Chapter 6

Summary, conclusion and perspectives
6.1 SUMMARY

The occurrence of ADRs is one of the main reasons for the high attrition rates in the drug discovery and development pipeline and contributes to the high costs for the pharmaceutical industry to bring new drugs to the market. A comparison of the various reasons for drug attrition between 1991 and 2000 is depicted in Figure 6.1. [1] Next to efficacy and commercial reasons, toxicology issues are an important reason for attrition, with even a significant increase between 1991 and 2000. A more recent study of drug failures in phase II and phase III clinical trials performed in 2007-2010 shows that drug safety continues to be a major issue in drug development accounting for 22% of the overall failure rate of drugs. [2]

The assessment of the metabolic stability of drugs and the characterization of metabolites has been recognized as an important aspect in the production of safer drugs. [3] In these so-called MIST (Metabolites in Safety Testing) guidelines, the monitoring and toxicity assessment of (re)active drug metabolites throughout the whole drug development process is regulated. Characterization of reactive metabolites is often performed using trapping agents, such as glutathione, in order to gain a better understanding of the metabolic pathways behind drug (bio)activation. [4-5] However, from an industry point of view, the potential for metabolic activation of drugs should be minimized because this is seen as the first step towards drug toxicity. [6]

As outlined in Chapter 1, there are several theories on the mechanism behind ADRs. One of the assumptions underlying this thesis is that the covalent binding of reactive metabolites to proteins plays an important role in ADRs. This is known as the hapten theory. [7] In current practice, covalent binding of reactive metabolites to liver proteins is only assessed on a global level using radiolabeled drugs and radioactivity.

assays. A conservative threshold value of 50 pmol drug equiv/mg total liver protein has been suggested to aid in the selection of suitable drug candidates [6], but less stringent requirements may apply for drugs for severe diseases that are difficult to treat or incurable [8]. However, studies have shown that not all drug–protein adducts may induce an immune response, for instance, comparable covalent binding levels of acetaminophen (APAP) and its non-toxic regioisomer 3’-hydroxyacetanilide in vivo in mice showed that only APAP led to hepatotoxicity. [9] This finding may indicate that only adduct formation of to certain critical proteins plays a role in organ toxicity. However, the same regioisomer was found to be equally or more toxic than APAP in rat and human in an ex vivo study using precision-cut liver slices. [10] Therefore, a detailed investigation is needed to assess which drug–protein adducts are formed and if they are related to ADRs. Current strategies for the identification of hepatic protein targets of reactive metabolites involve the use of a radiolabeled drug in animal experiments followed by analysis of the liver proteins with two-dimensional gel electrophoresis combined with radioactivity assays and mass spectrometry. [11] The list of identified protein targets continues to grow, but the identification of the specific reactive metabolite, protein target and modification site using such strategies is a significant challenge due to the failure in detection of the adducted peptides. [12]

The aim of the research described in this thesis was to develop an advanced analytical methodology, based on proteomics approaches, to study drug–protein adducts in vivo in biofluids and eventually tissues. The essential difference between a global strategy and our envisaged approach is that we wanted to approach the topic at protein level in order to obtain absolute proof of drug–protein adduct formation. The aim was to use targeted proteomics approaches to establish the identity of the drug–protein adducts at the (single) protein level and use this information to (semi-)quantitatively assess the level of adduct formation. The general workflow in a proteomics approach consists of a number of steps: (1) separation and/or fractionation of a complex protein sample, (2) denaturation, reduction and alkylation of the isolated protein (mixture), (3) enzymatic digestion of the proteins into peptides, (4) LC–MS/MS analysis of the peptides, and (5) data processing using bioinformatics tools. We considered the conversion of the proteins into peptides to be one of the critical steps in the complete procedure and decided to optimize this step. In order to be able to adequately monitor this optimization, all sample preparation steps were optimized to obtain optimum results.

