and fatty acid ethyl esters (FAEE). EtG showed a strong concentration decrease through bleaching, perming, and dyeing [47-49]. Thermal straightening also degrades the content of EtG in hair [50]. Ethanol in cosmetic products like hair spray on the other hand did not lead to false-positive results [47] since EtG is only formed in the liver. On the other hand, a few EtG containing commercial herbal hair products were identified that produced false-positive results after continuous application [51-53]. EtG is formed during ethanolic

extraction of plants in such products [53]. For the other alcohol marker FAEE different effects of cosmetic treatment were demonstrated [47]. Bleaching, dyeing and perming did not lead to a strong degradation [54] while ethanol containing hair products (wax, spray) led to an increase of FAEE concentration in hair [47, 54, 55]. The proposed mechanism is a diffusion of the topically applied ethanol into the sebum glands where it is metabolized to FAEE and then incorporated into the hair [54].

The ongoing research on the effects of cosmetic treatment led to consequences for the practical applications of hair analysis. It is recommended that the past cosmetic treatment should be assessed prior to sampling [8]. For examinations in the context of drugs in driving in Germany cosmetically treated hair must not be used for a proof of abstinence from alcohol [56]. In that case, leg, beard or breast hair may be analyzed while the use of pubic or axillary hair is not allowed due to a possible contamination and sweat-related washout effects [56]. For drugs of abuse a treated hair sample may be analyzed if a second analysis of 6 cm untreated hair is directly followed [56]. However, investigators should be aware that the use of aggressive hair treatment is possible tool of manipulation [5]. In other contexts, like postmortem hair analysis, information on past hair cosmetic use is rare and investigators should consider these consequences in their interpretation of results.

2.2 Nail analysis as an alternative to hair analysis

Over the past 30 years, the analysis of hair has become established and intensively researched for retrospective substance detection in many fields of application in forensic toxicology. Nevertheless, there are some situations where hair is not available for investigation. Due to genetic predisposition or disease, there may be very little hair growth. In some cases, aggressive hair cosmetics also prevent adequate hair analysis, or the hair has been deliberately shaved off. In the context of postmortem toxicology, hair cannot be collected in some cases due to severe rotting, mummification or in burned bodies. Nails, which also consist of keratinized cells, have been discussed as a possible alternative matrix for retrospective substance detection [57] over the past years. The examination of nails became known for the first time for the detection of arsenic poisoning or other heavy metal exposure [58-59]. In 1984, the detection of methamphetamine in nail was the first report for the detection of drugs of abuse [60]. A study by Krumbiegel et al. [18] revealed that qualitative screenings in matched hair and nail analyses led to comparable results. To date, the main applications of nail analysis have been long-term monitoring of drug and alcohol abuse, workplace drug testing, drug adherence monitoring, drug-facilitated crimes (DFC), detection of prenatal drug exposure and postmortem toxicology [61].

2.2.1 Anatomy, physiology and formation of nails

Nails are located at the tips of the fingers and toes and have two functions: protection and the enhancement of the sense of touch [62]. A nail is categorized into six different parts illustrated in Figure 2: germinal matrix or nail root, lunula, nail bed, hyponychium, nail plate and nail folds [63]. The nail is formed in the nail root by the keratin cells in the germinal matrix, which lies under the skin and is protected by the proximal nail fold [64]. During the formation of new keratin cells, the old ones are pushed out through the cuticle and undergo a cytoplasmic condensation which results in the formation of the nail plate [64]. This formation goes in two directions: forward and upward. Thus, growth takes place not only in length but also in height. The length of the nail root determines the shape of the nail: the longer the matrix, the more cells can be produced and the thicker the nail plate becomes [64]. Tightly knitted and interlocked dead keratinous cells build the nail plate which is approximately 0.5 mm thick. Also, the presence of only a few melanocytes in the germinal matrix leads to a lack of pigmentation of the nail [64].

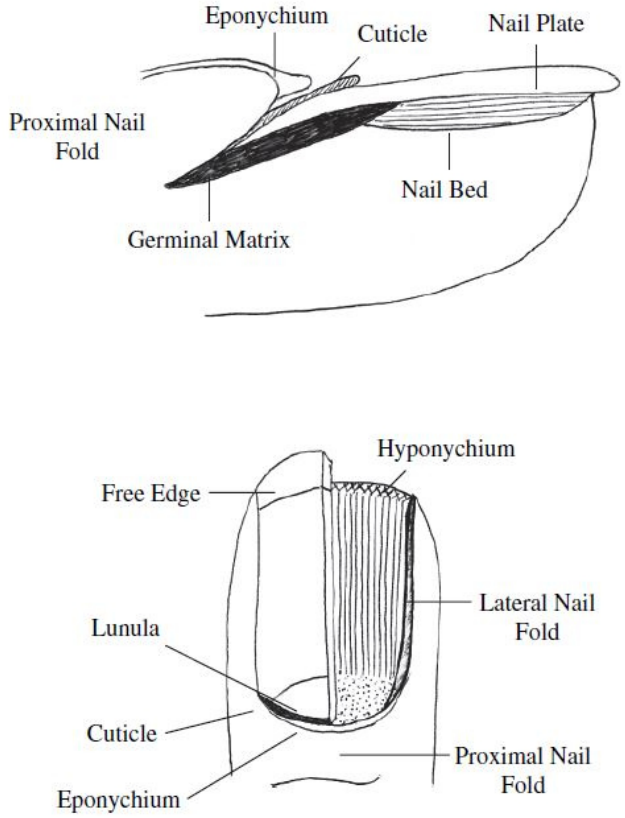

Figure 2 - anatomy of a human nail, reprinted with permission from Garside et al. [64]

It is assumed that the nail plate is thinner at the free edge than the plate over the nail bed [65]. The nail bed is a flat, blood vessel rich surface that adheres the nail plate. It extends from the lunula to the hyponychium, which forms the transition between the nail bed and the normal epidermis.

Unlike hair, nails do not grow in cycles [64]. Fingernails and toenails differ in their growth rate. The average growth rate of fingernails is 0.1 mm per day (3 mm per month) in contrast to 0.03 mm - 0.04 mm per day for toenails [66, 67]. The complete replacement of a new fingernail takes about 6 months while it takes about 12-18 months to replace a toenail due to the one third slower growth of the toenails [64]. In addition, growth depends on many individual differences such as gender, age and state of health. It is assumed that nails are formed 80 % in the germinal matrix and 20 % during growth via the nail bed [64]. The second growth process mainly contributes to the growth of thickness and occurs through the formation of ventral layers during growth from the lunula to the free nail end [65, 68].

2.2.2 Incorporation of xenobiotics into nails

The incorporation of substances into the nail has so far only been clarified to a limited extent. The described ventral thickness growth contributes to a large extent to the kinetics of the deposition of substances in the nail, since the connection to the capillaries in the nail bed provides a large-area access over the entire nail [64]. From these capillaries a diffusion of the substances into the nail can take place, both during growth in the germinal matrix of the nail root and via the nail bed [57]. If the growth only passed through the germinal matrix, it could be assumed that a thin band of deposited substances would henceforth move from the lunula to the free end of the nail [62]. The detection of a substance in the edge of the nail would then prove an uptake about 3 - 5 months ago [57]. On the contrary, some studies have shown that the substances are detectable much earlier after an administration in the nail bed [62]. It was also shown that zolpidem, a sedative, was detectable in fingernails after a single oral dose very early [69]. Thus, three incorporation pathways were proposed [69]: a) contamination from sweat, detectable 24 h after administration, b) incorporation from the nail bed, detected after 2 weeks and c) incorporation from the growth in the germinal matrix, with a maximum level 3 months after the administration. Like hair, incorporation from an external source e.g. body fluids (sweat, sebum, saliva), powder or other substances can also occur in nails in the sense of contamination [57, 61]. The risk of an unintended external contamination seems to be greater for nails as touching of substances or body fluids can occur easily. It is still not well investigated to what extent a cosmetic treatment (e.g. acetone use

or polish) affects the concentration in the nail and should be clarified in the future to determine the suitability of nail clippings for abstinence control or the detection of long-term use [57].

In summary, the examination of nail clippings thus represents a time window of up to 5 months due to the growth and incorporation pathways in the nail [57, 69]. Due to the slower growth of the toenails, they represent a time window of about 8-14 months [57]. The concentration obtained represents a mixture of internal and external incorporation processes [57].

2.3 Chromatography and mass spectrometry in forensic toxicology

2.3.1 Liquid chromatography with triple quadrupole mass spectrometry (LC-QQQ-MS)

Mass spectrometry in general has become an integral part of forensic toxicology over the past decades. Many different devices with different techniques (gas chromatography with mass spectrometry (GC-MS), LC-QQQ-MS, liquid chromatography with time-of-flight mass spectrometry (LC-TOF-MS/LC-QTOF-MS) fit certain purposes within forensic laboratories. For the intended goal of presenting quantitative data on antidepressants and antipsychotics in hair, a LC-QQQ-MS device was used. This technique offers a wide range of linearity for the simultaneous quantification of very low concentrations and high concentrations. The analytes are dissolved in a liquid mobile phase which moves through a stationary phase (column) where the analytes are separated via interaction with the column material. After this separation, the mobile phase containing the mix of analytes is then transferred into the mass spectrometer where it is ionized via electrospray ionization (ESI). An electrical field is applied to transfer the mix of ions into the mass spectrometer where they are separated by their mass-to-charge ratio (m/z) using quadrupoles as mass analyzers [71]. The most sensitive and selective mode is the multiple reaction monitoring (MRM). In this setup the first quadrupole (Q1) is used as a mass filter, only letting ions of a specific m/z (precursor ions) pass into a second quadrupole or octopole unit (Q2). Here, the Q2 serves as a collision cell where the filtered ions collide with inert gas molecules into new fragment ions. This mix of fragment ions is further transferred into the third quadrupole (Q3) which is again used as mass filter, only letting ions of a predefined mass (product ions) pass into the detector to produce a signal. The combination of specific precursor ion selection and explicit product ion selection offers a very specific detection of the compound. For each analyte of interest, a fragmentation profile, also called MRM transition, can be detected simultaneously during an analytical run. A main disadvantage of this measurement is that only transitions that were predefined in the method are acquired. If there is new information and the toxicologist wants to detect another compound, a new measurement with the respective predefined MRM transition must be done. On the other hand, this approach is useful for the analysis of complex biological matrices since it does not scan the whole mass range nor detects co-eluting matrix components of a different mass. Usually the MRM transition (quantifier) with the highest abundance is used for quantification while the second or third most abundant transitions are used as qualifiers. The ratio of quantifier and qualifier as well as the retention time must be in a predefined range to confidently identify a substance for forensic purposes.

