Integrated Analytical Strategies for Drug Discovery

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Chapter 1. Introduction:

The role of mass spectrometry in drug discovery analytics

Drug discovery and development

The discovery and development of a new drug is a multidisciplinary research effort where the interaction between the different scientific disciplines is crucial for a successful outcome of this complex trajectory. First of all, one should understand the therapeutic area or the disease to be targeted. Amongst others, the involvement of physicians, clinical experts, biologists and molecular pharmacologists will lead to the selection of a certain protein to be used as the drug target. This target, often a receptor or enzyme, has to be validated as a viable model for disease intervention using the new drugs to be developed [1]. Once a drug target is validated, the finding of chemical structures that interact with this target will be initiated and will result in the formation of a library of lead compounds. These lead compounds subsequently undergo an iterative process to be optimized based on several parameters such as metabolic stability, toxicity, pharmacological activity and absorption [2,3]. This process is an interplay between chemistry, biology, pharmacology and toxicology and eventually provides the so-called drug candidate which then will be used in the development stage. A schematic overview of the overall process is given in figure 1. The development phase addresses all processes involved to develop the drug molecule into a registered product. This includes pre-clinical and clinical development, formulation studies as well as the process to get the drug approved by the regulatory authorities.

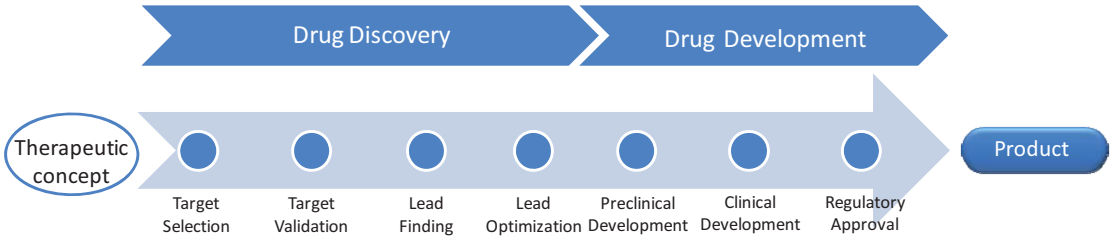

Figure 1: Overview of the drug discovery and development process starting with the therapeutic concept definition up to the product finalization, based on [7].

The role of analytical chemistry in drug discovery

Whether it is to elucidate the chemical structure of a newly synthesized drug molecule, to determine the pharmacokinetics of a drug candidate or to verify the

formulation of the product, analytical chemistry is a crucial discipline for the drug discovery and development process. The continuous development of advanced analytical technologies and methods are of utmost importance to sustain, innovate and increase the efficiency of a drug discovery and development program. Moreover, high quality analytical chemical data supports or protects patent claims and secures the integrity of the products made by so called originators.

In the following sections, the role of analytical chemistry, and especially of mass spectrometry as an advanced analytical tool, in the drug discovery process is highlighted.

Drug discovery by medicinal chemists

The starting phase of drug discovery for a medicinal chemist is the so-called hit identification phase [4]. The characterization and identification of chemical structures that can form a starting point for the creation of lead libraries greatly depends on the screening methods and synthetic feasibility [5]. Additionally, nature has always been an important source of bioactive compounds and many hits can be found when screening natural products from plant extracts, the marine world or animal venoms and toxins [6]. The screening of natural products for potential drugs has resulted in several success stories and is still often done in parallel with the screening of synthetic compound collections. In the last decades, the approach in lead library formation has mainly been dominated by combinatorial chemistry. Combinatorial chemistry has undergone many redefinitions since its discovery. Starting with automated solid-phase synthesis of peptides and small organic molecules, the number of compounds to be screened increased dramatically [7]. Although the screening libraries were expanding very rapidly, the hit rate in these libraries did not evolve as quickly as hoped for [8]. With understanding the biologically relevant chemical space, medicinal chemistry efforts were more guided by *in silico* molecular design [9]. This involved structural information on the target and existing ligands to construct computer models for structure-based drug design to create so-called focused screening libraries [10]. All these different approaches for lead library generation require diverse screening approaches.

Synthesis and screening

Being responsible for the formation of new chemical entities, medicinal chemists need to verify the chemical structure of the product and synthetic intermediates. This is often done by a combination of analytical techniques. Synthesis products are mainly purified using column chromatography in the organic synthesis lab. For purification of the final synthesis products, mainly preparative chromatography (LC) with ultraviolet detection (UV) or mass spectrometry (MS) triggered fraction collection is used [11]. Of the collected fractions, nuclear magnetic resonance (NMR) spectra are recorded, often in combination with high resolution mass spectrometry data (HR-MS) [12]. In this way, the chemical structure of the synthesis products can be confirmed. Once structures are determined and impurity levels are profiled, the newly synthesized compound series are tested for biological activity towards the intended drug target using a variety of biological activity screening assays.

In order to support this lead finding and optimization process, data on structure and activity relationships has to be acquired. Although there are many ways to obtain this information, this field is dominated by high throughput screening (HTS) technologies that are mostly based on micro titer plate assays [13,14]. For reasons of throughput and automation, so-called mix and measure assays are highly preferred and are often based on fluorescence readout [15], radio ligand binding assays, or activity based chemiluminescence or bioluminescence readout [16]. Important factors in this are speed, efficiency and data quality as well as optimized target protein consumption. The development of specific assays is either done by the pharmaceutical company itself, or is outsourced to a contract research organization but is a delicate matter since especially in early discovery new drug targets may be pursued. Although the actual affinity screening and data acquisition is fast in an industrial setting, usually time-slots are allocated to specific drug target screens. This means that it might take up to one month to actually obtain feedback on the biologic activity of a compound series [17]. Alternative methods for affinity determinations could close the gap between HTS and other elaborate and mechanistically detailed screening technologies [18].

