Development of the First Metabolite-based LC-MS^n Urine Drug Screening Procedure

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Pro utilitate hominum

„So eine Arbeit wird eigentlich nie fertig, man muss sie für fertig erklären, wenn man nach der Zeit und den Umständen das mögliche getan hat.“

(Johann Wolfgang von Goethe)

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1 GENERAL PART

1.1 INTRODUCTION

Intoxications and poisonings had occurred within living memory. In order to detect, identify, and measure toxic compounds or drugs (TCD), a scientific field called analytical toxicology came aside using broad screening procedures [1]. The involved TCD must be known to achieve optimal treatment for intoxicated or poisoned patients. Screening for TCD in body samples was and is one of the major tasks in clinical and forensic toxicology as well as in doping control. Urine is still the best sample for comprehensive screening, as most of the TCDs are excreted more or less metabolized in high concentrations [2]. Detection of various metabolites increases the selectivity, confirms the body passage and therby the intake of a particular TCD.

1.2 SCREENING PROCEDURES

Several screening procedures using different separation and/or detection systems were described [1]. While immunoassays allow a quick screening result for a limited number of targets, chromatographic methods like photodiode array detector coupled to liquid chromatography allows a broad screening for TCD. Nevertheless mass spectrometry (MS) is widely used in the field of analytical toxicology as this technique provides higher sensitivity and identification power. Different hyphenations of mass spectrometry and their current use in analytical toxicology are reviewed elsewhere [3-9] but will be shortly discussed as follows:

1.2.1 Gas Chromatography-Mass Spectrometry

Hyphenation of gas chromatography to MS (GC-MS) revolutionized the field of analytical toxicology, as this technique is robust, rather cheap and allows detecting TCD in low concentrations. Therefore, screening procedures and comprehensive reference libraries using electron ionization (EI) spectra and sophisticated search algorithms had been developed [4, 5, 7, 10-15]. GC-MS screening methods became “gold standard” in clinical and forensic toxicology and doping control thanks to

concentration- and instrument-independent EI spectra and excellent screening results [3, 4, 7]. Nevertheless, GC-MS is limited to volatile and more or less apolar compounds.

1.2.2 Liquid Chromatography-Mass Spectrometry

Hyphenation of liquid chromatography to MS (LC-MS) in the 1990s lead to high expectations in the field of analytical toxicology as this technique provides higher sensitivity for most of the TCD and overcomes the limitations of GC-MS (*vide supra*). As LC-MS spectra using in source fragmentation are - in contrast to GC-MS EI spectra - influenced by the concentration of a TCD and by the instrument type, the screening ability of LC-MS was limited and the high expectations were not fulfilled. That changed by introduction of tandem LC-MS techniques (LC-MS/MS). Using ion trap technology concentration-independent and reproducible collision-induced dissociation (CID) LC-MS/MS spectra could be obtained as a prerequisite for comprehensive screening. Nevertheless, these spectra are still restricted to a certain instrument type of a specific manufacturer [16]. Therefore, several LC-MS screening approaches using different instrumentation types have been developed [4, 6, 8, 9, 17-34].

In contrast to established GC-MS libraries containing parent compound and metabolite spectra [10-12], current commercialy available LC-MS/MS libraries [10, 24, 31] were lacking of metabolite reference spectra. This limits their applicability for urine screening.

2 AIMS AND SCOPES

The aim of this dissertation was to develop the first metabolite-based LC-MS^n screening procedure suitable for urine screening complementing the current “gold standard” GC-MS approach [11, 12].
Ultra-high performance-liquid chromatography (UHPLC) should be used for sufficient separation of the huge number of potential TCDs and/or their metabolites in urine.
A linear ion trap (LIT) apparatus should be used providing good identification power and highly reproducible CID spectra.
For collecting reference spectra of TCD metabolites and the development of the screening approach, urine samples of rats should be used after administration of the corresponding TCD. Urine samples of patients submitted for toxicological analysis should be used for confirmation.