2.3.2 Method validation according to GTFCh guidelines

Forensic laboratories must meet an extraordinary quality standard when their results are used for legal purposes. The used techniques need to be highly reliable, based on the current state of art and they must be “defendable” in court. Two instruments are now commonly used to ensure quality in forensic toxicological investigations: the norm DIN EN ISO/IEC 17025 [72] which regulates general requirements for the competence of testing and calibration laboratories and the guidelines for the validation analytical methods from the GTFCh [15, 73]. Although the foundation for the GTFCh guidelines is within the DIN ISO norm (e.g. personal qualifications, laboratory layout, laboratory safety, quality control samples) the focus will be on the validation guidelines for the purpose of this thesis. The guideline recommends validation parameters and their acceptance criteria together with the required statistical background and is based on current state of knowledge [15]. Several parameters must be tested and documented to show that an analytical method gives accurate, precise and robust results [15]:

- *Selectivity as the capability of the method to detect and identify substances unambiguously and without interferences from endogenous or exogenous substances* and
  Specificity as the capability to detect a single substance without being affected by other substances in the sample
  In a series of blank samples from different batches, also spiked with an internal standard or other substances than the validated ones, no interfering peaks or signals should occur.
- *Linearity of calibration as the capability to produce a directly proportional relationship between the response and the concentration of a substance within a certain concentration range.*
  The range of calibration should cover vast majority of expected concentrations and includes upper and lower concentrations for which acceptable precision, accuracy and linearity has been shown. Repeated measurement (six-fold) of the chosen calibrators (at least five) and subsequent plotting of the peak area ratios (substance/internal standard) allows further statistical testing. Outliers must be identified by the Grubbs-test (significance level 95 %). The homogeneity of variance is tested using the Cochran-test (significance level 99 %). If homoscedasticity is evident, a simple regression is used, and the fit is tested with the Mandel’s linearity test. In case of heteroskedasticity two options are possible: (a) limitation of the calibration range until homoscedasticity is reached or the application of a weighted calibration model (e.g. $1/x$ or $1/x^2$) for compensation of the heteroskedasticity. After application of the weight factors, the fit is again tested by the Mandel’s linearity test.

- *Accuracy is measured as the difference between an individual result and a reference value that originates from systematic (bias) and random errors (precision)*

  The repeated measurement of quality control samples with known concentrations on eight different days allows the statistical evaluation of the bias (acceptance criterion: bias ±15% and ±20% near the limit of quantification). The precision expressed as the repeatability (acceptance criterion: relative standard deviation (RSD) ≤ 15 % and 20 % near the limit of quantification) and the intermediate precision (acceptance criterion: RSD ≤ 15 % and 20 % near the limit of quantification) are also tested with this approach. Furthermore, the accuracy is tested as the combination of bias and precision and expressed as the 95 % β tolerance interval (acceptance criterion: ±30% and ±40% near the limit of quantitation).
- *Stability as documentation that a substance is chemically stable in a prepared sample over a given time interval under certain conditions*

  At least six measurements over a time window corresponding routine laboratory workaround are tested with a linear regression. Only the peak areas are used and the maximum acceptable decrease over the testing period is 25% (for deuterated standards) and 15% in other cases (20% near the limit of quantification).
- *Limit of detection as the lowest possible concentration to identify a substance*

  Limit of quantification as the lowest concentration of a substance that can be measured with an acceptable bias (±20 %) and precision (RSD=20 %)

  Both parameters can be tested using the DIN ISO 32645 [74], where both limits are calculated based on at least five calibration levels near the expected limit of detection.
- *Matrix effects that lead to a suppression or enhancement of the ion abundance by the presence of a co-eluting substance*

  Recovery as the complete transfer of the analyte into final extract after sample preparation

  In LC-MS/MS methods, the recovery and matrix effects can be tested together. At least five different spiked matrix samples, spiked matrix extract and neat standard solutions at different concentrations are compared. The recovery is calculated as the ratio of the peak areas of the spiked matrix samples to those of the corresponding spiked extracts (acceptance criterion: more than 50 %). The matric effect is expressed as the ratio of the peak areas of the spiked extracts to those of the neat standard solutions (acceptance criterion: 75-125 % and max. 25 % standard deviation when deuterated standards are used)

All acceptance criteria and statistical tests (e.g. Grubbs Test, Mandel's F Test) must be passed and documented for every analyte to fully validate a method.

3 Results

3.1 Manuscript I

Hair analysis of antidepressants and antipsychotics - Overview of quantitative data

3.1.1 Summary

Hair analysis is widely used today for various issues in the field of forensic toxicology, such as workplace testing, driving ability assessments, post-mortem toxicology, drug-related crimes, doping control, therapy compliance testing, evaluation of drug history or chronic intoxication and excessive alcohol consumption testing [1]. Because of their sedative properties [10, 11], their relatively high toxicity [12] and their broad prescription frequency [13], psychotropic drugs (antidepressants, antipsychotics) are often part of these questions. According to Pragst [75], the biggest pitfall for such investigations lies in the interpretation of the results and not in the actual execution of hair analyses. This requires a critical examination of the basics of hair analysis, but also an understanding of how the tested substances are incorporated into the hair. The aim of the present publication was therefore to show the extent to which psychotropic drugs have been researched in hair and at the same time to help colleagues in forensic toxicology to interpret their own cases with the comparative presentation of quantitative hair concentrations.

A comprehensive literature search revealed about 60 publications, roughly divided into three types of publication: a) publications dealing with the development and validation of detection methods, b) individual case reports in which psychotropic drugs were detected and c) controlled dose studies. In order to prove the applicability of the validated methods (a), a few hair samples were often examined within the scope of these publications, but unfortunately often without any information on dosage or frequency of intake in the time before sampling. In the case reports (b), the dosage information was very variable from publication to publication. Furthermore, the hair lengths used in these two types of publications were very inconsistent, which greatly reduced the comparability of the data collected. The statistical power was also very low due to the small number of cases in these types of publications. In contrast, numerous controlled dose studies (c) could be found, where the conditions of investigation were much more comparable. They were

characterized by higher case numbers, precise dosage information and the analysis of uniform hair lengths. In these studies, collectives of patients with psychiatric disorders were often examined in whom a stable and long-term intake of psychotropic drugs could be assumed. The substances were selected based on the annual medication prescription report of the health insurance companies in Germany [13]. These include some substances that have been marketed for decades but have now been replaced by newer, more effective substances and are therefore prescribed less frequently. In addition, there were also substances that are currently very frequently prescribed. It became very clear that there were big differences in the research results for the different substances. For some substances that are very common today, there was sometimes little comprehensive data available, while some older substances that are less relevant today have been intensively researched in hair over the last 20 years.

Amitriptyline, a tricyclic antidepressant (TCA), and mirtazapine, a tetracyclic antidepressant (TECA), will serve as comparative examples to illustrate this imbalance. Discovered in the 1950s and 1960s [75], TCA quickly became very common in the treatment of depression. However, due to their strong binding to the histamine H1 receptor [10, 11], TCA have a very strong side effect profile, characterized by strong sedative components and cardiotoxic components. The relatively high toxicity associated with this side effect profile [12] led to the continuous development of psychotropic drugs with fewer side effects. The TECA mirtazapine was approved in the USA in 1996 and has significantly fewer side effects with the same antidepressant effect [76]. This development is also evident from the prescription rates in Germany [13]. There, the prescription rate of mirtazapine, at 183 million defined daily doses (DDD), is considerably higher than that of amitriptyline, at 81.4 million DDD [13]. On the other hand, mirtazapine also interacts with the histamine H1 receptor, albeit less strongly than TCA. This binding can also cause sedative side effects, but less strongly and less toxic than TCA [12, 77]. Now one could assume that mirtazapine could potentially occur frequently in forensic toxicological investigations for DFC or overdose deaths due to this wide prescription rate and mild sedative effect. However, the present review showed that there is a very limited data for the detection of mirtazapine in hair compared to the detection of amitriptyline in hair. While five publications with case numbers of more than five cases could be identified for amitriptyline, none of the publications for mirtazapine has a higher case number than three cases. It was also found that the hair lengths used varied widely between the investigations and only rarely a dosage was indicated. For amitriptyline, four studies with a high case number and dosage were identified, while for mirtazapine there was only one study with the stated dosage and a number of two cases. In summary, this results in a solid data basis for amitriptyline and a very weak basis for mirtazapine.

Against the background of numerous analytical aspects, the work also critically examines the comparability of quantitative data in hair. A successful method validation can already ensure a high degree of quality and comparability. It should be noted, however, that the production of calibration and quality control samples can hardly simulate real incorporation into hair. Likewise, the extraction method chosen can have a strong influence. Each laboratory has developed its own extraction procedure, so that even here a low level of comparability between the results is given. Today, comparability of results is ensured by so-called interlaboratory tests (or proficiency testing), in which a large number of laboratories examine hair samples with known concentrations. Spiked blank hair matrix is usually used for these tests although samples with an authentic incorporation from drug intake would be favorable. To date, unfortunately, no interlaboratory tests for psychotropic drugs in hair are available but would be urgently needed to demonstrate the suitability of the methods. Successful validation must therefore be considered sufficient as proof of confidence in the accuracy of the data collected. When using such data for own interpretations, these aspects should be taken into account.

Through this work it could be shown that there is a lack of comprehensive quantitative data in hair for numerous psychotropic drugs. Furthermore, it was shown that the publications hardly showed comparable extraction protocols or examined hair lengths, despite the recommendations of numerous professional societies.

In addition, the paper provides an important overview of the available scientific literature, which can provide other scientists with a quick and comprehensive overview and thus possibly assist them in their case work.

3.1.2 Publication

Methling M, Krumbiegel F, Hartwig S

Hair analysis of antidepressants and antipsychotics - Overview of quantitative data. *Drug Test Anal. 2020; 1– 18.* https://doi.org/10.1002/dta.2784

3.2 Manuscript II

Toxicological findings in suicides – frequency of antidepressant and antipsychotic substances

3.2.1 Summary

Several evidence matrices including blood and hair samples are routinely taken during autopsies for further toxicological investigations in postmortem cases. Such samples are released for research by the public prosecutor's office and can be used for retrospective studies. In contrast, prospective studies with a prescribed drug intake on individuals are of high ethical concern. Retrospective studies are therefore a valuable way to achieve greater understanding in forensic research. As mentioned in the aims and objectives chapter, hair analysis is a time and cost intensive technique and therefore the selection of samples should be undertaken wisely. Therefore, identification of postmortem cases with a possible long-term intake of antidepressants or antipsychotics prior to death was chosen to approach this issue and to achieve an effective case selection. During the retrospective evaluation of earlier postmortem cases investigated, it became apparent that psychoactive substances were very frequently detected in suicides. With the emerge of the selective serotonin reuptake inhibitors (SSRI) in the early 1990s and 2000s controversy about suicidality-inducing effects was raised by some case reports [78-80]. This hypothesis was then addressed by many different perspectives but remained a controversy due to divergent results from different approaches. It cannot be denied that these substances have a relatively high toxicity and broad side effect pattern although they are a major factor in the treatment of mental disorders [81, 82]. Out of this observation a cross-sectional study was conducted to not only identify possible positive cases for a later hair analysis but to give insight on the prevalence of antidepressants and antipsychotics in suicides, at least in this region.