Drug Metabolism and Pharmacokinetics (DMPK)

The administration of any xenobiotic will lead to a reaction of the body towards this chemical entity. In the case of the administration of a drug, the drug will follow all steps from the ADME principle, being Absorption, Distribution, Metabolism and Excretion [19]. All these processes are depending on the physicochemical properties of the drug molecule. A very important parameter is the LogP, a measure for lypophilicity. In general, lypophilic compounds are difficult to excrete from the body. One way to improve the excretion of these drug molecules is to change their physicochemical properties. This is mostly done in the liver, where cytochrome P450 enzymes (CYPs) are important for detoxification of xenobiotics [20]. CYPs comprise a wide family of enzymes with different isoforms [20]. These enzymes catalyze several types of oxidation reactions, i.e., the so-called Phase I biotransformation reactions [21]. These involve hydroxylation of an aromatic or aliphatic carbon, epoxidation of a double bond, heteroatom oxygenation and N-hydroxylation, heteroatom dealkylation and dehydrogenation [22]. Also cleavage of esters and oxidative group transfer are CYP mediated reactions. The selectivity of these enzymes towards substrates is caused by the different isoforms of CYPs. The distribution and polymorphism of these isoforms is an important aspect in e.g. interracial or interindividual differences in metabolism or toxicity of drugs and other xenobiotics [23]. So-called phase II biotransformation enzymes largely increase the hydrophilicity of drugs and with that the excretion of the metabolites from the body. Important phase II reactions are conjugation with glutathione, glucuronic acid, sulfate and with amino acids. Although acetylation and methylation are also phase II reactions, they do not contribute to the increase in hydrophilicity. Since drug metabolites can have a pharmacological as well as a toxicological effect *in vivo*, it is of great importance to both quantify and identify the metabolites already in an early stage of drug discovery [24,25].

The *in silico* prediction of drug metabolites is a relatively new tool to support the identification in *in vitro* and or *in vivo* experiments. The software packages are based on either rule/knowledge based methods [26] or the statistical analysis methods [27]. Integration of the software to predict potential metabolites and the mass spectrometric experiments to actually detect the observed metabolites can significantly speed up the identification process [28]. Having a preferred list of

accurate *m/z* values of predicted metabolites increases the chances of acquiring the necessary data to support the first structure identification steps [29]. The SyGMa (Systematic Generation of potential Metabolites) algorithm of Ridder *et al.* [30] may serve as an example of the approach. It combines the use of expert knowledge and empirical scoring for the prediction of metabolites. The interfacing between predictive software and the data-acquisition programs should be without much manual interference to enhance the throughput for application in a routine setting.

Metabolites in Safety Testing

The importance of metabolite identification and quantification is stressed by the guidelines published in 2008 by the US food and drug administration (FDA). The so-called Metabolites in Safety Testing (MIST) guidelines describe the studies recommended to perform on metabolites having more than 10 % systemic exposure of the parent drug to support human safety. A decision diagram of this is depicted in figure 2.

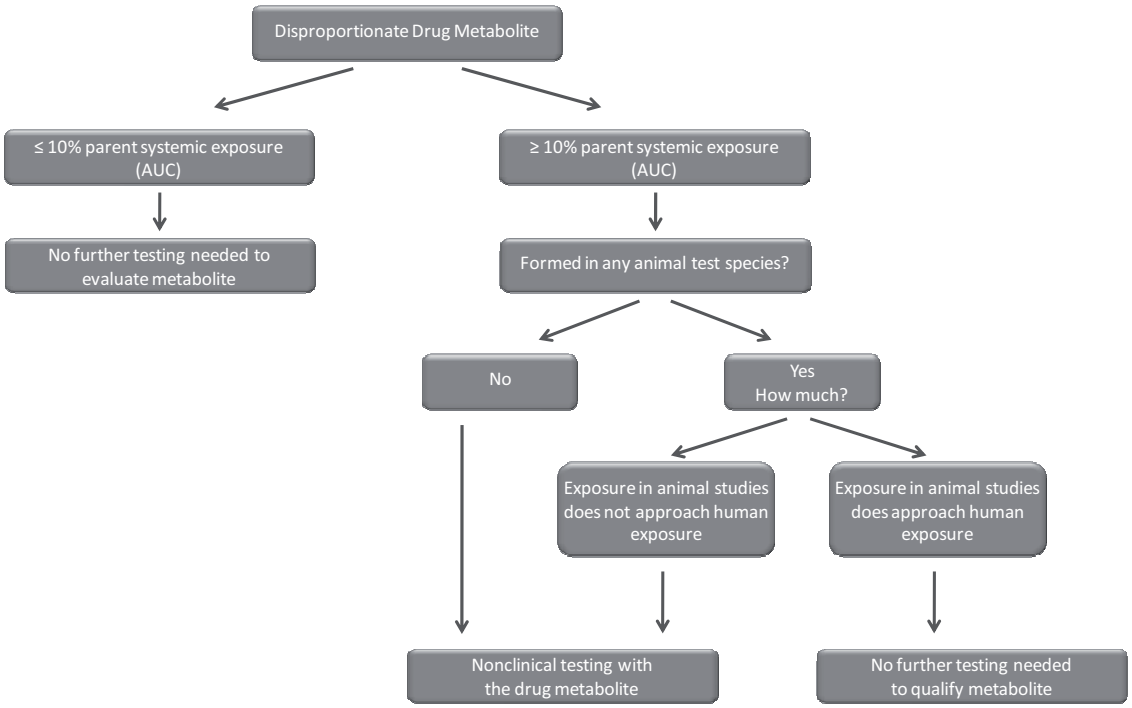

Figure 2: Decision tree flow diagram of the MIST guidelines as determined by the FDA, based on [31].

These guidelines have resulted in strategic discussions within the pharmaceutical industry on the nature and timing of metabolism studies in the discovery and development process [32,33]. Moreover, it challenges the development of analytical technologies dealing with the quantification and identification of drug metabolites [34] in an efficient and intelligent way.