The following steps had to be performed:

- → Collection of urine samples
- → Development of a simple and fast sample workup
- → Development of a fast and sufficient separation system
- → Development of the MS settings for the screening concept to record reproducible LC-MS/MS spectra
- → Identification of the TCD metabolites in urine
- → Recording of reference MS^2 and MS^3 spectra for the reference library
- → Transfer of the screening and reference library to a QTrap apparatus
- → Establishing a data evaluation system for automated screening

3 PUBLICATIONS OF THE RESULTS

The results of the studies were published in the following papers:

3.1 DEVELOPMENT OF THE FIRST METABOLITE-BASED LC-MS^N URINE DRUG SCREENING PROCEDURE-EXEMPLIFIED FOR ANTIDEPRESSANTS

(DOI: 10.1007/s00216-010-4398-9) [35]

3.2 DRUGS OF ABUSE SCREENING IN URINE AS PART OF A METABOLITE-BASED LC-MS^N SCREENING CONCEPT

(DOI: 10.1007/s00216-011-5032-1) [36]

3.3 TOWARDS A UNIVERSAL LC-MS SCREENING PROCEDURE – CAN A LINEAR ION TRAP (LIT) LC-MS^n SCREENING APPROACH AND REFERENCE LIBRARY BE USED ON A QUADRUPOLE-LIT HYBRID INSTRUMENT?

(DOI: 10.1002/JMS.2027) [37]

4 LC-MS^n LIBRARY OF DRUGS, POISONS, AND THEIR METABOLITES

Mass spectra of the parent compounds were recorded from methanolic stock solutions (1 mg/L) and those of the metabolites in rat or human urine after workup and LC separation. They were stored in the library using the NIST (National Institute of Standards and Technology, Gaithersburg, MD) library formate by the NIST mass spectral search program.

The organization of such an entry will be exemplified for JWH-073 as depicted in Figure 1. For example, the MS² spectrum of JWH-073 is implemented to the library including the name “MS2_JWH-073_wideband35” stored in the name field. Therefore, this field contains information about the MS stage (here MS²), the compound (here JWH-073) and the applied collision parameters (here wideband activation using 35% normalized collision energy). In addition, the empirical formula (here Formula: $C_{23}H_{21}NO$), the molecular weight (here MW: 327), the CAS number (here CAS#: 208987-48-8) and the chemical structure is stored in the corresponding fields. According to the corresponding empirical formula the calculated exact molecular mass (here Exact Mass: 327.162313) is given by recent versions of the the NIST mass spectral search program (NIST 2.0 g). In the ID number field (here ID#: 6603) an identification number is given, which is automatically associated with the compound embedded in the corresponding database (here DB: ms2ms3_wb_pol_switch). The comment row (Comment:) contains the scan filter information “ITMS + c ESI d w Full ms2 328.10” summarizing the MS parameters; here ion trap MS (ITMS) using electrospray ionization (ESI) in positive ionization mode (+), centroid (c) data dependent (d) full scan product ion scans (Full) acquisition using wideband activation (w). Additionally, the MS stage (here ms2) and the measured M+H mass of the compound (here *m/z*=328.10) is given. Up to the ten most abundant fragments and their relative abundance are stored in the next line (10 largest peaks). If a compound has any synonymously used name, or this compound is also a metabolite of another drug, a synonym entry was created and in addition to the compound name also stored in the synonym field. These entries were generated in order to simplify any database search.