Additionally, the LC–MS/MS analysis had to be optimized, especially considering the fact that new rapid-resolution LC using columns packed with (in those days) innovative 1.8 μm ID particles would be used. Under such conditions, it is important to find the best compromise between speed of analysis and information content. In fact, when considering information content in relation to LC–MS–MS performance, optimization can be directed at optimum response for a particular drug–protein target or at the highest possible sequence coverage of the protein(s) analyzed. The former would be especially important in targeted analysis, whereas the latter would possibly enable the discovery of yet unknown drug protein adducts. The same line of thinking can also be applied to enzymatic digestion. In most bottom-up proteomics experiments, the emphasis lies on achieving high protein coverage for accurate protein identification, whereas detection and identification of the modification and the modification site, i.e., only a certain part of the protein sequence, is paramount for confirmation of drug–protein adduct formation.
However, the in vivo concentrations of drug–protein adducts are generally very low, as typically less than 1% of the protein is adducted. Thus, a well-designed and optimized analytical methodology is required to meet the challenge in the detection of low-abundant drug–protein adducts. Chapter 3 describes the optimization of all phases of sample preparation and analysis specifically for drug–albumin adducts with a main focus on enzymatic digestion. The effect of different enzymes and digestion conditions on the identification of drug–albumin adducts in terms of protein coverage and detection of a specific peptide containing the modification was evaluated through the innovative application of a Design of Experiments. This approach allows for a more efficient and more accurate determination of the optimum digestion conditions through simultaneous optimization of multiple variables and responses, and avoids the disadvantages associated with the traditional one-variable-at-a-time approach. The digestion conditions were optimized with a model adduct of monochlorobimane to human serum albumin (MCB–HSA), and the optimized conditions were applied to an adduct of N-acetyl-p-benzoquinoneimine (NAPQI), the reactive metabolite of APAP, to HSA (NAPQI–HSA). In this study, it was shown that optimization of digestion conditions for a specific application is very meaningful because it leads to increased efficiency and sensitivity for the detection and identification of NAPQI–HSA adducts. In particular for the less specific and less well-described enzyme thermolysin, both the protein coverage and sensitivity for the adduct modification site was improved several fold.

The approach described above is termed “bottom-up proteomics” because it involves digestion of the proteins into peptides and subsequent identification of the peptides in order to establish the identity of the original protein, thus from the bottom up. Despite increasing popularity of other proteomics approaches, bottom-up proteomics remains the method of choice for the analysis of proteins (and their modifications). Protein digestion is the most crucial step in such an approach and can be performed in a variety of ways using proteolytic enzymes for enzymatic digestion or using chemicals for non-enzymatic digestion. Many different enzymes and reagents exist for this purpose, but traditional digestion protocols often apply trypsin, the gold standard in enzymatic digestion, and are performed during an overnight incubation. Protein digestion is often the bottleneck in terms of time consumption and, in recent years, much effort has been invested in the development of techniques for the acceleration of this process. Alternatively, increased throughput can also be achieved through incorporation of the digestion step into online systems, which allows for automation and reduces sample handling. These state-of-the-art protein digestion strategies are reviewed in Chapter 2 of this thesis and may reduce protein digestion time from hours to as short as seconds, which greatly reduces the time needed for sample preparation.

As in vivo analysis of drug–protein adducts was aimed at, another important aspect of sample preparation is the extraction of the target protein from a complex biological matrix. As stated above, less than 1% of a protein is modified by reactive drug metabolites, which means that the very similar, non-adducted protein is present in at least a hundred-fold excess. Selective purification methods for extraction of only the adducted proteins are not yet available, meaning that, following extraction of the target protein, a mixture of adducted (<1%) and non-adducted (>99%) protein is obtained. A sufficient amount of target protein needs to be extracted, otherwise the presence of the drug–protein adduct may be masked by the excess of non-adducted protein. Chapter 5 describes the development of a dedicated sample preparation, digestion and analysis protocol for the in vivo identification and quantification of drug–albumin adducts in serum. This protocol consists of a number of steps: (1) affinity chromatography for the extraction of albumin from
serum, (2) buffer exchange of the extracted albumin sample to denaturation buffer using a gel-filtration column, (3) disulfide reduction and cysteine alkylation to achieve complete unfolding of the extracted albumin, (4) buffer exchange of the unfolded albumin sample to pure water using a gel-filtration column, (5) freeze-drying to concentrate the sample and to bring the NAPQI–albumin adduct within the detectable range, (6) enzymatic digestion using the optimized conditions from Chapter 3, (7) LC–MS/MS analysis of the generated peptides, and (8) data analysis, both manually and with the aid of database search software, for identification of drug–albumin adducts. For final application of the developed protocol to actual biological samples, collaboration was sought to one of the partners in the larger project group that performs animal experiments, studying (among other) immunoactivity of drug–protein adducts in mice models. For our project, we received serum samples from a drug exposure study in which the mice received a high dose of APAP on either a single or seven consecutive days. Since the mouse is a small animal, the serum samples are only available in very limited amounts, which also added to the challenge.