The federal state of Berlin has a unique structure in the processing of death investigations where the autopsies are performed at two institutes of equal standing: the Institute of Legal Medicine and Forensic Sciences at the Charité University Medicine Berlin and the Berlin State Institute of Legal and Social Medicine. This study only observed cases from the Institute of Legal Medicine and Forensic Sciences at the Charité University Medicine Berlin during the period of 2012 - 2015 with

about 4000 performed autopsies. Generally, an autopsy is not ordered by the public prosecutor's office in every case of suicide, therefore not all suicides during that time were covered. In 2600 of the cases from the Charité University Medicine Berlin a comprehensive toxicological investigation was performed. Venous blood, heart blood, urine and other organ tissues as well as hair samples are routinely taken as evidence during an autopsy. For a systematic toxicological analysis preferably blood or urine samples are investigated (if available), although organ tissue can also be analyzed to prove an acute (brain tissue) or subacute exposure (liver, kidney) to a substance. The toxicological investigations were performed with published and validated high-performance liquid chromatography with diode-array detection (HPLC-DAD) [83], LC-QTOF-MS [14], LC-QQQ-MS and gas chromatography flame ionization detection (GC-FID) [84] methods. All cases with positive findings for TCA (amitriptyline, clomipramine, doxepin, opipramol, trimipramine), SSRI (citalopram, fluoxetine, paroxetine, sertraline), TECA (mirtazapine, maprotiline), atypical neuroleptics (ATYP: amisulpride, clozapine, olanzapine, quetiapine, risperidone), typical neuroleptics (TYP: chlorprothixene, haloperidol, melperone, promethazine, zuclopenthixol) and other substances with antipsychotic or antidepressant effects (tranylcypromine and venlafaxine) were included in this study. Other information like gender, age, cause of death, mental disorders, location of death and blood alcohol level were also included.

Overall n = 447 cases with positive results for antidepressants and antipsychotics were identified and divided into the non-suicide cases (n = 212; male n = 117, 55.2 %; female n = 95, 52.5 %) and suicide cases (n = 235; male n = 149, 63.4 %; female n = 86, 36.6 %). The classification was based on the final investigation results of the public prosecutor's office and the final autopsy report, including the cause of death. An evaluation of the gender distribution showed a ratio of 2:1 for male to female cases which is in line with nationwide trends [85] and other cases from the institute. A similar trend was evident for the age distribution [85]. The included cases therefore appeared to be a suitable cross section of the national population. Higher frequencies were found for almost all drug classes in the suicide group and may be explained by the higher number of cases with more than one drug detected in the suicide group (37.5 % suicide group vs. 22.7 % non-suicide group). Several reasons for this observation were discussed: combination of treatment regimens or histories of failed and non-effective treatment regimens that led to a higher access to such drugs for some patients. A gender evaluation of the suicide group showed similar results for males and females except for ATYP that were more frequently detected in males. Our findings were compared to the results of a group from Munich [86] that investigated an earlier time period. The observed changes in the frequencies were discussed as possible reflection of the changing prescription rates. A mental disorder was known for 22.9 % of the suicide cases and the most

common location of death was at home. The most prevalent causes of death were drug overdose, followed by polytrauma and hanging. The portion of drug poisonings included more female than male cases (41.2 % female vs. 31.2 % male). Split by drug class it was evident that TCA were the most frequently detected drugs by far (followed by SSRI, ATYP, TYP, TECA and others). That observation was discussed and related to the individual toxicity of the substances which is, expressed as the fatal toxicity index in overdose, far higher for TCAs than for the other classes [12]. The detailed evaluation of the substances showed the five most frequently detected substances found in the suicide group: doxepin (20 %), citalopram (15.3 %) mirtazapine (14.9 %), quetiapine (13.6 %) and amitriptyline (12.3 %). It was discussed that this ranking might be related to a complementary effect of the individual toxicity [12] and the prescription frequency [13] (for example citalopram with high prescription rate, but a low toxicity). In contrast, this ranking was different in the non-suicide cases: citalopram (15.6 %), doxepin (13.7 %), mirtazapine (12.3 %), amitriptyline (8.5 %) and melperone (8.5 %). Alcohol and suicidal behavior are somehow related in a complex way since drinking alcohol in a phase of suicidal ideation can remove inhibitions and lower the resistance to committing suicide [87]. In this study, alcohol was detected in about one third of the suicide cases compared to about one fifth of the non-suicide cases. Comparable proportions were also found by other studies [88-90].

The comprehensive investigation of a wide number of substances is a major strength of this study as previous studies did not include so many drugs. Also, often only classes of drugs were presented and not detailed data on single substances. The preselection bias that is based on a regional bias (only half of the autopsies in Berlin were included due to the structure of two equal forensic institutes) and the fact that not all suicides are investigated was intensively discussed. Finally, the advantages of this complementary approach to assess the risk of suicide during medical treatment and its possible role in suicide prevention were pointed out.

3.2.2 Publication

Methling M, Krumbiegel F, Hartwig S, Parr MK Tsokos, M

Toxicological findings in suicides – frequency of antidepressant and antipsychotic substances. Forensic Sci Med Pathol 15, 23–30 (2019) https://doi.org/10.1007/s12024-018-0041-4

3.3 Manuscript III

Concentrations of antidepressants, antipsychotics and benzodiazepines in hair samples from postmortem cases

3.3.1 Summary

The aim of this work was to comprehensively expand the data set for psychotropic drugs in hair based on the analysis of hair samples from selected postmortem cases. Sensitive, reliable and reproducible detection methods are essential for determining quantitative data in hair. The first step of this work was therefore the development and validation of an LC-QQQ-MS method for the detection of psychotropic drugs in hair and the subsequent validation to prove the suitability of the method. In the second step, 442 postmortem hair samples were examined with this method to present quantitative data for psychotropic drugs in hair based on sufficiently high case numbers.

The selection of the substances to be detected was based on the annual prescription report of the health insurance companies for psychotropic drugs [13] and included antidepressants, antipsychotics and benzodiazepines. In total, 52 analytes were included in the method development, including 8 metabolites to consider possible metabolite-parent drug relationships. After successful optimization of the mass transitions required for the LC-QQQ-MS method, a gradient elution was established, which allowed the clear separation of the analytes. Subsequently, the method was comprehensively validated according to the guidelines of the GTFCh [15]. The parameters described in 2.3.2 were all fulfilled, so that the method could be successfully validated for all 52 analytes. The linear calibration range for most substances was between 0.005 ng/mg and 2.5 ng/mg, with some analytes showing slightly smaller linear ranges of 0.05 ng/mg - 2.5 ng/mg. The range of the limits of quantification was very low with 1.2 - 37 pg/mg in hair.

Based on the previously conducted observational study (chapter 3.2. [91]), a large number of cases with confirmed intake of psychotropic drugs, at least at the time of death, could be identified. In addition, cases were included in the study where the investigation file showed that the patient had taken psychotropic drugs in the past. In total, hair samples from n = 442 cases were then included in the study and examined.

For sample preparation, the hair samples were segmented into two segments (0-2 cm and 2-4 cm) close to the head, if the quality of the sample allowed this. In this way n = 258 samples could be examined segment by segment, while the remaining hair samples were too short or too knotted and were examined in their entire length. Little or no reliable background information on the dosage taken was known for the individual cases. Even if there is evidence from the investigation file in this regard, there is still a residual uncertainty whether these drugs were taken properly. In postmortem cases it is also very difficult to determine whether aggressive treatment of the hair was present. For this reason, hair samples with aggressive treatment of the hair may also be among the cases examined.

From the n = 442 investigated cases, n = 22 hair samples were negative and n = 420 hair samples revealed quantitative data for 41 substances and 8 metabolites. In order to address the differences in the investigated hair lengths and the uncertainty in the intake, a presentation of the data in percentiles was chosen. This approach was also found suitable by Musshoff et al [92], Tsanaclis et al. [17] and Wang et al. [93] and allows to show strong variations in hair length or the context of the samples of the study. It was also suggested [92, 94] that each laboratory should keep its own statistics based on this approach. The results were then divided into two groups, the group of segmented cases and the group of segmented and non-segmented cases. In the group of segmented cases, numerous data from comparable examination lengths and time periods could thus be presented. The group containing the segmented and non-segmented samples lost this comparability but was able to present much more additional data for many substances (e.g. amisulpride: n = 26 in group 1 vs. n = 43 in group 2).

From the previous study it was known in which cases antidepressants or neuroleptics were detected at the time of death. This allowed the comparison of the acute and chronic intake of many substances. It was found that there were cases where something was found only in the hair but not at the time of death, as well as cases where these substances were detected in both matrices. However, the results differed widely between the substances, so that no real trends could be deduced. As possible reasons for the sole detection in a particular matrix, adherence in the context of drug therapy or the frequent change of medication were discussed.

External contamination of the hair can occur very quickly in postmortem hair and is a major problem for the interpretation of such findings. Kintz et al. [95] discussed the postmortal incorporation of drugs into the hair from body fluids (e.g. blood, sweat, or putrefaction fluid), from environmental pollution, or excessive sweating during a long agonal phase in the process of dying. It was suggested that the results of segmental analysis could indicate contamination if the

concentrations are homogenous or consecutive. The investigator should be aware of these mechanisms in order to avoid false interpretation of long-term exposure. In addition, metabolite parent drug ratios are also used as possible indicators for the distinction between external contamination and systemic incorporation [96]. As part of the work, these mechanisms and possibilities of interpretation were shown for four selected cases. The results of the blood tests, the circumstances of the cases, the results of the segmental hair analysis and metabolite-parent drug ratios were discussed in detail to illustrate the complexity of the topic. Without the metabolite-parent drug ratios collected in this study, such a comprehensive consideration would be impossible.

Despite the limitations regarding the uneven hair lengths, the unknown hair cosmetics (which can decrease hair concentrations) and the missing information on the ingested dose, this work has made a valuable contribution to the expansion of data on psychotropic drugs in hair. For some substances very high case numbers (partly more than 100 cases) compared to other studies were presented and in addition 8 metabolite-parent drug ratios with also high case numbers were reported.

3.3.2 Publication

Methling M, Krumbiegel F, Alameri A, Hartwig S, Parr MK Tsokos M

Concentrations of Antidepressants, Antipsychotics, and Benzodiazepines in Hair Samples from Postmortem Cases. SN Compr. Clin. Med. (2020) 2: 284–300 https://doi.org/10.1007/s42399-020-00235-x

3.4 Manuscript IV

The use of nails as an alternative matrix for the long-term detection of previous drug intake: validation of sensitive UHPLC-MS/MS methods for the quantification of 76 substances and comparison of analytical results for drugs in nail and hair samples.

3.4.1 Summary

In the work of Krumbiegel et al. [18] it was shown that nails can be used as an alternative retrospective examination matrix for undirected search analyses. The present work continued the comparative investigation of hair and nails using quantitative data. For this purpose, two LC-QQQ-MS methods for the determination of 76 analytes (narcotics, psychotropic drugs) in hair and nail were first developed and successfully validated according to the guidelines of the GTFCh [15]. The LLOQ values ranged between 1.23 and 98.6 pg/mg for hair samples and between 0.95 and 46.7 pg/mg for nail samples, providing very sensitive detection.