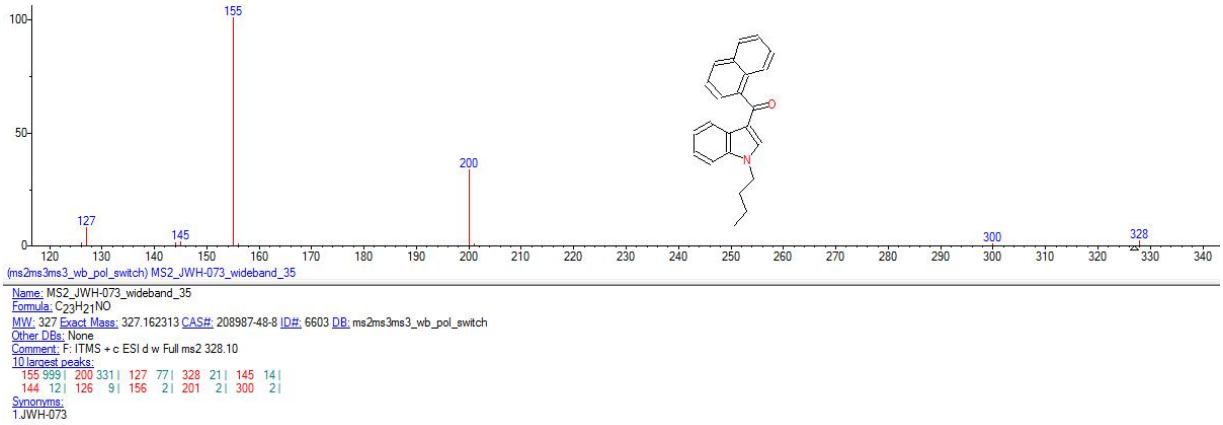

Figure 1. Typical library entry (exemplified for JWH-073).

The corresponding MS^3 spectra (most and second most abundant fragments) were stored in the library as exemplified for the most abundant fragment (*m/z*=155) of JWH-073 as depicted in Figure 2:

Name field, “MS3_155_Compound_refer_to_synonyms_wideband40”; MW field, 155 [u]; comment line, F: ITMS + c ESI d w Full ms3 328.23@cid35.00 155.00@cid40.00. A proposed structure of the fragment *m/z*=155 is also given by applying typical fragmentation rules. The synonym line contains the name of the compound where this fragment was firstly observed (here “JWH-073”). As the fragment *m/z*=155 is a common structure element, it is also observed for various other drugs or metabolites. These were also given in the synonym field as follows: JWH-018, JWH-073-M (N-dealkyl), JWH-018-M (N-dealkyl), JWH-073-M (N-dealkyl-HO-indole-glucuronide), JWH-018-M (N-dealkyl-HO-indole-glucuronide), JWH-073-M (N-dealkyl-HO-indole), JWH-018-M (N-dealkyl-HO-indole), JWH-073-M (HO-indole-glucuronide), JWH-073-M (HO-indole), JWH-018-M (HO-indole-glucuronide), JWH-018-M (HO-indole), JWH-015, JWH-019, JWH-200, Unknown (JWH compound? Found in pipe 82409), Unknown (JWH compound? Found in pipe 82409, tobacco 82464), Unknown (JWH compound? Found in tobacco 82464).