Metabolite Identification

In order to support or confirm the structural identification of metabolites, several alternative approaches have been reported for the production of reference standards. The traditional way mainly relies on organic chemistry for the formation of synthetic standards. In other cases, biosynthetic approaches are used for the production of standard metabolites [35,36]. This includes recombinant enzymes [37,38], specific bacterial strains [39], or simply the upscaling of *in vitro* incubations if other systems are not available [40]. Chemical approaches include electrochemistry [41-44], oxidation under harsh conditions with low pH [45], peroxide reactions and oxidation by light [46].

For the structural elucidation of drug metabolites, high resolution mass spectrometry (HR-MS) is the technique of choice [29]. Combined with a separation method, e.g., high-performance, ultra-performance, or nano liquid chromatography (HPLC, UPLC, nLC), this enables the accurate mass determination of the metabolites formed as well as of their fragmentation patterns in tandem mass spectrometry (MS–MS) [47]. The use of hyphenated mass spectrometry provides a relatively high throughput and sufficiently low detection limits for most drugs and metabolites to allow the use of conventional microtiter plate experiments for the generation of drug metabolites [48]. Moreover, the sample pretreatment needed is relatively straightforward and can be automated [49]. The speed of analyte separation determines the total throughput of the analysis. Recently, Wright reviewed the use of mass spectrometry for metabolite identification from its starting point in the 1970s [50]. Clearly, the use, the speed, the sensitivity and the confidence in mass spectrometry has increased tremendously in time. An overview of different mass analyzers and their use in metabolite identification studies is given in Table 1.

Mass analyzer	Mass resolution*	Mass accuracy**	Mass range	Tandem MS	Advantages	Disadvantages	Year first published	Popular for metabolite identification
Magnetic sector	100,000	5 ppm	10,000	MS^2	High mass resolution	Expensive; limited sensitivity; not robust; high vacuum so difficult to interface with HPLC	1934	Up to late 1980s
Quadrupole	4000	100 ppm	4000	MS	Inexpensive; robust; newer designs are fast scanning; tolerates higher pressures -facilitates interfacing with HPLC	Low mass resolution; not specific	1953	Limited application for metabolite identification
Triple quadrupole	4000	100 ppm	4000	MS^2	Inexpensive; robust; sensitive; newer designs are fast scanning; tolerates higher pressures - facilitates interfacing with HPLC	Low mass resolution	1978	Late 1980s to present day
Ion trap	4000	100 ppm	4000	MS^n	Inexpensive; robust; sensitive;	Product ion mass range limited by "one third rule"; low resolution	1953	1980s
TOF	8000	100 ppm	300,000	MS	Inexpensive; robust; sensitive; fast scanning	Low resolution; cannot perform constant neutral loss and precursor ion scans	1946	Limited application for metabolite identification
TOF reflectron	15,000 (V geometry) 30,000 (W geometry)	10 ppm (V geometry) 1–5 ppm (W geometry)	10,000	MS^2	Inexpensive; robust; sensitive; fast scanning; high resolution	Cannot perform constant neutral loss and precursor ion scans	1973	Late 1990s to present day
Quadrupole-TOF	15,000 (V geometry) 30,000 (W geometry)	10 ppm	10,000	MS^2	High mass resolution; sensitive	Cannot perform constant neutral loss and precursor ion scans	1984	Late 1990s to present
IT-TOF	10,000	5 ppm	1000	MS^n	High mass resolution; fast scanning; fast polarity switching	Cannot perform constant neutral loss and precursor ion scans	2001	Mid-2000s to present day
Quadrupole-trap	4000	5 ppm	4000	MS^n	Robust; sensitive; fast scanning	Low mass resolution; product ion mass range not limited	2002	Mid-2000s to present day
FTMS	500,000	1–5 ppm	10,000	MS^n	Excellent resolution; good sensitivity	Expensive; limited sensitivity; not robust; high vacuum can be more difficult to interface with HPLC; not fast scanning	1974	Mid-2000s to present day

*Mass resolution: the ability of the mass spectrometer to distinguish between ions of different mass-to-charge ratio. For example, if an ion at m/z 500 has a peak width at half height of 0.5 will have a resolution of 1000 (500/0.5).
**Mass accuracy: a measure of the accuracy of mass determination. For example: accurate mass = 500.000; measured = 500.002; difference = 0.002; error = 0.002/500 or 4 ppm.
IT, ion trap; FTMS, Fourier transform mass spectrometer; HPLC, high-performance liquid chromatography; MS, mass spectrometry; TOF, time-of-flight instruments.

Table 1: Characteristics of MS systems used for metabolite identification from 1971 to 2011 by Wright [50], reprinted with permission.

Although Table 1 shows an overview of most instruments used in metabolite identification, several instruments are missing. The orbitrap, which was a revolution in the field of FTMS systems, the combined linear ion trap – Orbitrap (LTQ-orbitrap) and the more recently introduced ion mobility separation – TOF mass spectrometers (IMS-TOF-MS) are worthwhile to mention in this context. The latter has the ability to rapidly separate isomeric species based on differences in their physical size and shape in the gas phase which can provide potential structural information. Recently, Dear *et al.* used this technology in combination with molecular modelling for the identification of aromatic hydroxylated metabolites of ondansetron [51].