For the comparative study of hair and nails, 7 postmortem cases with a known history of drug abuse were selected. In each case, samples were taken before autopsy, with whole nails being taken in five highly putrefied cases and clippings in two cases. The special focus of this work was the chosen segmentation of both hair and nail samples. This allowed similar examination periods to be considered for both matrices and helped to estimate possible ways of drug incorporation in the nail. By segmenting whole nails, it was possible to investigate areas in which different incorporation mechanisms are important. A whole nail sample was divided into four different segments: A (nail clipping), B (only the bottom of the nail plate), C (segments of nail plate excluding the bottom), D (segments of the complete nail plate). It was shown that the substance concentrations in the individual segments in hair and nails are not comparable. For all cases, nail and hair concentrations of drugs of abuse where found, while also quantitative data for four antidepressants (doxepin, trimipramine, mirtazapine, opipramol) and methylphenidate was found. Based on the investigation results of the individual cases, different ways of incorporation in the nail were then discussed. Especially in the clippings, higher concentrations were often found than in other nail segments, which was hypothesized to be based on an acute intake prior to death. The incorporation of drugs from sweat or external contamination into the free edge of the nail were

discussed as possible reasons for this observation. Due to the highly variable concentrations between the individual nail segments, it was assumed that incorporation via the geminal matrix must be one of the main routes. A drug incorporation from the nail bed into nail following drug intake (via the blood) appeared to be negligible. One case showed positive findings for heroin metabolites in only one nail segment and in the clipping. It was assumed that this was due to heroin uptake a long time ago. A drug incorporation via blood during the formation of the nail at the nail root was discussed in this case. The significantly lower concentrations in the clipping were attributed to a possible external contamination for a longer time before death. Furthermore, concentration ratios of metabolites and parent substances for some drugs were considered and compared for hair and nails. The metabolite ratios for (3,4-methylenedioxyamphetamine) MDA/ (3,4-methylenedioxymethamphetamine) MDMA, 2-(ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) EDDP/methadone, and the metabolites of tilidine bisnortilidine/nortilidine were similar in the nail and hair samples in five of the different cases. It was assumed that this points to a similar incorporation, probably via the blood during the formation of the matrix. In one case, however, the ratio for EDDP/methadone was significantly higher in nails compared to hair. This observation was discussed against the background of increased sweating under methadone substitution. In this constellation, increased incorporation due to sweating could lead to altered incorporation rates. Differences in the concentration ratios of hydrolytically sensitive analytes such as cocaine or 6-acetylmorphine were discussed on the basis of sample preparation. This was shown in more detail in case 5, were the results of hair cut into small pieces were compared with grounded hair. The concentration in the cut hair was significantly higher than in the grounded hairs. The heat generated during the grinding process was discussed as a possible reason for hydrolysis and the associated loss of substance.

In summary, quantitative data for substances in nails were presented and compared with hair in temporally similar segments. Despite the non-comparable substance concentrations in both matrices, it was assumed that the incorporation pathways in hair and nails are comparable, as similar metabolite ratios were determined for some substances. In addition, quantitative data on some antidepressants and methylphenidate was presented.

3.4.2 Publication

Krumbiegel F, Hastedt M, Westendorf L, Niebel A, **Methling M**, Parr MK, Tsokos, M

The use of nails as an alternative matrix for the long-term detection of previous drug intake: validation of sensitive UHPLC-MS/MS methods for the quantification of 76 substances and comparison of analytical results for drugs in nail and hair samples. Forensic Sci Med Pathol (2016) 12: 416-432. https://doi.org/10.1007/s12024-016-9801-1

3.5 Manuscript V

Ethylglucuronide as a biomarker for alcohol consumption – a comparison between hair and nails (publication in german language)

3.5.1 Summary

It has already been pointed out in the previous chapter 2.2 that retrospective examination of hair is not always possible in cases of sparse hair growth or intentionally shaven hair. In addition, in postmortem toxicology there may cases of prolonged decay or severe burning of the body. In order to have a possibility to assess the long-term drug intake in such cases, the research of alternative matrices is all the more important. The present work dealt with the incorporation of the direct alcohol consumption marker EtG into nails in the context of postmortem toxicology. In addition to nail edges, hair samples were also examined. First, an LC-QQQ-MS method for the detection of EtG in nails and hair was developed and successfully validated according to the guidelines of the GTFCh [15]. For the validation, hair and nail edges of children were used as blank matrix. A linear calibration range of 6.5 - 208 pg/mg for hair and 6.5 - 417 pg/mg for nails was achieved. The limit of quantitation was 1.49 pg/mg for hair and 3.49 pg/mg for nails.

Following successful validation, a collective of living subjects (n = 18) and a collective of postmortem cases (n = 19) were examined. The living subjects answered a questionnaire on their drinking behavior over the last 3 months before inclusion in the study, whereupon only subjects with normal drinking behavior were included. Furthermore, six of the test persons stated that they lived abstinently. Only cases were included in the group of deaths in which public prosecutor's investigation files provided reliable evidence of chronic excessive alcohol consumption in the last months before death.

From both groups a 0-3 cm long proximal hair section and the protruding free nail edge ("clipping") were examined. In the postmortem cases, samples were taken before the autopsy in order to avoid possible contamination by the body fluids leaking during the autopsy.

The results of the study of the group of living normal drinkers showed on average a higher concentration of EtG in nail clippings than in hair, although the extent of the increases is still subject to fluctuations. However, it could be shown from the samples of the abstinent subjects that

the negative findings of the hair analysis could also be confirmed by nail analysis. Previous studies by Berger et. al [97] and Morini et. al [98] also showed higher average EtG concentrations in the nail. In the group of the postmortem cases a much more diffuse picture with highly fluctuating EtG concentrations was evident. The concentrations in the nail were partly significantly higher than in the hair, but not consistently in all cases examined.

Various reasons for these results were compared and discussed. Many aspects can play a role here, such as different examination time periods for nails and hair, external contamination by putrefactive or bodily fluids or influences by unknown hair cosmetics in postmortem cases. Since external contamination is a known problem in post-mortem cases, it seems likely that it can also affect the concentration in the nail [95]. The paper also examined the hitherto little understood incorporation mechanisms for substances in nails and discussed the measured concentrations as a combination of internal incorporation and external incorporation over a longer period of about 3-5 months [57]. Studies to clarify the incorporation mechanisms of EtG in nails were not known at this time. Because of its high water solubility, the deposition of EtG in hair was assumed mainly external through sweat [1]. It was discussed to what extent this can be transferred to nails in the sense of accumulation of sweat under the nail edge. As limitations of the study, non-comparable examination periods of nails and hair were discussed as well as the lack of clarity about the actual consumption of alcohol in fatalities.

3.5.2 Publication

Methling M, Neumann M, Krumbiegel F

Ethylglucuronid als Alkoholmarker – Haare und Fingernägel im Vergleich. *Blutalkohol* (2017), Vol 54 No. 6, 337-45; https://www.bads.de/media/1376/ba_06_2017.pdf

4 Delimitation of own contribution

In the following the author's own contributions to the individual publications, which are the basis for this cumulative work, should be presented in detail.

Table 1 - delimitation of own contribution

manuscript	conception [%]	performance [%]	data evaluation [%]	manuscript preparation [%]
I	90	90	90	90
II	80	60	90	90
III	80	50	90	90
IV	10	40	40	10
V	50	30	30	90

5 Discussion

5.1 Contribution of the results to the scientific community

Forensic toxicological investigations in many contexts can require a retrospective investigation of a substance use over a longer period of time. Such contexts can include: Parental custody disputes, abstinence issues, assessment of past substance use, DFC cases, assessment of habituation in postmortem examinations, assessment of past alcohol consumption and compliance control. Within these contexts, questions may arise that require a distinction between abstinence, single intake or frequent intake or substance habituation. Within certain limits, hair and nail analysis can help to assess these questions. Psychotropic drugs (antidepressants, antipsychotics, benzodiazepines, alcohol) are a class of substances that may appear frequently in the daily routine of a forensic toxicologist, due to their widespread prescription frequency [13], easy accessibility (alcohol) and relevant toxicity [12]. However, most routine hair tests in forensic toxicology laboratories usually only include drugs of abuse or the examination of drinking behavior using direct alcoholism markers. The investigation of nails as an alternative matrix, in cases where no hair is available, is established to an even lesser degree in most laboratories. Situations in which psychotropic drugs are used, for example, as sedatives with criminal intent or in the context of postmortem examinations, where it is decisive how the previous intake was, are thus a great challenge in everyday forensic work. To meet this challenge, it is necessary to consider various research aspects: a) the availability of comparable data, b) the ethically correct and as uncomplicated as possible collection of data with reliable detection methods, c) the assessment of the validity of such data, d) the knowledge of substance incorporation and associated pitfalls and interpretation approaches. The general aim of this work was to take a comprehensive look at these aspects to ensure a very well-founded interpretation of the retrospective detection of psychotropic drugs in hair and nails. This was achieved by means of various types of studies and newly developed and validated analytical methods.

In a general overview of the available research, it turned out that psychotropic drugs were examined in some studies, but that both the comparability among the studies was very poor and some substances were examined very rarely. The lack of comparability was mainly due to the inconsistent use of hair lengths as well as the often-missing information on the actual intake or dosage. On the other hand, methods with very low limits of detection were presented and validated, allowing the detection of smallest amounts in low pg/mg concentration range. This is necessary if a discrimination is to be made between a single exposure or a long-term exposure

using hair analysis. One reason for the poor examination of many substances may be the ongoing development and marketing of new psychotropic drugs. However, this results in an imbalance in the data when there are some well-researched substances that may not be prescribed frequently, in contrast to drugs that are frequently used today and have hardly been researched in hair analysis. The available data for amitriptyline, a TCA, and mirtazapine, a TECA, in hair were compared and discussed to illustrate the situation. The mentioned lack of sufficient data on mirtazapine in hair may become fatal if the detection and interpretation of mirtazapine in hair becomes necessary. With increasing prescription rates and sedative effects, this situation may become more common in the future. This imbalance has been shown for several other substances and in the long run leads to a much more difficult interpretation of the detection of psychotropic drugs in hair. A different focus in the use and prescription of psychotropic drugs across countries can further exacerbate this situation for individual substances. In situations where no hair is available, interpretation of such forensic cases is very difficult without proper data. For the analysis of nails, the situation is even worse, as almost all available studies focus on drugs of abuse.

This hence revealed imbalance of quantitative data was to be counteracted within the framework of this thesis. Various considerations were made to find an appropriate study design. Controlled retrospective studies with comparable hair lengths and known dosages may be the most profound way to obtain data of high quality. Such data collection in the context of research work can only be done indirectly with patients that already take this medication. Ethical considerations forbid that healthy living test persons are affected in their health by the intake of medication only for the purpose of research on hair concentrations. These retrospective studies can only be realized in the context of clinical co-operations with psychiatric institutions. A co-operation requires adherence to strict ethical standards and extensive planning and time. After successful approval of the performance of such a retrospective examination, the recruitment of suitable patients takes a lot of time. Tight inclusion criteria such as sufficient hair length, no use of aggressive hair cosmetics and a proper intake of the prescribed medication for at least three months mean that the selection of potential patients is already very limited. The relationship of trust between recruiting physician and patient is also crucial for successful recruitment, since patients are likely to have a mental disorder when taking psychotropic drugs. In order to avoid the major hurdles in conducting such studies, an alternative approach was chosen. Data collected in the context of forensic toxicological investigations of postmortem cases may be used for retrospective research purposes with the permission of the Berlin public prosecutor's office. A disadvantage of this study approach is the often-insufficient information about the existing medication of the deceased and the uncertainty about the actual intake in the last months before death. This requires special