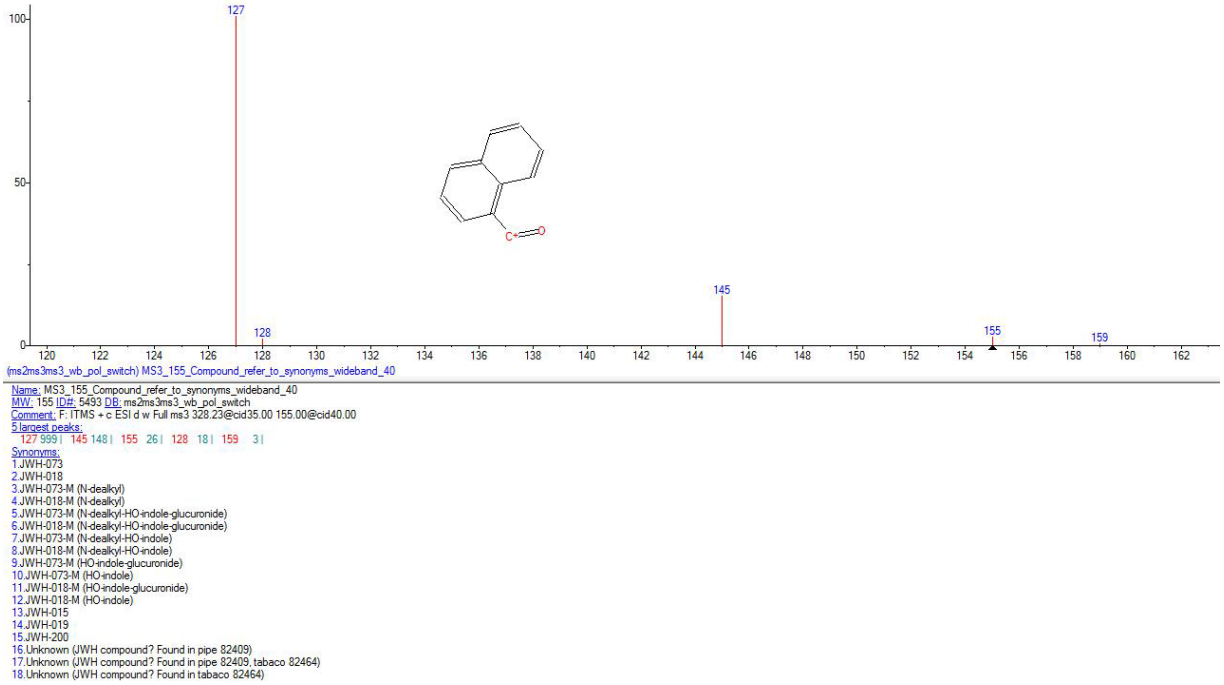

Figure 2. MS^3 spectrum of the most abundant fragment *m/z*=155 in the JWH-073 MS^2 spectrum depicted in Figure 1.

The MS^3 fragment spectra can be used to identify unknown compounds/metabolites with similar structural elements as shown here for JWH-018, JWH-073-M (N-dealkyl), JWH-018-M (N-dealkyl), JWH-073-M (N-dealkyl-HO-indole-glucuronide), JWH-018-M (N-dealkyl-HO-indole-glucuronide), JWH-073-M (N-dealkyl-HO-indole), JWH-018-M (N-dealkyl-HO-indole), JWH-073-M (HO-indole-glucuronide), JWH-073-M (HO-indole), JWH-018-M (HO-indole-glucuronide), JWH-018-M (HO-indole), JWH-015, JWH-019, JWH-200, Unknown (JWH compound? Found in pipe 82409), Unknown (JWH compound? Found in pipe 82409, tobacco 82464), and Unknown (JWH compound? Found in tobacco 82464) in Figure 2.

If a compound does not provide a second reproducible MS^3 spectrum under the used MS conditions (e.g. trimipramine) the library entry for that compound exists of one MS^2 and one MS^3 spectrum; otherwise one MS^2 and two MS^3 spectra were stored in the library for each compound. Abbreviations used in the library are listed in Table 1.

Table 1. List of abbreviations used for the LC-MS^n library.

Abbreviation	**Meaning**
Artifact ()	() artifact
Artifact (+K)	Ionization artifact +K
Artifact (+Na)	Ionization artifact +Na
Artifact (+NH4)	Ionization artifact +NH_4
Artifact (dimer)	Ionization artifact dimer formation
Artifact (double charged)	Ionization artifact double charged
Artifact (-H2O)	Artifact formed by dehydration
HO-	Hydroxy
HOOC-	Carboxy
-M ()	() metabolite
-M (HO-)	Hydroxy metabolite
-M (HOOC-)	Carboxylated metabolite
-M (nor-)	*N*-Demethyl metabolite
-M/artifact ()	() Metabolite or artifact
ME	Methylated
Unknown (? Found in)	Unknown substance (proposed compound class? Found in sample matrix and number)
X-conjugate n	Unknown conjugate (*m/z*= n)

The library consists of MS^2 or MS^3 spectra of over 1,000 toxicologically relevant parent compounds, over 2,700 metabolites or artifacts, about 100 endogenous biomolecules and impurities, and about 50 unknown compounds (e.g. “Unknown (JWH compound? Found in tobacco 82464)”) containing common structure elements of compounds stored in the library. Table 2 summarizes the number of entries of the current library sorted by drug categories.