The mouse study was designed to study the kinetics in ADRs via the different treatment groups, thus, it was also pertinent to develop a quantification strategy to assess whether a relationship to the formation of NAPQI–albumin adducts exists. Several options, such as the use of albumins of other species and synthetic NAPQI–albumin adducts, were evaluated, but the best approach was found to be a relative quantification via comparison of the peptide peak areas obtained from the adducted and non-adducted albumin. One other factor that demanded consideration was the presence of two free cysteines on the surface of mouse serum albumin (MSA), which is different from human albumin where only one surface cysteine is present. In the end, the developed strategy allowed for successful identification of NAPQI–MSA adducts in vivo in mouse serum for the first time. NAPQI modification was only detected on one of the free cysteines, which could be due to an in vivo preference for this modification site. On the other hand, despite the large dose of 300 mg APAP/kg that was administered to the mice, only extremely low levels of NAPQI–albumin binding of 0.2% of the total albumin were detected, which may indicate that low-level NAPQI-adduct formation to the other modification site may have been below the detection level. Furthermore, similar adduct levels were detected in both treatment groups, which may suggest that a single high dose of APAP may already severely affect liver function and, thus, the production of both reactive drug metabolites as well as serum albumin.

As described above, sample preparation protocols for bottom-up proteomics can be very laborious and time-consuming, especially when a large number of samples need to be treated. Although the use of gel-filtration columns in the previous study was effective, the number of samples that could be prepared simultaneously with this individual sample clean-up procedure was limited for practical reasons. When large sample sets need to be analyzed for comparative studies or quantitation purposes, the sample preparation and analysis should ideally be performed in one batch. Chapter 4 describes the development of a high-throughput sample preparation approach based on 96-well filter plates that allows for the simultaneous preparation of 96 samples. The use of a molecular weight cut-off membrane has several advantages, including a reduction of the number of sample transfer steps, thereby reducing the potential for sample loss, and enabling simultaneous sample concentration. The latter also avoids the use of a freeze-drying step and increases the overall sensitivity. The particular protocol described in this chapter was developed for global cellular proteomics and performed significantly better than the gel-filtration protocol.
in terms of the number of identified peptides and proteins. This difference in performance was already visible using the rapid-resolution LC–QTOF system for analysis, but was far more evident when the filter plate methodology was combined with more advanced nanoLC–Orbitrap MS analysis. The final filter plate and LC–MS analysis protocol resulted in the identification of more than 400 cellular proteins. This number may still be increased through the use of two-dimensional LC to improve peptide separation and detection. The developed methodology can also be used for targeted protein analysis and may easily be applied to other proteomic samples.

6.2 DISCUSSION AND PERSPECTIVES

The project plan provided basically two general lines of research, one directed at the offline and online targeted analysis of specific drug–protein adducts, e.g., NAPQI–albumin, and another one at non-targeted analysis, enabling screening for (circulating or tissue) proteins that are susceptible to adduction. The proposed online targeted approach was envisaged based on previous research in our group, involving the development of an online system for the analysis of target proteins in complex matrices using immunoaffinity selection of the target protein, subsequent digestion of the protein in an immobilized enzyme reactor, collection of the resulting peptides on an SPE column, and finally LC–MS/MS analysis of the peptides [13-14]. The project plan turned out to be too ambitious for application to drug–protein adduct analysis in vivo, given the high analytical challenges that have to be met.