attention in the subsequent evaluation and interpretation of such data compared to controlled dose studies. T, a retrospective investigation of hair samples from postmortem cases with known psychotropic drug use from the Institute of Legal Medicine and Forensic Sciences at the Charité University Medicine Berlin was conducted. A pre-selection of cases was necessary because the sample preparation of hair and the sample extraction is very time consuming and costly. Due to the high effort involved, hair analyses are often not routinely performed in postmortem investigation procedures. Even in the investigation files of the public prosecutor's office there is only rarely any indication of psychotropic drug use. It can at least be assumed that a person who was under the influence of psychotropic drugs at the time of death may have been taking these substances for a longer time, and consequently the hair sample may give a positive result. These cases can then be selected for a comprehensive examination of the hair samples. The retrospective evaluation of postmortem cases for this pre-selection process showed a high number of suicides with positive findings for antidepressants and antipsychotics. This observation was pointing to the urge for an investigation of the prevalence of antidepressants and antipsychotics in suicides and non-suicides. In this cross-sectional study, it was shown that the toxicity and prescription frequency of certain substances was reflected in the frequency of their detection in postmortem cases. In addition, numerous cases with a certain intake of psychotropic drugs close to the time of death were identified for further hair analysis. Psychotropic drugs are a special type of medication because on the one hand they are very important in the treatment of mental disorder, but on the other hand they have strong side effects. In addition, in the 1990s there were the first reports of an increase in the risk of suicide when taking such substances [78-80]. Since then, research into this risk has been a continuous focus of various study approaches. The presentation of prevalence data at the actual endpoint, death, represents an important further approach. The differentiation of the results into the group of suicides and non-suicides also showed higher detection rates for suicides. Otherwise, an examination of gender differences in the group of suicides showed no major differences in the detection frequencies. In summary, TCA were found most frequently, followed by ATYP, SSRI, TYP, TECA and venlafaxine in both the suicide and non-suicide groups. Several cautious conclusions can be drawn from these results. First, that people who committed suicide were more likely to be under drug treatment. On one hand, this is a positive development, as it shows that many of these people at risk of suicide were under treatment. The distribution of the respective groups of therapeutic agents can represent both their prescription frequency and their actual toxicity. This was made clear by the example of TCA, which are the most frequently detected, even though they are less frequently prescribed than SSRI in terms of numbers [13] but are significantly more toxic due to their side effect profile [81, 82]. It can therefore be assumed that these two effects can cause these results. It was also shown that the

deceased in the suicide group were more often under the influence of several substances at once. Since drug therapies are often changed in the context of mental disorder until the desired effect occurs, it is quite possible that some of the deceased were treated with many different drugs in the near time of death. It was also found that alcohol was detected in about one third of all suicides, which was consistent with previous studies [88-90]. A limitation of this investigation, however, remains the regional bias and the bias that arises, since not all deaths and suicides in Berlin are included in this investigation. The results of the sample studied can therefore only be applied with limitations to a larger population. In studies with a similar approach, individual classes of substances were usually examined, but rather rarely the evidence of individual substances was presented [88, 99, 100]. The joint consideration of the most common antidepressants and neuroleptics has not yet been published in this form. The comprehensive investigation of the postmortem detection of antidepressants and neuroleptics in suicides and non-suicides in the context of this publication thus contributes as a complementary approach to research into the risk of suicide while taking psychotropic drugs. With this study, numerous cases with secured psychotropic drug use were identified for a further hair analysis.

These analyses were conducted with a newly developed method for the quantification psychotropic drugs in hair. The LC-QQQ-MS method for the detection of 52 substances (antidepressants, antipsychotics, benzodiazepines) in hair was successfully validated according to the guidelines of the GTFCh [15]. The successful validation proves that the method provides precise and comparable results. However, some analytical considerations concerning the preparation of calibration samples and quality control samples for hair analysis must be addressed. Cuypers et al. [101] and Xiang et al. [94] point out that the preparation of such samples can be done in three different ways, each with its own problems. Both the spiking of washed negative hairs with analyte solution and the soaking of negative hairs (fortyfing) in analyte solution do not exactly produce extracts of authentic hairs in which the substances have been incorporated. Even the approach of decontaminating and homogenizing authentic hair samples bears the risk of losses during decontamination and thus altered hair concentrations. External verification of the method by means of proficiency testing would provide further assurance against. Unfortunately, the available proficiency tests in hair do not yet include any of the psychotropic drugs investigated. These limitations must always be considered for hair analyses, despite the great performance of the methods used.

The consecutive analysis of hair samples (n = 442) from the previously identified cases with known psychotropic drug intake before death and samples with an assumed past drug use provided

quantitative data for 41 substances and 8 metabolites. High case numbers for some substances and above all new data for substances that have been rather poorly investigated so far were presented. This study thus makes an enormous contribution to counteracting the insufficient data basis for many substances. Due to the postmortem context, very little information is often available on how long a deceased person took which drug, in which dosage (and which application form) and how regularly he or she took it. Such information cannot be verified with certainty in postmortem cases either, since there is always a residual uncertainty concerning the actual intake. As already criticized in the previous sections, the knowledge about dosage and compliance is extremely important in order to use quantitative data for comparison for later interpretation of own hair analyses. However, as this remaining uncertainty on dosage cannot be excluded for this type of investigation, and correlation with dosage is therefore impossible, a different approach for the statistical evaluation of data was chosen. All detected hair concentrations were displayed in percentiles as strong variations concerning the context of the sample or variations in the used hair length can thereby be addressed. Other authors [16, 17, 92, 93] outlined the usefulness of this approach as well and propose that each laboratory should build its own statistics around it. The concentration ranges obtained in this way can be used for a rough and comparative estimation of a past intake frequency. The individual ranges between the percentiles can be assigned and interpreted the following way [16]: the lower range between the minimum to the 25th percentile, the middle above the 25th to the 75th percentile and the upper range above the 75th percentile. For drugs of abuse and pharmaceuticals the individual ranges were interpreted differently: the lower range is to be predictive of moderate use of drugs of abuse and the upper range to be linked to a heavy consumption [16]. Middle range concentrations were assigned for drug misuse while concentrations above the 95th percentile could suggest excessive consumption [16]. For pharmaceuticals and legal drugs, the lower range is associated with a misuse in the recent past or a current proper use [16]. The middle range may suggest an actual administration of high doses or a misuse while the upper range can be associated to administration of unusual high doses, dependency or tolerance [16]. To what extent this applies to psychotropic drugs cannot be clarified at this point, but nevertheless this proposal provides an important basis for interpretation. For the investigated postmortem cases within this study, concentrations above the 95th percentile may also indicate cases with possible external contamination. A comparison of the present work with the work of Musshoff et. al [16] shows a satisfactory conformity of the concentration ranges for numerous substances with partly similar case numbers. Although the hair samples originated from a different context (criminal offense or custody cases) and a different sample preparation was used in this publication [16], similar concentration ranges are achieved in both laboratories. The comparison also shows that the investigation of larger numbers of cases and the associated

statistical significance can lead to better comparability of results. This is particularly exciting, as significantly higher concentrations may occur in postmortem cases, due to external contamination. The comparison may thus suggest that the concentration ranges presented in this work from postmortem cases may be artificially increased only to a small extend by cases with possible external contamination. Thus, a good comparability and transferability of the presented data to samples from living subjects seems acceptable. Nevertheless, hair concentrations from controlled dose studies should have the greatest benefit for an estimate of past frequency of use. Despite uniform validation guidelines, each laboratory uses its own decontamination and extraction procedures, so that the detected concentrations may differ between two laboratories. Due to the aforementioned lack of proficiency tests to ensure similarity, it is even more important that laboratories use statistics as described above. Despite different methods in other laboratories, the concentrations determined in this study can help other scientists to interpret their own findings cautiously, also in view of the fact that they themselves may perform such analyses too rarely and thus obtain sound statistics. However, since very few laboratories carry out hair analyses at all and even fewer laboratories study psychotropic drugs in hair, it is very important to share the results of this work. Since many cases would have been excluded from the study due to differing hair lengths, the results were divided into the group of segmented samples and the combined group of segmented and non-segmented samples. The grouped presentation of the results for the hair analyses allows comparable hair lengths for the group of segmented samples. By including the non-segmented samples, many more cases could be presented, but their results are to be considered within the limits of the non-comparable hair lengths and investigation periods. A major limitation of this study is the often-unknown hair cosmetic treatment in postmortem cases. Aggressive hair cosmetics such as bleaches and dyes can lead to a significant reduction of the analyte concentration in the hair. In living persons, it is therefore essential to ask for and record hair cosmetic treatments when taking samples. In the postmortem cases, this inquiry is rarely possible, although the extracted sample buffer can be colored if certain cosmetic treatment was applied. This limitation should be considered when interpreting the data. Despite the limitation of the study mentioned above, the approach of indirectly obtaining quantitative data via retrospective postmortem studies is still very useful in order to further explore the research area on an ethically justifiable basis.

The examination of hair samples from previously selected cases with an intake of psychotropic drugs at the time of death allows a comparison of the acute intake and the long-term intake of these substances. In the examined collective, a full agreement of the results in both examined matrices up to an exclusive detection in the hair was shown, depending on the substance. If the

results from blood (or urine and organs) positive for a substance, but negative in the hair sample this may indicate that a medication has been discontinued as part of a failed drug treatment. The clarification of this history of use would not be possible without a hair analysis and may be helpful for further investigation in certain cases. However, it is not possible to derive trends for individual substances with regard to their efficacy or hazardousness from these data.

The already mentioned lack of sufficient studies on psychotropic drugs in hair also concerns their metabolites. Only a few studies at all have dealt with these degradation products and presented data on them [35, 93]. With the present work and the presentation of high case numbers for 8 metabolites and the corresponding metabolite-parent drug ratios, an important contribution was achieved. In the course of the study it was also shown how a possible external contamination by body fluids can be clarified by using a segmental hair analysis [95] and the investigation of metabolite/drug ratios. This would never have been possible without the metabolite-parent drug ratios identified in the study and the segmental analysis. Especially in forensic investigations it is of enormous importance to avoid false positive results. They can occur due to technical reasons, for example sample mix-ups or problems during analysis, or they can be caused by passive exposure to drugs. The topic of external contamination and thus the possible falsification of interpretation in hair analysis results is currently being intensively discussed [96]. Various investigations have shown that the deposition of substances from the outside onto the hair, in the sense of external contamination, is possible by different scenarios. It has been shown that staying in rooms where cannabis products are smoked [103] or contact with cocaine dusts can lead to positive results in hair analysis [103]. Intensive washing protocols are often performed during sample preparation. The detection of a substance in the washing solution can be an indication of external contamination. The detected concentration in the hair should be a factor of 10 higher than the concentration in the wash solution to exclude contamination [112]. In addition, the study of different metabolites and their relation to the parent substance was proposed to distinguish true uptake from external contamination The search for suitable metabolites to detect systemic uptake into the hair is therefore still an intensively investigated field of research [104, 105] and the investigations in this thesis have contributed significant data for this purpose.