Table 2. Number of library entries (parent compound, metabolite, artifact) sorted by drug categories.

Category	Parent Compounds	Metabolites	Artifacts
Alkaloid	21	84	2
Anabolic	7	4	0
Analgesic	28	50	28
Anesthetic	7	8	5
Anorectic	12	13	0
Antagonist of benzodiazepines	2	0	0
Anthelmintic	5	6	0
Antiadiposita	2	1	0
Antiamebic	2	3	0
Antianginal	2	12	0
Antiarrhythmic	14	38	0
Antiasthmatic	1	0	0
Antibiotic	48	47	14
Anticholinergic	1	0	0
Anticoagulant	8	9	1
Anticonvulsant	15	33	10
Antidepressant	49	460	53
Antidiabetic	15	42	4
Antidiarrheal	2	9	0
Antiemetic	7	14	0
Antiestrogen	1	0	0
Antigonadotropin	1	0	0
Antihistamine	45	56	8
Antihyperlipic	5	2	3
Antihypertensive	37	79	18
Antihypotensive	1	2	0
Antiinflammatory	1	0	0
Antilipemic	1	0	0
Antimalarial	6	7	0
Antimigraine	7	15	6
Antimycotic	14	41	7
Antineoplastic	1	3	1
Antiparkinsonian	20	16	2
Antirheumatic	23	28	7

Category	Parent Compounds	Metabolites	Artifacts
Antisepticum	1	0	0
Antispasmotic	17	45	1
Antitussive	10	27	1
Aromatase inhibitor	2	0	0
Benign prostatic hyperplasia drug	5	12	0
Beta-Blocker	30	95	3
Bronchodilator	15	4	3
Ca Antagonist	14	83	10
Cannabimimetic	21	58	2
Cannabinoid	4	3	0
Capillary protectant	2	0	0
Cardiotonic	2	0	2
ChE inhibitor for M. Alzheimer	3	10	0
Chemical	5	1	0
Chemotherapeutic	3	1	0
Cholinergic	1	0	0
Coronary dilator	4	0	0
Dermatic	1	0	0
Designer drug	67	211	24
Diagnostic aid	1	1	0
Diuretic	21	12	3
Dopamin agonist	1	0	1
Doping agent	2	13	0
Emetic	3	0	0
Endogenous biomolecule/impurity	101	0	1
Expectorant	2	2	6
Fungicide	6	0	0
GABA-Antagonist	0	0	2
Gestagen	4	0	0
H2-Blocker	3	10	0
Hemostatic	1	0	0
Herbicide	9	0	2
Hypnotic	18	35	5
Immunosuppressant	2	0	3
Incontinence drug	1	1	0
Ingredient of black pepper	3	0	0
Ingredient of cannabis	1	0	0
Ingredient of nutmeg	1	1	1
Ingredient of opium	1	6	2

Category	Parent Compounds	Metabolites	Artifacts
Insecticide	6	0	5
Laxative	2	8	1
Local anesthetic	16	23	1
Muscle relaxant	9	50	11
Mydriatic	1	0	0
Neuroleptic	52	217	7
Nicotine replacement therapeutic	1	1	0
Nootropic	1	4	0
Opioid antagonist	5	4	0
Ovulation stimulant	1	0	0
Parasympatholytic	8	16	3
Parasympathomimetic	5	2	0
Pesticide	0	0	1
Potent analgesic	29	122	7
Potent antitussive	5	21	0
Preservative	1	0	1
Psychedelic	4	7	0
Rodenticide	3	0	2
Rubber additive	1	0	0
Rubefacient in pepper spray	1	2	1
Sedative	5	2	0
Selective estrogen receptor modulator	1	2	0
Serotonin antagonist	1	1	0
Softener	3	0	0
Steroid	18	8	8
Stimulant	24	36	7
Sympatholytic	1	0	0
Sympathomimetic	14	10	10
Thromb.aggr.inhib.	5	18	2
Toccolytic	1	0	0
Tranquilizer	39	49	12
Tuberculostatic	1	2	1
Ulcus therapeutic	3	12	0
Unkown compound	50	0	0
Uricosuric	0	0	1
Urinary antiseptic	1	0	0
Vasoconstrictor	11	3	0
Vasodilator	19	38	7
Virustatic	22	33	0