In the same review, a nice overview is given on the general characteristics of the ion sources used for metabolite identification studies and their advantages and disadvantages [50]. In the beginning of 1970s, mainly electron ionization (EI) and chemical ionization (CI) were used in combination with introduction of the analytes via a solids probe. In the 1980s, fast atom bombardment (FAB) and thermospray ionization (TSP) were introduced. The latter allowed the hyphenation of LC which opened up many opportunities for the MS detection of compound in mixtures. The introduction of atmospheric pressure ionization techniques in the 1990s was the basis for the currently most widely used ionization techniques such as electrospray ionization (ESI) and atmospheric pressure chemical Ionization (APCI). Currently, most metabolite identification studies are done with ESI-HR-MS instruments, especially using Q–TOF and linear-ion-trap–orbitrap hybrid instruments enabling MS/MS experiments. The use of MS-MS in drug metabolite quantification and identification is of utmost importance. In order to elucidate the structure of a metabolite, only the accurate mass of the parent ion is not sufficient. The ability to perform precursor ion scans and neutral loss scans in triple-quadrupoles and Q-TOF instruments allows the search for metabolites that contain a characteristic fragment with the parent compound. No prior knowledge on the full structure of the metabolite is needed and these scan modes can therefore be used to screen for unknown and known conjugates and other metabolites. Usually in a second experiment, the precursor *m/z* list generated from the precursor ion scan and neutral loss scan is used to

obtain a product ion scan of the metabolites detected. These fragmentation spectra can significantly help in the elucidation of the metabolite structure.

Another mode of data acquisition to support metabolite identification is the data dependent approach, also known as automated precursor ion selection. In this mode, the instrument cycles between full scan mode and MS-MS or even MS^n to provide an extensive data set in a fast way. The selection of precursor ions can be done based on a prior set ion intensity threshold or sometimes even by a specific isotopic pattern [52]. Other settings include the time to exclude a specific ion for new fragmentation cycles, an exclusion list for e.g. interfering background ions or a list of preferred ions [53]. This list of preferred ions is usually based on the parent drug molecule and the knowledge on metabolic reactions or input is provided by predictive drug metabolism software. This will increase the chance of fragmenting the target metabolites and provides the data needed for the first structural information.

The advances in mass spectrometry and software development have led to an increase in tools to assist metabolite identification. Nowadays, high resolution mass spectrometry is commonly used for metabolite identification which allows the use of accurate mass determination and therefore mass defect filtering, high resolution isotope filtering techniques and other algorithms to give input during data acquisition to the instrument as well as assisting the processing and interpretation of the data afterwards.

The use of high resolution isotope filtering enables the search for a specific isotopic pattern in a complex chromatogram containing many interferences from biological matrices. Figure 3 shows the high resolution ESI-MS spectrum of Clozapine with the characteristic isotopic pattern of Chlorine enabling high resolution isotopic pattern filtering algorithms.

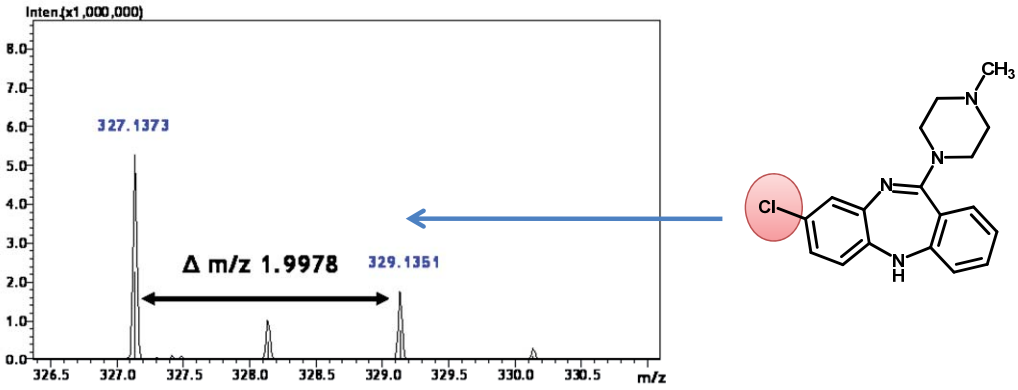

Figure 3: ESI-IT-TOF-MS spectrum of Clozapine with characteristic 3:1 Chlorine isotopic pattern for high resolution isotopic filtering algorithms. De Vlieger *et al.*, unpublished data.

Although this high resolution isotope filtering technique quickly provides a filtered chromatogram with all peaks containing the selected isotopic pattern, one has to be cautious when only focusing on the peaks that are displayed. Metabolic reactions sometimes eliminate the specific element responsible for the isotope pattern, e.g., the halogens, from the drug molecule and these metabolites will therefore not show up in the filtered chromatograms. LeBlanc *et al.* used brominated glutathione in reactive metabolite trapping experiments in order to take advantage of the isotopic filtering features related to bromine [54].

Another feature of high resolution MS in drug metabolite studies is mass defect filtering (MDF), which does not require the modification of metabolites before detection. This concept was first published by Zhang *et al.* in 2003 [55] and ever since an increasing number of modifications and applications is described. Since the atomic weight of every element in nature has a unique nonintegral mass using the scale relative to the exact defined 12 Dalton of ^{12}C, its mass defect is the residual mass of the atom [56]. The mass defect of a molecule can therefore be defined as the difference between the exact *m/z* and the nominal *m/z* of a molecule [57]. Based on known metabolic transitions, a mass defect filter can be constructed which filters out all *m/z* that have a mass defect too large to be associated to the parent drug or its common metabolites [58]. For instance, common Phase I metabolic reactions will results in at maximum a 0.04 Da shift in

the mass defect of the parent drug, whereas the mass defect shift in common Phase 2 reactions is only 0.07 Da [59]. Holcapek *et al.* published an extensive review on mass defects of many biotransformation reactions [60]. Table 2 shows, for example, the mass defects of several metabolic reactions that are observed frequently.

Table 2: Changes in nominal mass, exact mass and in the mass defect of common phase I and Phase II biotransformation reactions, based on [59,60].