With the help of the comparative work on nails and hair in two further investigations, important aspects about the incorporation pathways in nails and the application of nail analysis could be gained. The successful development and validation for the detection of EtG as well as drugs of abuse and psychotropic drugs in nails and hair provides a good basis for further quantitative investigations. Due to the low detection limits of both methods, it is possible to detect even very

low concentrations and thus possibly also one-time recordings of substances. In the case of DFC cases, this is highly desirable in order to provide decisive information for the solution of such cases. Both publications showed that the concentrations in the nail edges are not comparable with the hair sections that were comparable over time. In accordance with results from other authors higher EtG concentrations were found in the nail clippings compared to 3 cm of proximal hair [97, 98, 106]. It was shown that fingernails are suitable as an alternative matrix for the investigation of drinking behavior over a longer period of time. It was discussed whether EtG could be deposited in the nail mainly via sweat and the different time windows investigated. Nail clippings cover about 3-5 months before sampling compared to 3-6 months for 3 cm hair [57]. It should be noted, however, that the concentration found in nail clippings is a mixture of internal and external deposition via sweat [57] and thus seems difficult to compare with the usual cut-offs for EtG in hair. Cappelle et al. [106] mentioned similar implications from the observation of higher EtG concentration in nails: a) analysis of nails can be interesting for expected low EtG concentrations and b) established hair analysis cut-offs by the SoHT [8] are not valid for nail analysis and future studies should focus on establishing cut-off values for EtG which are relevant for nails. However, a good agreement between the stated drinking amount and EtG concentration in nail and hair was found in the presented work and further work [106]. Recently, the SoHT proposed a lower cut-off of 5 pg/mg EtG in hair for the discrimination of abstinence and normal drinking behavior [107]. In anticipation of this lower limit, the detection method for EtG in hair has now been established on a new LC-MS/MS device with a better performance in the low limits of detection. Nevertheless, the examination of nail clippings is suitable for the investigation of total abstinence, as could be shown by our results. The detection of EtG in fingernails in postmortem cases has not yet been presented in any other study. The very diffuse results obtained here once again illustrate the strong influence of possible external contamination by body fluids. For the drugs of abuse and psychotropic drugs, there were also differences between the concentrations of hair and nails. Based on the results of both investigations, however, it is not possible to say whether the concentrations in the nail are generally higher than in the hair. The successful segmentation of whole nails allowed important insights into the incorporation pathways in the nail. Since the respective substance concentrations between the segments varied widely, metabolite ratios were used. Here it was shown that the concentration ratios (with some exceptions) in the nail and hair are approximately the same, as could also be shown by Madry et al. [108] for MDMA and its metabolite MDA. Therefore, it can be assumed that the deposition rate for nails and hair can be similar. Cappelle et al. [109] recently published a study where they investigated hair and nail samples from patients in drug withdrawal therapy. In accordance with the presented work, the authors found higher cocaine concentrations in hair than in the nails and higher concentrations for

its hydroxylated metabolite benzoylecgonine in the nails compared to hair. Both findings led to a higher benzoylecgonine/cocaine ratio in hair. While in the presented work it was discussed that this finding is related to a possible heat-driven hydrolysis during grinding of the samples, Cappelle et al. [109] raised the idea that the lack of melanin in the nails leads to a lower accumulation of cocaine. The detection of heroin in only a single nail segment and not in the other nail and hair segments may be an indication of phased incorporation into the nail.

Unfortunately, due to the small number of cases and available evidence material, no comparisons between finger and toenail concentrations could be made in both studies. However, toenails should be less restricted by cosmetic influences. A study by Engelhart et al. [110] showed that the substance concentrations of finger and toenails differ. In addition, a comparison of the substance concentrations in the nail with those in a washing liquid showed similar conditions in fingernails and toenails. It was therefore assumed that the influence of external contamination on the nails in this study was rather small.

An important analytical aspect became clear from the sample preparation. In both investigations the hair samples were ground in a ball mill in the same way as nail samples. In routine investigations for drugs of abuse, the hairs are usually cut into small pieces while for EtG the hair samples are milled. For one of the presented cases it could be shown that a loss of substance for hydrolysis-sensitive substances such as 6-acetylmorphin, cocaine or benzoylecgonine can be observed when grinding the hair. This was already pointed out by Pragst et al. [1]. To circumvent this problem, cooled ball mills may be better suited, or the samples may be frozen before processing. Since both matrices, hair and nails, were ground, this effect is included in both investigations, therefore the concentrations obtained remain comparable.

In summary, this thesis, with its investigations, methods used and critical discussions, is in line with current research and makes an important contribution to retrospective studies of psychotropic drugs. It provides many other researchers with a quick overview of the current data and research situation regarding the detection of psychotropic drugs in hair and has made a very important effort to expanding the database of psychotropic drugs in hair by means of a larger statistical case numbers. For some substances even high two-digit to three-digit case numbers could be presented. This high number of cases, only rarely achieved in other studies, provides a great statistical certainty. In the introduction to this work, several situations from the everyday life of a forensic toxicologist were outlined. With the help of the collected data, these situations can now be dealt with in a much more profound way. Clearly, the data and critical discussion of the

interpretation of such results can meaningfully help other toxicologists with their own forensic investigations. The comparative studies of hair and nails made important contributions to basic research in this field, especially regarding concentration differences, sample extraction and incorporation pathways in the nail.

6 Outlook

The present work importantly contributes valuable data to extend the detection of psychotropic drugs in hair. However, the lack of comparable data still exists and the research in the coming years should make efforts to overcome this situation. The chosen approach of the investigation of post-mortem cases with known psychotropic drug intake has led to the presentation of comprehensive data. The comparison with recently published data showed highly similar results despite different contexts of the hair samples. Thus, the collected data may be an important aid for forensic toxicologists, facilitate their daily work, and support them in interpreting concentrations of psychotropic drugs in hair. Nevertheless, the lack of certainty regarding the history and frequency of use remains a limitation of this approach. Although major hurdles are involved, controlled retrospective studies with comparable hair lengths and known dosages may help to overcome this uncertainty. With the developed and established LC-QQQ-MS method it is now possible to conduct retrospective clinical studies with controlled dosage in co-operation with psychiatric institutions. Through these types of studies, more profound data on psychotropic drugs in hair will be obtained, which will provide valuable assistance in interpreting the results of hair analyses. Since in previous publications contradictory results have been found regarding the correlation of dose and hair concentration [111], such studies are very valuable to investigate to what extent the ingested dose and concentration in hair can perhaps be correlated at some point in time by new techniques or statistical models. Once such correlations at some point will be well-founded and scientifically accepted, they will certainly offer an exciting and above all non-invasive alternative to blood level determinations in the context of adherence controls.

A study on living individuals with known drug intake was proposed and approved by the ethics committee. Recently, a co-operation with the Clinic for Psychiatrics and Psychology of the Charité University Medicine Berlin was initiated and is currently recruiting patients with a stable and long intake of antidepressants or antipsychotics. The foundation stone for further research work has thus been laid.

Measurement results of hair analyses can vary to a certain extent depending on the extraction, decontamination and measurement technique used. The quality of such detection methods is proven on the one hand by validation and on the other hand by comparative proficiency testing. These are more important for hair analyses, since control samples used in routine analysis cannot completely mimic hair material into which substances have been naturally incorporated. Even if currently hair analysis for the detection of psychotropic drugs is rather rarely applied, it will be

important in the future to establish proficiency tests that address the analysis of psychotropic drugs in hair. To date, such proficiency tests are not commercially available. Alternatively, laboratory comparisons could be carried out using samples from controlled dose studies. However, due to the voluntary nature of the participation in such studies, cosmetic compatibility should be considered when taking samples, so that no large sample quantities could be obtained. Thus, only a few laboratories could participate in a comparison. These two testing options would drastically increase the comparability of methods and results from different laboratories and thus contribute to overall evaluation of measurement certainty.

The investigation of the prevalence of certain psychotropic drugs in suicides and the comprehensive presentation of toxicological data may continue to represent a complementary approach to the risk assessment of these substances. It would be desirable to collect such data nationwide, as is the case in Sweden, for example. The accredited toxicological center in Linkoping investigated all post-mortem cases of Sweden and data is collected at a national health register for research purposes [112, 113]. This approach allows for more comprehensive statistical studies that are not limited to regional districts. However, the federal structure of Germany with its division into independent forensic institutes and the resulting great effort of a comprehensive exchange of data is currently an obstacle.

A segmentation of whole nails and the comparative examination of nail segments with hair segments has not yet been reported by other research groups for drugs of abuse. Further of such, admittedly very costly, investigations would be necessary to confirm the results obtained from other sources. Although nails have been researched to some extent in recent years, there have been few controlled dose studies of individual substances. Research in this area should continue under controlled conditions in a similar approach to that for hair. Capelle et al. [114] point out that the relationship between the concentration in the nail and the concentration in other matrices (e.g. blood, urine, hair) should be brought more into focus. From an analytical point of view, efforts should be made to harmonize sample preparation, as this work also showed a strong influence of the preparation (grinding vs. cutting). Furthermore, proficiency tests for nails should be developed to ensure a comparable analytical quality [114]. In addition, extensive controlled studies should be carried out in the next years to establish cut-off values [114]. Some authors already proposed cut-off values for the detection of EtG in nails on the basis of small collectives [97, 106]. However, they all point out, that more the major problem for nail analysis is the lack of research that must be continued in the future.

Both presented nail studies in this work were preliminary investigations on very small case numbers and only few data on psychotropic drugs in the nail was found. Nevertheless, some important quantitative data were obtained for some substances. With the help of the validated methods now available, more extensive, at best comparative controlled dose studies should be carried out to detect psychotropic drugs in hair and nails.

7 Summary

Psychotropic drugs are a class of substances that are widely used in Germany and worldwide in the treatment of mental disorder and are therefore frequently prescribed. Like any drug, they have a certain side-effect profile, mainly comprising of cardiac and sedative components. Some of these substances, such as the tricyclic antidepressants, also have a high toxicity in case of overdoses. Due to these properties, the broad prescription and their toxicity, these substances may occur in different scenarios of forensic toxicological work. Their sedative properties and easy availability also make them substances that could potentially be used in crimes. To a certain extent, with the analysis of nails and hair it is possible to draw conclusions about the past frequency of use. The main objective of this thesis was to collect and meaningfully expand the currently available data on the detection of psychotropic drugs in hair in order to allow a comprehensive interpretation of such findings in daily forensic work. Furthermore, comparative studies of hair and nails should provide a deeper insight into the incorporation of substances in the nail and their use as an alternative matrix to hair.

A comprehensive literature review showed that there are clear gaps in research on psychotropic drugs in hair, especially quantitative data. Three categories of publications were found: (i) method validations for the detection of numerous substances in hair, (ii) case reports and (iii) controlled dose administration studies. In the first two categories, often only small case numbers, very variable hair lengths and only very rarely information on the dosage taken was available. This was not the case in the controlled dose studies, which showed comparable hair lengths, at reported doses and assumed compliance. Furthermore, it was shown that the data basis for some, rather older, substances (e.g. amitriptyline) is very extensive through controlled dose studies, while many newer, now much more frequently prescribed substances (e.g. mirtazapine) were investigated in only a few studies. The publications also showed large differences in the sample preparation and extraction method selected. So far, no interlaboratory tests for psychotropic drugs in hair are available. For future studies a harmonization of the hair lengths and extraction methods used would therefore be welcome.