The in vivo concentrations of drug–protein adducts are generally very low, as typically less than 1% of the protein is adducted (Chapter 5). NAPQI–HSA is the most well-known example of drug–protein adduct formation and only results in ADRs after an overdose of APAP, which leads to increased levels of NAPQI resulting in depletion of GSH and detectable levels of NAPQI–albumin adducts. [15] Most types of adverse reactions are considered to be dose-related, see Chapter 1, thus may also result in detectable drug–protein adducts. However, some drugs may already result in immune-mediated ADRs after a single low dose or after extended use at low dose, which means that the level of drug–protein binding may be even lower. In addition, the work in this thesis was mainly focused on the analysis of drug-adduct formation to albumin, which is the most abundant serum protein. Other protein targets of reactive drug metabolites will have lower abundance, which may also imply that the drug–protein adducts will be even more difficult to detect, especially in global proteomics studies. Another factor that may influence the in vivo concentration of drug–protein adducts is the generation of multiple reactive metabolites. Although APAP only produces one reactive metabolite, which can react with cysteines in two different ways, other drugs, such as diclofenac and clozapine, are bioactivated to multiple reactive species, which may lead to the formation of different adducts at the same protein. In addition, the multiple reactive metabolites may target more than one protein, which has also become evident from the in vivo covalent binding studies with radioactive labeled drugs. Both issues may result in a “dilution” effect, which leads to failure in the detection of drug–protein adducts. [12]
Furthermore, if a small-molecule drug is covalently bound to the protein, only very minor changes in mass and/or physicochemical properties result. Thus, in order to analyze the drug–protein adduct within a complex biological matrix containing an at least hundred-fold excess of the very similar, non-adducted protein requires highly specific sample pretreatment as well as extensively optimized steps in the analytical methodology. The online digestion systems are more suitable for the analysis of concentrated, purified proteins and this is difficult to achieve for drug–protein adducts. Therefore, the focus was shifted more towards offline methodologies.

In the end, we succeeded in performing semi-quantitative analysis of low-level APAP-mouse serum albumin adducts in vivo using an offline approach (Chapter 5). The strategy was extensively optimized, but may be further improved through the development and application of selective drug–protein adduct extraction. Adduct formation to HSA by systemic electrophiles as well as drugs takes place on its single free cysteine residue on position 34. Therefore, a method for the purification of modified HSA using a thiol affinity resin has been suggested. [16] Only HSA that does not contain any modifications to this cysteine binds to the resin. The non-bound fraction, containing the modified HSA, can subsequently be processed and analyzed. This approach could be added to the developed strategy following HSA extraction from serum to enrich drug-HSA adducts prior to digestion. However, it is not selective for drug–protein adducts because other cysteine modifications, such as oxidation (non-mercaptalbumin), will be co-enriched as well. Additionally, the applicability of this enrichment technique is limited when protein targets of reactive metabolites possess multiple free cysteines, such as glutathione-s-transferase p1 [17] and mouse serum albumin [18].

Therefore, there still is a need for truly selective enrichment strategies for drug–protein adducts. One possibility may be the use of drug–protein adduct-specific antibodies, used either at the protein or the peptide level. Antibodies are raised in animal models, to obtain polyclonal antibodies, or in immortalized cell lines, to produce the more selective monoclonal antibodies. These antibodies could be raised against the intact drug–protein adduct to enrich on the protein level, but may also be raised against signature peptides containing the adduct formation site in order to perform enrichment on the peptide level following digestion of the drug–protein adduct. Antibody enrichment on the peptide level has already proven its usefulness in protein biomarker studies as part of the Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) approach, in which also isotopically labeled, synthetic peptides are used for quantitation of the enriched target peptides. [19] Such an approach could potentially be very valuable in drug–protein adduct analysis.

The results obtained in Chapter 4 show that a sample preparation approach based on molecular weight cut-off (MWCO) membranes performed significantly better for global cellular protein profiling than the gel-filtration procedure, which was used in Chapter 5. Sample handling and transfer, and thus, sample loss is minimized with the filter plate methodology, which may be very beneficial for the global or targeted analysis of low-abundant proteins. Additionally, the study also showed that the type of LC–MS instrument used for analysis greatly affected the results. The high resolution and efficiency of the nanoLC–Orbitrap MS system improved the number of identified peptides and proteins in comparison with the rapid-resolution LC–QTOF system. The possibility of performing multiple MS/MS fragmentations in a short time period means that not only the highest abundant, but also less abundant peptide ions will be selected for fragmentation.
This feature, in combination with the increased sensitivity of nano-electrospray ionization (nano-ESI), may lead to the additional identification of low-abundant peptides, which are often related to low-abundant modifications.