Metabolic Reaction	**Δ nominal mass (Da)**	**Δ exact mass (Da)**	**Δ Mass defect (Da)**
Phase I			
- CH_2 (demethylation)	-14	-14.0157	-0.0157
+ O (hydroxylation)	+16	+15.9949	-0.0051
- H_2 (dehydrogenation)	-2	-2.0156	-0.0156
Phase II			
+ $C_{10}H_{15}N_3O_6S$ (glutathione)	+305	+305.0682	+0.0682
+$C_5H_7O_3NS$ (N-Acetylcysteine)	+161	+161.0147	+0.0147
+ $C_6H_8O_6$ (glucuronidation)	+176	+176.0321	+0.0321
+ SO_3 (sulfation)	+80	+79.9568	-0.0432

Multistep metabolism reactions result in the sum of the individual mass defect shifts. The difference in the mass defect is especially beneficial for the discrimination between ions of interest and interferences from biological matrices such as urine, bile, and feces. The groups of Mortishire-Smith [58,61,62] and of Zhu [63,64] have extensively published on the use of mass defect filtering in metabolite identification studies and this filter algorithm is now also implemented in standard software of several mass spectrometer vendors, e.g. Metabolynx from Waters, MetID Solution from Shimadzu and MetWorks from ThermoFisher Scientific. Cuyckens *et al.* recently described the combined use of MDF, neutral loss filtering and high resolution isotope pattern recognition [61] to extract metabolite ions from a matrix background.

The actual elucidation of the metabolite structures is done based on the fragmentation spectra obtained. Although software tools like e.g. HighChem Mass Frontier [65,66] are used to facilitate the structure elucidation based on the

fragmentation data [67], this field still heavily relies on expert knowledge and their interpretation of the data. Stranz *et al.* reported the combined use of computational metabolite prediction and automated structure based analysis of MS fragmentation data to identify drug metabolites [68]. Pelander *et al*. describe the use of *in silico* metabolite prediction and the use of ACD Fragmenter software for the identification of quetiapine metabolites in urine using LC-TOF MS [69]. Although these approaches are very interesting and eventually might be implemented in a routine setting [70], this is still at the beginning of its development and the application and rate of success is still limited.

Despite all efforts in mass spectrometry and the development of software tools to assist this process, not every metabolite can be identified based on MS fragmentation data. This can be due to scarce fragmentation of the parent drug or to the fact that the position of hydroxylation in e.g. a aromatic ring is difficult to determine by MS^n experiments. Moreover, the absolute configuration of a molecule is important for its structure activity relationship and this cannot be determined by mass spectrometry. Therefore, in most cases, NMR spectra are acquired of the metabolites formed to determine full structural information [71]. This can be either done off-line as described in **chapter 5** of this thesis after preparative incubation and fractionation. Alternatively, this can be done in an on-line fashion where (stopped-flow) LC separation is hyphenated to flow-NMR [12], often with an SPE trapping step in between for analyte preconcentration and solvent exchange [72]. Although NMR is a very powerful tool for structure identification, there are some limitations within the metabolite identification field. The main drawback of the use of NMR is the sensitivity compared to MS. This means that unfortunately metabolite samples cannot be transferred easily from the MS experiments to NMR. In most cases, the metabolite generation has to be up scaled before NMR can deliver sufficient data for structure identification. This is not really an issue for *in vitro* experiments, but material from *in vivo* experiments can be difficult to obtain in sufficient quantities. By far, rat urine is most often used for these kinds of studies [73,74], although also bile is reported but has significant more issues with background interferences from the matrix. The use of plasma is also described but this requires significant more sample preparation than urine [75]. Advanced hyphenated systems combining LC-MS and NMR in one instrument are used for these kinds of experiments [72,76]. The availability of an accurate *m/z* of the metabolites significantly facilitates the

structure elucidation by NMR. Recent developments in NMR hardware and probe designs might eventually bridge the gap in sensitivity between MS and NMR in the future [77] although MS technology also keeps getting more sensitive over the years.

Metabolite Quantification

In early discovery, the quantification of drug molecules is mostly performed using a radiolabeled isotope of the drug. A combined analysis of LC with on-line radioactivity detection and mass spectrometry allows the so-called mass balancing of the drug and its metabolites [78]. In total, the radioactivity measured of all peaks individually should be the same as the radioactivity measured for the drug standard before metabolism. This approach has several advantages. Firstly, it assists in finding new unexpected metabolites in the chromatogram and allows the scientist to assign an accurate mass to the new metabolite. The second important characteristic is, that all metabolites can be quantified based on their radioactivity counts [79]. A disadvantage, however, is that the resolution of these radioactivity detectors is still rather poor and results in broad peaks which can influence the detection in the mass spectrometer. Moreover, often multiple metabolites are detected as one peak in the radioactivity detector which complicates the quantification of the individual metabolites. Strategies are reported to measure radioactivity and MS data in parallel or even performing radioactivity detection off-line [80,81]. Recent publications describe the development of advanced radioactive detection systems based on accurate radioisotope counting techniques [82,83]. When there are no radiolabelled standards available, other methods have to be used for the quantification of drug metabolites. Several strategies are reported where quantification is based on UV detection [84], but this easily leads to the over- or underestimation of metabolites since metabolic reactions can alter the chromophore in the molecule and with that the molar extinction coefficient [85]. The assumption that metabolites show the same absorption profile is therefore very difficult to make. To verify this, fast scanning photodiodearray detectors are implemented instead of the single wavelength UV detectors. This provides an absorption spectrum of each metabolite detected and this data can be used to verify the assumption made [86].