In order to expand the data situation for psychotropic drugs in hair, two retrospective studies were conducted on post-mortem cases. For the identification of cases with known psychotropic drug ingestion, the toxicological test results of all deaths from 2012-2015 were evaluated in the section property of the Institute for Forensic Medicine of the Charité University Medicine Berlin. All cases with positive findings for a selected number of psychotropic drugs in blood, urine or tissue samples

were included, as these findings indicate acute or subacute influence at the time of death. Furthermore, the blood alcohol test was also evaluated in all cases. In these cases, it was assumed that they have taken psychotropic drugs for a longer period before sampling. It quickly became apparent that many of the cases with positive psychotropic drug evidence at death were suicides. From this observation, as well as from case reports [94] that found an increased risk of suicide under psychotropic drug use, the idea of conducting a comparative observational study was born. All included cases were divided into suicides (n=235) and non-suicides (n=212) on the basis of the final public prosecutor's investigations. In the group of suicides, higher frequencies were found for all substance classes compared to the group of non-suicides. The most frequently detected class of substances in the suicide group were TCAs, followed by SSRIs, ATYPs, TYPs, TECA and other substances. Among the individual substances, doxepin (20%), citalopram (15.3%), mirtazapine (14.9%), quetiapine (13.6%) and amitriptyline (12.3%) were detected most frequently. Alcohol was detected in about one third of the suicides compared to one fifth in the non-suicide group, in line with data from other authors [104-106]. This first study served to identify cases for further hair analysis with known ingestion of psychotropic drugs in the time before death.

The next investigation dealt in the first step with the development and validation of a sensitive LC-QQQ-MS method to determine psychotropic drugs in hair. Based on the current prescription report of the health insurance companies for psychotropic drugs [13], 52 relevant substances were included in the method. The method was successfully validated based on the guidelines of the GTFCh for the validation of analytical methods [15, 73] for most analytes in a working range of 0.005 - 2.5 ng/mg in hair. All validation requirements were tested and met. The range of the limits of quantification was very sensitive with 1.2 - 37 pg/mg in hair. Hair samples (n = 442) from the cases of the study on the frequency of antidepressants and antipsychotics in suicide together with cases in which psychotropic drug use was indicated in the public prosecutor's investigation file were examined in this study. A special feature of the study was the segmentation of the hair whenever possible. In those cases where segmentation was not possible due to the quality of the sample, the entire length of the hair was examined. In summary, quantitative data in hair were determined for 41 substances and 8 associated metabolites using n = 420 samples. The hair samples of twenty-two included cases were negative. In order to present the variations in the hair lengths, investigated population and the missing dose information in a statistically representable way, the quantitative data was presented in percentiles. With results of the previous study it was also possible to show that the detection in hair did not always correspond to the results in blood, urine or organs. Similar to the previous study, it was shown that in many cases more than one substance was often detected. The study further addressed the influence of external

contamination in postmortem hair analysis and its proposed interpretation based on the results of four cases. Taking into account the discussed limitations of the study, this work made an important contribution to the expansion of the data situation for psychotropic drugs in hair based on case numbers, some of which are in the three-digit range.

In two comprehensive studies comparing hair and nails, valuable information was obtained on the incorporation of substances into the nail and the applicability of nail analysis in postmortem cases. After successful development validation of an LC-QQQ-MS method for the detection of EtG in nails and hair according to GTFCh guidelines [15, 73], fingernail edges and hair samples from living subjects with normal drinking behavior (n = 18) and from postmortem cases with chronically excessive drinking behaviour (n = 19) were examined. In agreement with other authors [113, 120], it was shown that the concentrations in the nail margin were higher than in hair. The different detection windows and a strong deposition of EtG via sweat in the nail margin were discussed as possible reasons for this. On the basis of four abstinent volunteers it was shown that nails are also suitable for abstinence control. In the postmortem cases, significantly different results were obtained between hair and nails. This observation was also confirmed in a further study, which dealt with a comparative segmental examination of nail and hair samples of selected postmortem cases. Two LC-QQQ-MS methods for the detection of 76 narcotics and psychotropic drugs in nails and hair were developed and validated according to GTFCh guidelines [15, 73]. Hair and nail samples from seven selected postmortem cases with known drug abuse in the past were examined and both hair and nail samples were comprehensively segmented. Similar to the results for the alcohol marker EtG, significantly different concentrations of narcotics were found in nails and hair. Additionally, the metabolite ratios for some substances were examined. It was found that some of the ratios of MDA/MDMA, EDDP/methadone and bisnortilidine/nortilidine were similar in hair and nails. Higher cocaine values in hair compared to nails and higher benzoylecgonine values in nails compared to hair were evident. Furthermore, quantitative data for four antidepressants (doxepin, trimipramine, mirtazapine, opipramol) and methylphenidate in nails and hair was presented.

In summary, an important collection and extensive expansion of the data basis for numerous psychotropic drugs in hair was realized within the scope of this dissertation. This basis can be of decisive importance for daily forensic work and can serve as an important support for the interpretation of hair findings. The thesis also demonstrated that nails as alternative examination matrix to hair are in principle suitable within certain limitations and should remain the subject of further research.

8 Zusammenfassung

Psychopharmaka sind eine Klasse von Substanzen, die national und weltweit eine breite Anwendung in der Behandlung mentaler Erkrankungen finden und dementsprechend häufig verschrieben werden. Wie jedes Arzneimittel besitzen sie ein gewisses Nebenwirkungsprofil, welches hauptsächlich herzwirksame und sedierende Komponenten umfasst. Einige dieser Substanzen, wie etwa die trizyklischen Antidepressiva, weisen zudem eine hohe Toxizität bei Überdosierungen auf. Aufgrund dieser Eigenschaften, der breiten Verschreibung und ihrer Toxizität können diese Substanzen in verschiedenen Szenarien von forensisch toxikologischer Arbeit vorkommen. Die sedierenden Eigenschaften und leichte Verfügbarkeit machen sie zudem zu Substanzen, die potenziell in Verbrechen eingesetzt werden können. Bis zu einem gewissen Grad ist es mit der Analyse von Nägeln und Haaren möglich, Rückschlüsse auf die zurückliegende Einnahmehäufigkeit zu ziehen. Das Hauptziel dieser Arbeit war es, die derzeit verfügbaren Daten über den Nachweis von Psychopharmaka im Haar zu sammeln und sinnvoll zu erweitern, um eine umfassende Interpretation solcher Befunde in der täglichen forensischen Arbeit zu ermöglichen. Weiterhin sollten vergleichende Untersuchungen von Haaren und Nägeln einen tieferen Einblick in die Einlagerung von Substanzen in den Nagel und die Verwendung als alternative Matrix zu Haaren geben.

Eine umfassende Literaturstudie zeigte, dass die Forschung zu Psychopharmaka im Haar, im speziellen zu quantitativen Daten, deutliche Lücken aufweist. Die verfügbaren Veröffentlichungen gliederten sich grob in drei Kategorien: (i) Methodenvalidierungen für den Nachweis von zahlreichen Substanzen im Haar, (ii) Fallberichte und (iii) kontrollierte Dosisstudien. In den ersten beiden Kategorien lagen oftmals nur kleine Fallzahlen sowie sehr variierende Haarlängen vor und nur äußerst selten wurden Informationen zur eingenommenen Dosierung angegeben. Dies traf auf die kontrollierten Dosisstudien nicht zu, die vergleichbare Haarlängen und bekannte Dosierungen bei angenommener Compliance vorwiesen. Weiterhin zeigte sich, dass die Datengrundlage für einige, eher ältere, Substanzen (z.B. Amitriptylin) durch kontrollierte Dosisstudien sehr umfangreich ist, während viele neuere, heute wesentlich häufiger verschriebene Substanzen (z.B. Mirtazapin) in nur wenigen Studien untersucht wurden. Die Veröffentlichungen zeigten auch große Unterschiede bei der gewählten Probenaufarbeitung und Extraktion. Bisher sind keine Ringversuche für Psychopharmaka im Haar verfügbar. Für zukünftige Studien ist daher eine Harmonisierung der verwendeten Haarlängen und Extraktionsverfahren erforderlich und wünschenswert.

Zur Erweiterung der Datenlage für Psychopharmaka im Haar wurden daher zwei retrospektive Untersuchungen im Rahmen von Sterbefällen durchgeführt. Zur Identifizierung von Fällen mit bekannter Psychopharmaka-Einnahme wurden im Sektionsgut des Instituts für Rechtsmedizin der Charité - Universitätsmedizin Berlin die toxikologischen Testergebnisse aller Todesfälle der Jahre 2012-2015 ausgewertet. Alle Fälle mit positivem Befund für eine ausgewählte Anzahl von Psychopharmaka im Blut, Urin oder Gewebeproben wurden einbezogen, da diese Befunde auf einen akuten oder subakuten Einfluss zum Todeszeitpunkt hinweisen. Bei diesen Fällen wurde angenommen, dass sie auch längere Zeit vor der Probenahme Psychopharmaka eingenommen haben könnten. Dabei zeigte sich sehr schnell, dass es sich bei vielen der Fälle mit positivem Psychopharmaka-Nachweis zum Zeitpunkt des Todeseintritts um Suizide handelte. Aus dieser Beobachtung heraus, sowie aus Fallberichten [94], die ein erhöhtes Suizidrisiko unter Psychopharmaka-Einnahme feststellten, entstand die Idee eine vergleichende Beobachtungsstudie durchzuführen. Alle eingeschlossenen Fälle wurden anhand der abschließenden staatsanwaltschaftlichen Ermittlungen in Suizide (n=235) und Nicht-Suizide (n=212) eingeteilt. In der Gruppe der Suizide zeigten sich für alle Substanzklassen höhere Häufigkeiten im Vergleich zur Gruppe der Nicht-Suizide. Die am häufigsten nachgewiesene Klasse von Substanzen in der Gruppe der Suizide waren trizyklische Antidepressiva (TCA), gefolgt von selektiven Serotonin-Reuptake Inhibitoren (SSRI), atypischen Neuroleptika (ATYP), typischen Neuroleptika (TYP), tetrazyklischen Antidepressiva (TECA) und weiteren Substanzen. Unter den einzelnen Substanzen wurden Doxepin (20 %), Citalopram (15,3 %), Mirtazapin (14.9 %), Quetiapin (13.6 %) und Amitriptylin (12.3 %) am häufigsten detektiert. Alkohol wurde in Übereinstimmung mit Daten anderer Autoren [104-106] in ca. einem Drittel der Suizide im Vergleich zu einem Fünftel in der Gruppe der Nicht-Suizide nachgewiesen. Diese erste Studie diente dazu, Fälle für weitere Haaranalysen mit bekannter Einnahme von Psychopharmaka in der Zeit vor dem Tod zu identifizieren.