The work in this thesis was focused mainly on the targeted analysis of drug–albumin adducts, but the other envisaged research line was the development of a non-targeted screening methodology for the identification of potential target proteins. A first step in this direction was made by setting up an approach for the identification of proteins containing free surface cysteines that are accessible to reactive drug metabolites using fluorescent labeling. This approach consisted of (1) albumin depletion from human serum, (2) thiol-reactive fluorescent labeling of the free cysteine-containing proteins in the depleted serum, (3) size-exclusion chromatography (SEC) in combination with fluorescence detection for fractionation and collection of the fluorescent protein fractions, (4) sample preparation and digestion using spin filters and previously optimized conditions, (5) offline LC–MALDI-TOF/TOF MS analysis of the digested fractions, and (6) data analysis (manual and using bioinformatics tools) for identification of labeled proteins. Using this approach, three labeled proteins were identified, but only one of the proteins, Ig alpha-1 chain C region, contained a fluorescent label on a cysteine. The other two identified proteins, Alpha-2-macroglobulin and Ig kappa chain C region, were found to be labeled on one or more histidines. The confidence in their identification was high and additional examination of the 3D-structures of the identified proteins, when available, showed that the identified labeling sites are on the surface of the protein and may, thus,
be accessible to the label or, possibly, a reactive drug metabolite. The fluorescent label used in this study was MCB, which is thiol-reactive. However, the large excess of the label and possibly other conditions, such as the long (4 h) reaction time, that were used may have led to the off-target labeling of histidine residues. Therefore, further optimization of the fluorescent labeling conditions is required to reduce this non-selective labeling. Furthermore, serum was chosen in this initial study, but, due to the complexity of this matrix and the wide dynamic range, only abundant labeled proteins were identified. The sensitivity for lower abundant proteins may be improved through depletion of the most abundant proteins, for instance, using a multiple affinity removal system [20], or a different fractionation technology, since the resolution of SEC is relatively low (see Figure 6.2). Additionally, another matrix, such as liver, may perhaps be a better option, considering the fact that reactive drug metabolites are formed in the liver. Although little is known about the mechanisms behind drug–protein adduct formation, the short half-lives of the highly reactive metabolites may imply that adduct formation takes place in the liver, which may also suggest that higher adduct concentrations are present in this matrix. Finally, whereas the use of the offline LC–MALDI-TOF MS system was already a significant improvement over the rapid-resolution LC–QTOF system, further improvements may be achieved through the use of even more efficient MS instruments, such as the Orbitrap MS.

All of the methods described in this thesis have been based on bottom-up proteomics. However, recent developments in MS instrumentation now allow for the detection of intact proteins and identification of their post-translational or other modifications. From these so-called top-down proteomics experiments, the intact protein mass is obtained. Proteins up to 50 kDa may also be fragmented to obtain sequence information and, thus, identification and localization of modifications may be achieved. Although the mass difference between a drug-adducted protein and its non-modified form is too small to achieve a separation by common chromatographic separation technologies, it is sufficient for mass spectrometric discrimination. In theory, low resolution instruments (R<5000) should already be able to distinguish both species of the protein. For example, the mass spectrometry discrimination of non-modified HSA (~66500 Da) and the NAPQI–HSA drug–protein adduct (+149.07 Da) requires a resolution (R=M/ΔM, full width measured at half-maximum) of ~500, based on the singly charged species. However, from our own experience, the large difference in concentration, the obtained peak widths and the presence of other, naturally occurring protein modifications (isoforms, post-translational modifications) result in baseline noise/crowding and obscure the low-abundant drug–protein adduct. In addition, small mass modifications to proteins may also be obscured by overlapping isotopic distributions of intact proteins. [21]