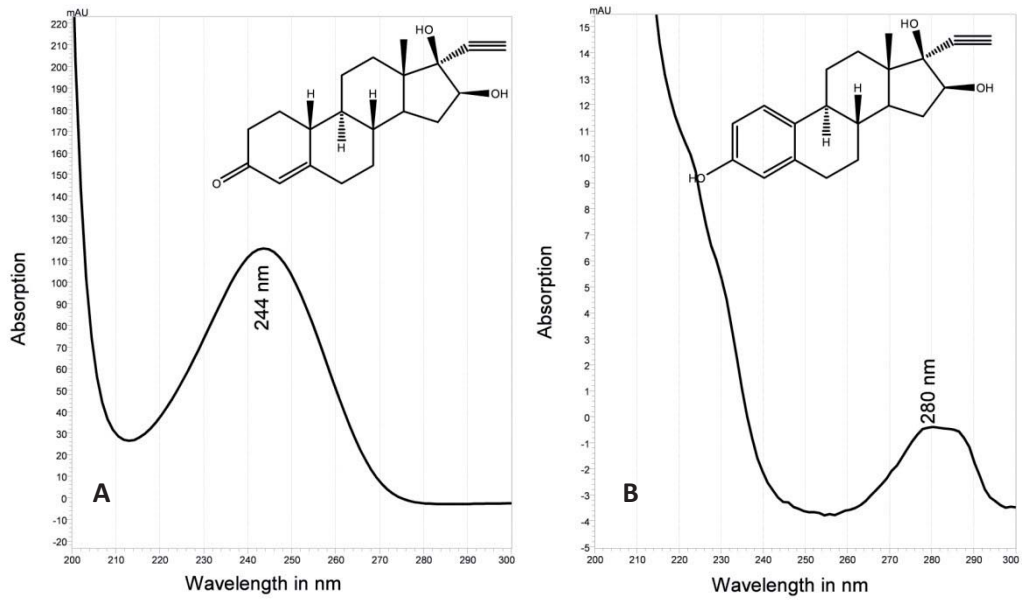

Figure 4: absorption spectra obtained with photodiode array detection after LC separation in a metabolite study. A) Absorption spectrum of the parent compound, 16β-OH Norethisteron. B) Absorption spectrum of a metabolite, 16β-OH Ethinylestradiol [86].

Simultaneously, it provides the first structural information on the metabolites as some functional groups in molecules have distinct absorption properties at specific wavelengths. As can be seen in figure 4, the metabolism of this particular steroidal structure leads to the aromatization of the steroid A ring. As a result, the absorption spectrum changes and if metabolite quantification was performed based on single wavelength UV detection, the concentration of this metabolite would have been greatly underestimated [86]. Another crucial aspect of quantification of drugs without any standard compounds is the response factor in ESI-MS [87]. A small change in structure can lead to a significant change in ESI-MS response and therefore hamper the (absolute) quantification. To address this, several approaches are reported where the MS response is calibrated based on another detection technique such as UV [88], chemiluminescence nitrogen detection (CLND) [87,89], evaporative light-scattering detection (ELSD) [90], inductive coupled plasma-mass spectrometry (ICP-MS) [76,79] and more recently the corona charged aerosol detection (CAD) [91]. Accelerator mass spectrometry (AMS) also is an equimolar detector and therefore provides quantitative data on

metabolites formed [92,93]. However, this technique does not provide structural information, requires radiolabelling of the parent drug molecule and the ionization techniques are mostly incompatible with liquid chromatographic types of analyses. Nevertheless, AMS is reported to obtain early quantitative data on pharmacokinetics to support the MIST guidelines of the FDA [94]. Some studies report the use of NMR for the quantification of metabolites [95]. Recently, Nedderman *et al.* published some key considerations when selecting an analytical approach for the quantification, see table 3 [32].

Table 3: Summary of some of the key considerations when selecting an analytical approach for metabolite quantification, reprinted with permission from [32].

Approach	Sample volume	Cost	Effort	Analytical output quality	Relative sensitivity[a]	Structural information
MS (without authentic standards)	Low	Low	Low	Relative ratio	High	High
Response factors (with ^{14}C)	Low/medium	Medium	Medium	Moderate	Medium/high	High
NMR	High	High	High	High	Low	Definitive
AMS	Low	Very high	Very high	High	Very high	None
Radiometric detection	Medium	Medium	Medium	Moderate	Med	None
Bioanalysis	Low	High	High	Very high	High	Confirmation

[a]MS sensitivity can vary widely by compound; however, this relative value is indicative for the majority of compounds.

All these developments indicate the search and need for an universal detector, being insensitive to structural changes. In reality, however, many of these detection methods suffer from either low sensitivity or incompatibility with the change of mobile phase during the analysis. The latter is typically the case in LC types of analyses where solvent gradients influence detector responses, as described by Pereira *et al*. [88]. Often, flow compensation or make up-flows are applied in these kinds of experiments to minimize fluctuations in the flow composition to maintain detector signal stability.