The total number of spectra is 6,779 because in some cases, only one or no MS^3 spectra could be recorded and some compounds formed the identical MS^3 spectra (*vide supra*).

5 CONCLUSIONS

The developed sample workup, UHPLC separation, and mass spectral detection allowed for the first time a metabolite-based LC-MS screening. An applicability study using over 140 patient urine samples proofed that it provided similar screening results as the well established GC-MS approach. It was more sensitive particularly for low-dosed drugs and for more polar drugs, while GC-MS was better e.g. for benzodiazepine screening because of the hydrolysis step. In conclusion, the new LC-MS approach is an excellent complement to the well established GC-MS approach and it is nowerdays an important part of the routine systematic toxicological analysis. Finally, this procedure and the reference library could successfully be transferred to another apparatus type raising the hope of a universial LC-MS library in future using sophisticated search algorithms.

6 SUMMARY

In the presented dissertation, the development of the first metabolite-based LC-MS screening approach is described. It consisted of establishing a simple and fast sample workup, fast and sufficient LC separation, MS settings for recording reproducible spectra, and a suitable screening concept. Using these methods, MS^2 and MS^3 spectra of parent drugs were recorded as well as those of their metabolites after having identified them in urine samples of rats and humans. By using the described methods, the new reference library (over 1,000 parent drugs, 2,700 metabolites, 100 biomolecules), and a sophisticated software tool, a new routine screening approach was established. This LC-MS screening approach is nowerdays an important part of the systematic toxicological analysis and showed excellent robustness and screening results in thousands of authentic patient samples. According to this, this LC-MS approach complements the established GC-MS approach. In addition, recent research projects are partly based on the developed workup, separation and/or detection methods. Finally, this procedure and the reference library could successfully be transferred to another apparatus type, which raised the hope of an instrument independent LC-MS reference library.

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8 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurde die Entwicklung eines Metaboliten-basierten LC-MS Screenings beschrieben. Dazu wurden eine einfache und schnelle Probenvorbereitung, eine schnelle und effektive LC-Trennung, MS Settings zur Aufnahme reproduzierbarer Spektren und schließlich ein geeigneten Screening-Konzept entwickelt. Damit wurden MS^2 and MS^3 Spektren von Muttersubstanzen und ihren Metaboliten aufgenommen, die zuvor in Ratten- oder Patientenurinen identifiziert worden waren. Mit Hilfe dieser Methoden, der neuen Referenzbibliothek (über 1000 Muttersubstanzen, 2700 Metaboliten, 100 Biomoleküle) und einer speziellen Auswertesoftware konnte ein neues Routine-Screeningverfahren etabliert werden. Das entwickelte LC-MS System ist heutzutage ein wichtiger Bestandteil der systematischen toxikologischen Analyse. Bei mehreren tausend Patientenproben zeigte es sehr hohe Robustheit und sehr gute Screening-Ergebnisse. Zusammenfassend lässt sich festhalten, dass dieses LC-MS-Verfahren das etablierte GC-MS-Verfahren ideal ergänzt. Zusätzlich stellten die entwickelten Aufarbeitungs-, Trennungs- und/oder Detektionsmethoden eine wichtige Grundlage für andere aktuelle Forschungsprojekte des Arbeistkreises dar. Schließlich konnte gezeigt werden, dass dieses Verfahren und die zugrunde liegende Referenzbibliothek auf einen anderen Gerätetyp erfolgreich übertragen werden kann. Dies lässt Hoffnung auf eine langersehnte geräteunabhänige LC-MS Referenzbibliothek zu.