Die nächste Untersuchung befasste sich im ersten Schritt mit der Entwicklung und Validierung einer LC-QQQ-MS-Methode zur Bestimmung von Psychopharmaka im Haar. Auf der Grundlage des aktuellen Verschreibungsreports der Krankenkassen für Psychopharmaka [13] wurden 52 relevante Substanzen in die Methode aufgenommen. Die Methode wurde auf der Basis der Richtlinien der GTFCh [15, 73] für die Validierung von Analysenmethoden für die meisten Analyte in einem Konzentrationsbereich von 0.005 - 2.5 ng/mg im Haar erfolgreich validiert. Alle Anforderungen an die Validierung wurden getestet und erfüllt. Die Bestimmungsgrenzen waren mit 1.2 - 37 pg/mg im Haar sehr niedrig. In dieser Studie wurden Haarproben (n = 442) von in der Studie über die Häufigkeit von Antidepressiva und Antipsychotika bei Suiziden identifizierten

Fällen zusammen mit Fällen untersucht, in denen in der Ermittlungsakte der Staatsanwaltschaft auf psychotropen Drogenkonsum hingewiesen wurde. Eine Besonderheit der Untersuchung war die Segmentierung der Haare, wann immer dies möglich war. In den Fällen, in denen keine Segmentierung aufgrund der Qualität der Probe möglich war, wurde jeweils die gesamte Haarlänge untersucht. Zusammengefasst wurden quantitative Daten im Haar für 41 Substanzen und 8 zugehörigen Metaboliten anhand von n=420 Proben ermittelt. Die Haarproben von zweiundzwanzig eingeschlossenen Fällen waren negativ. Um die Variationen in den Haarlängen, der untersuchten Population und die fehlenden Dosisinformationen statistisch darzustellen, wurden die quantitativen Daten in Perzentilen ausgewertet. Dies bildet einerseits die Unterschiede bezüglich der verwendeten Haarlängen, aber auch die Variationen innerhalb der untersuchten Zwecke und Populationen gut ab. Anhand der Ergebnisse der vorherigen Studie konnte auch gezeigt werden, dass der Nachweis in den Haaren nicht immer mit den Ergebnissen in Blut, Urin oder Organen übereinstimmte. Ähnlich wie in der Vorgängerstudie konnte gezeigt werden, dass in vielen Fällen oft mehr als eine Substanz nachgewiesen wurde. Ähnlich der zuvor durchgeführten Studie, zeigte sich, dass in vielen Fällen häufig mehr als eine Substanz nachgewiesen wurde. Die Studie befasste sich ferner mit dem Einfluss der äußeren Kontamination bei der postmortalen Haaranalyse und ihrer vorgeschlagenen Interpretation auf der Grundlage der Ergebnisse von vier Fällen. Unter Berücksichtigung der diskutierten Einschränkungen der Studie leistete diese Arbeit einen wichtigen Beitrag zur Erweiterung der Datenlage für Psychopharmaka im Haar auf der Basis von Fallzahlen, die zum Teil im dreistelligen Bereich liegen. Mit Hilfe dieser Daten wird die Interpretation von Psychopharmaka-Befunden im Haar wesentlich erleichtert und in konkreten Fragestellungen unterstützt.

In zwei umfassenden Studien zum Vergleich von Haaren und Nägeln wurden wertvolle Informationen über die Einarbeitung von Substanzen in den Nagel und die Anwendbarkeit der Nagelanalyse in postmortalen Fällen gewonnen. Nach erfolgreicher Validierung einer LC-QQQ-MS-Methode zum Nachweis von EtG in Nägeln und Haaren nach den Richtlinien der GTFCh [15, 73] wurden Fingernagelränder und Haarproben von lebenden Probanden mit normalem Trinkverhalten (n = 18) und von postmortalen Fällen mit chronisch übermäßigem Trinkverhalten (n = 19) untersucht. In Übereinstimmung mit anderen Autoren [113, 120] zeigte sich, dass die Konzentrationen im Nagelrand höher waren als in den in Haaren. Als möglicher Grund wurden dafür die unterschiedlichen Nachweisfenster und eine erhöhte Einlagerung des EtG über den Schweiß in den Nagelrand diskutiert. Anhand von vier abstinent lebenden Probanden konnte gezeigt werden, dass sich Nägel auch für eine Abstinenzkontrolle eignen. Bei den Sterbefällen zeigten sich deutlich unterschiedlichere Ergebnisse zwischen Haaren und Nägeln. Diese

Beobachtung wurde auch in einer weiteren Studie bestätigt, die sich mit einer vergleichenden und segmentweisen Untersuchung von Nagel- und Haarproben ausgewählter postmortem Fälle befasste. Zwei LC-QQQ-MS-Methoden zum Nachweis von 76 Betäubungsmitteln und Psychopharmaka in Nägeln und Haaren entwickelt und nach den Richtlinien der GTFCh [15, 73] validiert. Haar- und Nagelproben von sieben ausgewählten postmortalen Fällen mit bekanntem Drogenmissbrauch in der Vergangenheit wurden untersucht und sowohl Haar- als auch Nagelproben umfassend segmentiert. Ähnlich den Ergebnissen für den Alkoholmarker EtG zeigten sich deutlich unterschiedliche Konzentrationen für Betäubungsmittel in Nägeln und Haaren. Zusätzlich wurden für einige Substanzen die Metabolitenverhältnisse untersucht. Es wurde festgestellt, dass einige der Verhältnisse von MDA/MDMA, EDDP/Methadon und Bisnortilidin/Nortilidin bei Haar und Nägeln ähnlich waren. Es wurden höhere Kokainwerte im Haar im Vergleich zu den Nägeln und höhere Benzoylecgonin-Werte in den Nägeln im Vergleich zu den Haaren. Darüber hinaus wurden quantitative Daten für vier Antidepressiva (Doxepin, Trimipramin, Mirtazapin, Opipramol) und Methylphenidat in Nägeln und Haaren vorgelegt.

Zusammengefasst wurde im Rahmen dieser Dissertation eine wichtige Erfassung und umfangreiche Erweiterung der Datengrundlage für zahlreiche Psychopharmaka im Haar realisiert. Diese Grundlage kann für die tägliche forensische Arbeit von entscheidender Bedeutung sein und als wichtige Stütze für die Interpretation von Haarbefunden dienen. Weiterhin wurde dieser Arbeit gezeigt, dass sich Nägel als alternative Untersuchungsmatrix zu Haaren prinzipiell (mit bestimmten Limitierungen) eignen und Gegenstand weiterer Forschung bleiben sollten.

9 Publication portfolio

9.1 Publication in peer-reviewed international and national scientific journals

1. Methling M, Krumbiegel F, Hartwig S. Hair analysis of antidepressants and antipsychotics - Overview of quantitative data. *Drug Test Anal. (2020)*; 1– 18.

2. Methling, M., Krumbiegel, F., Alameri, A., Hartwig S, Parr MK Tsokos, M. Concentrations of Antidepressants, Antipsychotics, and Benzodiazepines in Hair Samples from Postmortem Cases. *SN Compr. Clin. Med. (2020)* 2, 284–300.

3. Pragst F, Krumbiegel F, Thurmann D, Westendorf L, Methling M, Niebel A, Hartwig S. Positive findings of ethyl glucuronide in hair of young children from families with addiction background. *Int J Legal Med (2020) 134*, 523–532.

4. Pragst F, Krumbiegel F, Thurmann D, Westendorf L, Methling M, Niebel A, Hartwig S. Hair analysis of more than 140 families with drug consuming parents. Comparison between hair results from adults and their children. *Forensic Sci Int.* (2019) 297:161-70.

5. Methling M, Krumbiegel F, Hartwig S, Parr MK Tsokos, M. Toxicological findings in suicides – frequency of antidepressant and antipsychotic substances. *Forensic Sci Med Pathol (2019)* 15: 23–30

6. Methling M, Neumann M, Krumbiegel F. Ethylglucuronide as a biomarker for alcohol consumption – A comparison between hair and nails (article in german language). *Blutalkohol (2017)* Vol 54 No. 6, 337-45

7. Methling M, Krumbiegel F, Hastedt M, Buschmann CT, Tsokos M. Extremely high post-mortem blood alcohol concentrations in a fatal stomach content aspiration combined with a mixed intoxication of alcohol and cocaine – a case report (article in german language). *Blutalkohol (2016)* Vol 53 No. 6, 415-426

8. Krumbiegel F, Hastedt M, Westendorf L, Niebel A, Methling M, Parr MK, Tsokos, M. The use of nails as an alternative matrix for the long-term detection of previous drug intake: validation of sensitive UHPLC-MS/MS methods for the quantification of 76 substances and comparison of analytical results for drugs in nail and hair samples. *Forensic Sci Med Pathol (2016)* 12: 416-432.

9.2 Oral Presentations and posters

1. Methling M. Zuviel oder nicht zuviel, dass ist hier die Frage? – Möglichkeiten der Forensischen Toxikologie. *5. Tag der offenen Tür im Kriminalgericht Moabit, June 2015*
2. Methling M, Westendorf L. Ablauf der Untersuchungen unter CTU-Kriterien im Institut für Rechtsmedizin. *Haarsymposium 2016 im Institut für Rechtsmedizin der Charité, Berlin, April 2016.*
3. Methling M, Neumann M, Krumbiegel F, Tsokos M. Nachweis des Alkoholmarkers Ethylglucuronid in Nägeln. *25. Frühjahrstagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM), Rostock May 2016.*
4. Methling M, Niebel A. Toxicology in Living and Deceased Individuals. *Introduction in Clinical and Post-mortem Forensic Medicine, Abu Dhabi, February 2017*
5. Methling M, Niebel A. Nachweis und Quantifizierung von Betäubungsmitteln in Haaren mittels LC-QTOF-MS und LC-MS/MS. *12. GTFCh – Fortbildungsveranstaltung für technische Angestellte in Berlin, April 2018*
6. Methling M, F. Krumbiegel, S. Hartwig, M. Tsokos. Nachweis von Antidepressiva und Neuroleptika bei Suiziden - Darstellung und Häufigkeit toxikologischer Befunde. *27. Frühjahrstagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM), Kiel May 2018*
7. Niebel A, Krumbiegel F, Methling M, Westendorf L, Thurmann D, Hartwig S, Pragst F. Prävalenz und Konzentrationen von Betäubungsmitteln in Haaren von Kindern und Erwachsenen. *27. Frühjahrstagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM), Kiel May 2018*
8. Methling M, Niebel A. Use of LC-QTOF and LC-QQQ in forensic toxicology. *Group seminar of working group Prof. Dr. M.K. Parr, June 2018*
9. Methling M. Einführung in die Forensische Toxikologie. *Deutsche Stiftung für internationale rechtliche Zusammenarbeit e.V. - Seminar zum Thema „Einführung in die Toxikologie“, Casablanca July 2018*
10. Methling M. Vor- und Nachteile der Haaranalytik. *Deutsche Stiftung für internationale rechtliche Zusammenarbeit e.V. - Seminar zum Thema „Einführung in die Toxikologie“, Casablanca, July 2018*
11. Methling M. Einführung in die Forensische Toxikologie. *Deutsche Stiftung für internationale rechtliche Zusammenarbeit e.V. - Seminar zum Thema „Einführung in die Toxikologie“, Agadir November 2018*
12. Methling M, Niebel A. Fallbeispiele. *Deutsche Stiftung für internationale rechtliche Zusammenarbeit e.V. - Seminar zum Thema „Einführung in die Toxikologie“, Agadir November 2018*

13. Methling M, Niebel A. Vor- und Nachteile der Haaranalytik. *Deutsche Stiftung für internationale rechtliche Zusammenarbeit e.V. - Seminar zum Thema „Einführung in die Toxikologie“, Agadir November 2018*

14. Methling M, Krumbiegel F, Hartwig S, Alameri A, Tsokos M. Psychopharmaka in Haarproben von ausgewählten Sterbefällen. *98. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin (DGRM), Hamburg September 2016.*

15. Methling M, Niebel A. The detection of antidepressants and antipsychotics in human hair.*Group seminar of working group Prof. Dr. M.K. Parr, February 2020*

16. Methling M. Haarige Angelegenheit - Haarprobennahme: Fallstricke und praktische Hinweise. *Treffen der Toxikologen Berlin-Brandenburg am 04. März 2020 in Berlin*

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11 Independence declaration

I hereby affirm that I have completed the present cumulative dissertation independently and without unauthorized assistance. No aids other than those listed in the text were used in the writing of the dissertation.

A doctoral procedure has never been completed at any other university or applied to another department.

Maximilian Methling

12 Appendix

12.1 List of figures

12.2 List of tables