Figure 6.3 shows a comparison of the protein profiles obtained from the analysis of an intact MCB–HSA adduct (mass shift of 190.07 Da) using MALDI-TOF MS and ESI-QTOF MS. The typical resolution of a MALDI-TOF instrument operated in reflectron mode is ≥10000 for analytes with a mass of <5000 Da, but larger molecules, such as proteins, can only be analyzed in linear mode with a significantly lower resolution of ~1000. Figure 6.3a shows that the effective resolution (m/z / FWHM) obtained for HSA actually is <100, most likely due to its higher mass. This resolution is not sufficient for resolving the various forms of HSA, including MCB–HSA, present in the sample. Since mass is measured as a function of charge, the production of multiply charged ions with ESI leads to multiple charge states of the protein that are detected in the lower m/z range where higher resolution can be obtained. In addition, the resolution of the MaXis
Figure 6.3 Results of the analysis of intact MCB–HSA; (a) MALDI-TOF MS spectrum obtained with a Bruker Ultraflex III instrument using sinapinic acid as matrix, (b) ESI-QTOF MS spectrum obtained with a Bruker MaXis QTOF instrument and (c) deconvoluted ESI-QTOF MS spectrum. The total protein concentration in this particular sample was 0.9 mg/mL and an estimated 10-20% of the HSA was modified by MCB at the cysteine 34 position (L. Switzer, unpublished data).
ESI-QTOF instrument used for this experiment is considerably higher (40000-60000) and is sufficient for the analysis of intact HSA. Even with this high-resolution instrument, the overlapping charge envelopes could not be completely resolved (effective resolution ~2000), probably due to the presence of other HSA isoforms, see Figure 6.3b. However, deconvolution of the obtained data does show a distinction between non-modified HSA and MCB-HSA and may even reveal the presence of a second MCB modification on HSA, see Figure 6.3c. Thus, high-resolution instruments or rather ultra-high resolution instruments, such as Fourier transform ion cyclotron resonance (FTICR) and Orbitrap MS, are required for analysis of intact drug–protein adducts. However, sensitivity may become an issue, in particular with increasing protein mass and lower modification levels. For example, the MCB modification level of HSA in this sample was approximately 10-20%, but the in vivo modification will be considerably lower. Therefore, selective drug–protein adduct purification methods will still be required for identification of drug–protein adducts from in vivo samples using top-down proteomics.

Ion mobility MS (IMMS) presents another possibility for the detection and identification of drug–protein adducts. In IMMS, proteins are separated based on size and shape (folding and conformation) rather than mass. In order to achieve separation in IMMS, covalent adduction of a reactive metabolite to a protein should induce a sufficient change in the shape of the protein (collision cross section) since the size may not change significantly due to the addition of a small metabolite to a relatively large protein. Many examples exist of the IMMS investigation of protein-ligand interactions, for instance, the interaction of the Bcl-2 related survival factor protein (Bcl-xL, ~26 kDa) and the small-molecule inhibitor ABT-737 (~800 Da). [22] Binding of ABT-737 induces a conformational change in Bcl-xL and the Bcl-xL/ABT-737 complex could be separated from Bcl-xL after optimization of instrument parameters. Although this example resembles the situation of covalent modification of proteins by reactive drug metabolites, the metabolites are generally smaller than 800 Da. It will need to be investigated whether they induce a change in conformation and/or collisional cross section large enough to achieve successful ion mobility separation. However, commercially available IMMS instruments can separate isoforms of large proteins, such as minor glycoforms of IgG (148 kDa), and have also shown to be able to achieve isotopic resolution for proteins up to 30 kDa. [23] These IMMS instruments also possess the option for top-down sequencing of proteins through ETD fragmentation. If ETD fragmentation could take place following ion mobility separation of the drug–protein adduct from its non-modified form, this would be an ideal instrument for confirmation and localization of adduct formation. However, the ETD cell is located in front of the ion mobility tube and only CID fragmentation can be performed following IMMS separation, which is not suitable for the fragmentation of large molecules.

When dedicated detection and identification strategies for individual drug–protein adducts become available, the covalent binding thresholds will need to be reevaluated. Currently, the threshold for global covalent binding is 50 pM [6], but when the identity of the specific drug–protein adduct(s) is known and the relationship to ADRs can be established, this threshold will need to be redefined for each drug or drug–protein adduct. Quantification of in vivo drug–protein adduct formation may be performed using existing methods, such as spectral counting or the above suggested modified SISCAPA approach. Other possibilities may include the use of isotopically labeled drugs in animal studies, or synthetic, isotopically labeled reference standards of drug–protein adducts for quantification in human samples from clinical trials. In the end, state-of-the-art LC–MS based proteomics methods for detection, identification and
quantification of drug–protein adducts may contribute to the establishment of the relationship of drug–protein adducts to ADRs and the levels at which they may cause toxicity, which in turn may aid to reduce drug attrition rates and the development of safer drugs.

REFERENCES