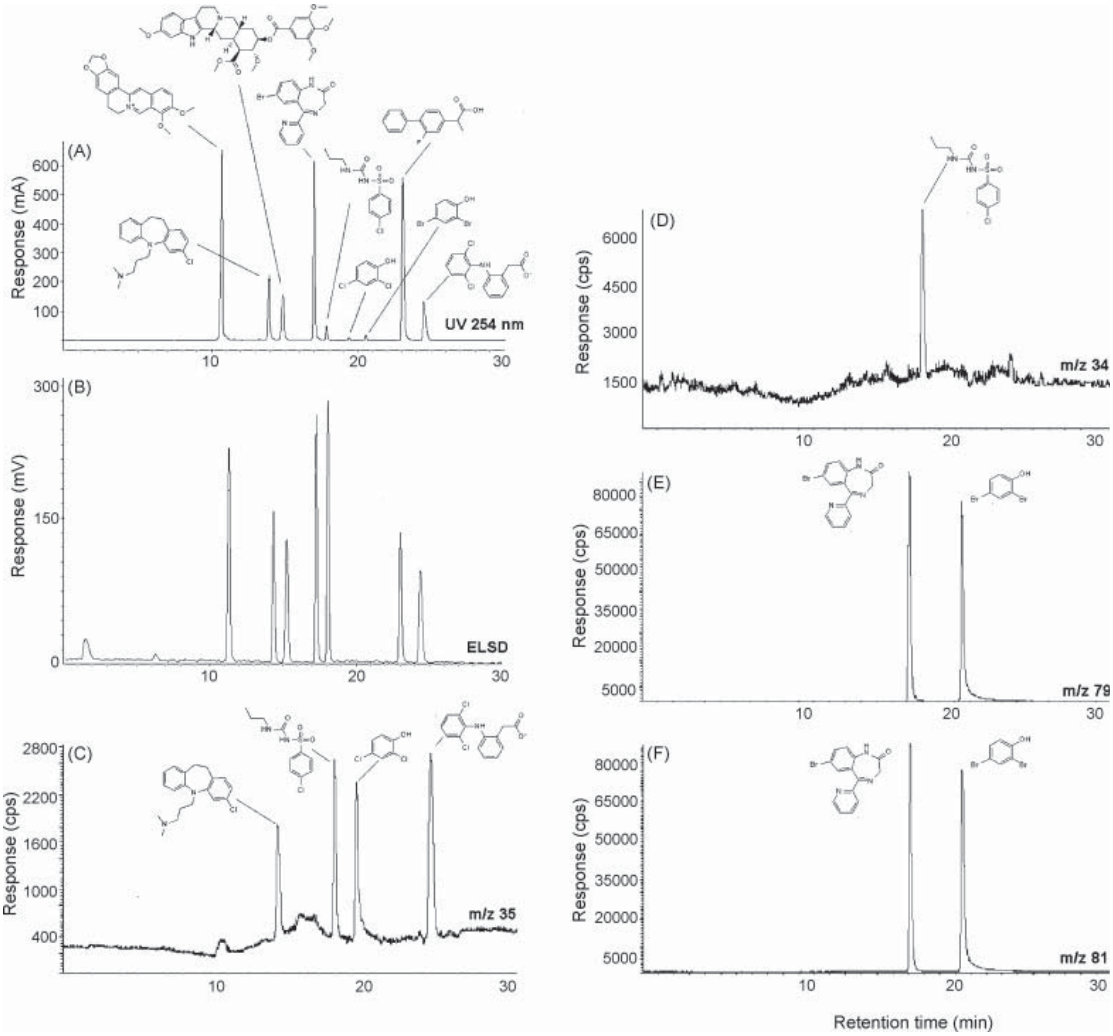

Figure 5: LC–UV–ELSD–ICP-MS chromatograms mixture of drug compounds. UV (A), ELSD (B), ICP-MS at *m/z* 35 for chlorine (C), ICP-MS at *m/z* 34 for sulphur (D), ICP-MS at *m/z* 79 for bromine (E), ICP-MS at *m/z* 81 for bromine (F). Adapted with permission from Pereira *et al.[88]*

Figure 5 shows the LC-UV-ELSD-ICP-MS chromatograms of a compound mixture of 10 µg/mL. The use of multiple detection techniques clearly shows the selectivity and response differences [88]. When comparing A (UV) and B (ELSD), the two standards 2,4-dichlorophenol and 2,4-dibromophenol were too small to be detected in the ELSD since they did not form particles in the aerosol. Clearly, the

response in the ELSD of the other compounds is much more comparable than with UV due to the different molar extinction coefficients at 254 nm. With selective detection of Cl, S and Br in figure C-F, compounds that contain one of these marker atoms are observed while the ones lacking these atoms are not detected.

Whenever a (bio)synthetic reference compound of the metabolites is available, the development of quantification assays is significantly less complicated. For routine analysis, normally triple quadrupole mass spectrometers are used [96]. Characteristics of these instruments are robustness, selectivity, sensitivity and they are relatively inexpensive. With a reference metabolite, the instrument can be tuned for specific fragmentation reactions to subsequently selectively screen for that transition (selected-reaction monitoring, SRM). The analysis of biological samples from *in vivo* studies can therefore be used for quantitative determination of pharmacokinetics where both the concentration of the parent drug and sometimes metabolites are monitored after administration. Numerous examples of this type of quantitative bioanalysis are available in the scientific literature [97,98].

An interesting example of the use of a triple quadrupole MS system for metabolite or parent drug quantification is implemented in the so called rapidfire system. This system relies on a very fast SPE step after injection from a 96 well plate. The sample is directly injected on a very small SPE cartridge and quickly flushed into the mass spectrometer, being a triple quadrupole or sometimes a time-of-flight type of instrument. The cytochrome P450 inhibition experiments described by Lim *et al.* [99] show the power of such an approach. By monitoring isoenzyme specific metabolic reactions with SRM, within 15 minutes they obtain data on the inhibiting effects of drug candidates on the selected CYPs [100]. In previous assays, the same data was obtained in a bit less than 4 hours [96]. This on-line SPE-MS/MS platform has also been successfully employed for label free screening of small molecules [101], kinase inhibitors [102] and phosphatidylserine decarboxylase activity [103]. Recently, the platform was modified to obtain other ADME parameters with P-Glycoprotein inhibition assays [104], Caco-2 assays and PAMPA assays [105].

Integration of biological interactions in analytical assays

Although the HTS platform in industry is the main source for information on drug-target interaction, other types of lower throughput systems offer interesting alternatives often with more mechanistic data on binding modes, kinetics or structural information on potential binders [13]. The use of mass spectrometry to obtain information on biological characteristics such as binding or activity towards a drug target is one of these alternative techniques. Basically, a differentiation can be made between techniques that study protein-ligand interactions as such, techniques using MS as readout for the biochemical assays and techniques that simply detect the binders after a complex sample pretreatment system. The first category is dealing with the infusion of the target protein with the ligand, also often referred to as non-covalent mass spectrometry. Examples of this are reported by Zenobi *et al.* on kinase inhibitors [106], the human carbonic anhydrase I [107], and the human estrogen receptor on a chip-based nano-ESI-MS system [108]. Most of the non-covalent MS interaction studies are performed on nano-scale ESI-MS for reasons of protein consumption, sensitivity and accuracy of the interactions studied [109].

The use of affinity selection mass spectrometry is recently reviewed by Jonker *et al.* [110] and mainly discusses the use of mass spectrometry to screen protein-ligand interactions in both direct and indirect manner. De Boer *et al.* reviewed the use of MS as a readout for enzyme inhibition assays [111]. Attention is paid to the advantages and limitations of the integration of biological systems and ESI-MS. The main bottleneck is the incompatibility of ESI-MS with physiological conditions mimicking buffers containing high concentrations of salts and other additives, pH differences and organic modifier concentrations. Nevertheless, some successful studies have been reported amongst others by de Jong *et al.* [112] and de Boer *et al.* [113] in which ESI-MS compatible buffers were used and enzymatic reactions were monitored by a combination of selected-ion monitoring (SIM) and full spectrum analysis.

Another important category of integration of biological interactions and analytical chemistry includes the use of separation methods prior to the affinity assessment. This is extensively reviewed recently by Kool *et al.* [114]. The main application areas of these techniques are the search for active drug metabolites and identification of active compounds in complicated mixtures such as natural

extracts. A division can be made between truly on-line affinity assays hyphenated with a separation method and e.g. mass spectrometry or the more at-line directed assays that include for instance micro fractionation strategies [115,116], bioassay guided identification [117], and bioassay guided fractionation strategies [118]. The desired drug-target determines the appropriate assay to be used. This mainly depends on the necessary incubation times to observe effects of the binders, inhibitors or activators. Normal binding assays have relatively fast kinetics and can therefore be used in an on-line hyphenated setting. Enzyme activity assays already require longer incubations times to observe sufficient product formation for detection. Generally, functional assays are too slow to be monitored in an on-line fashion, and if implemented in an hyphenated system, it must be performed at-line, as demonstrated in assessing microbial activity of random-modified neomycin antibiotics [119].

Scope

This thesis consist of two parts. **Part I** describes the development and application of innovative analytical technologies for the chemical analysis, mainly addressing metabolite identification related issues. In **Chapter 2**, the development of a high temperature liquid chromatographic (HTLC) method is described and its hyphenation to ESI-HR-MS and ICP-MS. Such a method can be applied for the identification and quantification of human relevant metabolites containing the halogens Cl, Br and I. This techniques allows mass balancing for oxidation products, as demonstrated for the of kinase inhibitor SB-203580-I. This chapter shows the importance of normalized detection methods for the quantification of drug metabolites and contributes to approaches for metabolite quantification when no reference standards are available.

In Chapter 3, the identification of phase I metabolites is described for the antibacterial agent trimethoprim (TMP). Based on the *in silico* prediction of metabolites by the SyGMa software package and subsequent high resolution MS/MS experiments, structure proposals are given for the metabolites generated by human liver microsomes and a drug metabolizing mutant M11 of cytochrome P450 BM3.

Subsequently, **Chapter 4** describes the identification of reactive intermediates of TMP by high resolution MS/MS experiments. In this chapter, the glutathione

adducts originating from the reactive metabolites are identified with Ion Trap and Ion Trap –Time of Flight MS^n experiments. Observation of large inter species difference for some metabolites and the formation of minor glutathione adducts by cytochrome P450 2D6 might implicate that genetic polymorphisms in CYP enzymes can play a role in the observed TMP-related adverse drug reactions in humans.

Part II of this thesis deals with the on-line and at-line implementation of biological interactions as an additional dimension in analytical chemistry. This means, that next to the chemical information of e.g. drug metabolites, also biological information is obtained. The incorporation of a separation method in combination with biological activity profiling is an effective method to assess the drug-target interaction of specific molecules in mixtures such as metabolic incubations, crude synthesis products, or natural extracts. Additionally, several methods of creating new possible hit compounds are described throughout this part.

Chapter 5 describes the formation and identification together with the parallel bioactivity profiling of human estrogen receptor (hER) α and hERβ binders. The use of biosynthetic enzymes, enabling the formation of new selective binders is described in detail. The use of 6 template steroidal molecules allows the formation of a significant set of compounds to be screened for affinity. The use of HR-MS is described as a quick tool to obtain first structure ID information, while 2D NMR is described for the full structure identification of a set of metabolites. This chapter shows the importance of monitoring changes in absorption spectra of the metabolites, since molecules with a steroidal core structure can easily undergo modification of the steroid A ring influencing the chromophore.

In **Chapter 6**, the development of a new high resolution screening assay is described. Based on the platform introduced in **Chapter 5**, the screening assay was adapted to the mitogen activated protein kinase p38α. Modifications to the hardware of the screening part are described to improve the resolution in the screening assay. This, for instance, decreases the non-specific binding of the generally lypophilic kinase inhibitors to the inner walls of the capillaries. **Chapter 7** subsequently describes the application of this p38α kinase binding assay in a fully on-line system incorporating electrochemistry for the formation of structures related to existing kinase inhibitors. By minimizing the manual sample handling steps, this can provide a fast overview on potential modifications of the parent

drug molecule and their binding towards the p38α kinase protein. HR-MS^n experiment provide structure information of the modified kinase inhibitors.

Chapter 8 describes a post-column microfractionation approach for the assessment of antibacterial activity of N-alkylated neomycin regio-isomers. These isomers are products from a one-pot reductive amination of neomycin. This illustrates the need for the hyphenation of separation technologies and bioaffinity assessment. In contrast to the screening assays described in **Chapters 5-7**, the effective readout of antibacterial activity is not compatible with on-line detection systems. Instead of seconds, the effect on bacterial growth is detectable after hours. As a results, such bioassays require longer incubation times and benefit from conventional plate reader type of assay formats.

To finalize, the conclusions of this thesis and future perspectives in the areas discussed can be found in **Chapter 9**.

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