Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 797 (2003) 63–90
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Separation and detection methods for covalent drug–protein adducts

Shufeng Zhou*

Department of Pharmacy, Faculty of Science, National University of Singapore, Science Drive 4, Singapore 117543, Singapore

Abstract

Covalent binding of reactive metabolites of drugs to proteins has been a predominant hypothesis for the mechanism of toxicity caused by numerous drugs. The development of efficient and sensitive analytical methods for the separation, identification, quantification of drug–protein adducts have important clinical and toxicological implications. In the last few decades, continuous progress in analytical methodology has been achieved with substantial increase in the number of new, more specific and more sensitive methods for drug–protein adducts. The methods used for drug–protein adduct studies include those for separation and for subsequent detection and identification. Various chromatographic (e.g., affinity chromatography, ion-exchange chromatography, and high-performance liquid chromatography) and electrophoretic techniques [e.g., sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), two-dimensional SDS–PAGE, and capillary electrophoresis], used alone or in combination, offer an opportunity to purify proteins adducted by reactive drug metabolites. Conventionally, mass spectrometric (MS), nuclear magnetic resonance, and immunological and radioisotope methods are used to detect and identify protein targets for reactive drug metabolites. However, these methods are labor-intensive, and have provided very limited sequence information on the target proteins adducted, and thus the identities of the protein targets are usually unknown. Moreover, the antibody-based methods are limited by the availability, quality, and specificity of antibodies to protein adducts, which greatly hindered the identification of specific protein targets of drugs and their clinical applications. Recently, the use of powerful MS technologies (e.g., matrix-assisted laser desorption/ionization time-of-flight) together with analytical proteomics have enabled one to separate, identify unknown protein adducts, and establish the sequence context of specific adducts by offering the opportunity to search for adducts in proteomes containing a large number of proteins with protein adducts and unmodified proteins. The present review highlights the separation and detection technologies for drug–protein adducts, with an emphasis on methodology, advantages and limitations to these techniques. Furthermore, a brief discussion of the application of these techniques to individual drugs and their target proteins will be outlined.

Keywords: Reviews; Drug–protein adducts

Contents

* Tel.: +65-6874-2931; fax: +65-6779-1554.
E-mail address: phazsf@nus.edu.sg (S. Zhou).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00399-4

1. Introduction

A protein–drug adduct is a protein complex that forms when electrophilic drugs or their metabolites(s) covalently bind to a protein molecule [1,2]. The Millers first found that chemical carcinogens (e.g., *p*-dimethylaminoazobenzene) were converted to electrophilic metabolites that covalently bound macromolecules including DNA and proteins in the 1940s [3]. In the 1970s the role of hemoglobin (Hb) adducts was initially identified in the toxicity of xenobiotics such as aromatic amines [1,4,5]. In addition, the work in 1970s by a few groups established that the covalent binding of acetaminophen (APAP) metabolite to hepatic proteins was closely associated with its hepatotoxicity [6–8]. All these data established initially an association between protein adducts and toxicity. In the last 20 years, a large body of data concerning the formation of protein adducts and its relevance to toxicity has emerged from in vitro and in vivo (animal and human) studies, suggesting a causative role for protein adducts in the toxicity of xenobiotics including many therapeutic drugs [9,10].

Drugs always require activation to form reactive metabolites [11]. Various cytochrome P450s (CYPs) are the major enzymes responsible for the bioactivation which are abundant in the liver [12–17]. The relative abundance of the hepatic CYPs in humans has been determined as: CYP1A2 (13%), 2A6 (4%), 2B6 (<1%), 2C (20%), 2D6 (2%), 2E1 (7%), and 3A4 (30%) [15,18]. Since individual CYPs differ significantly in their substrate specificity and regulation, the expression profiles of CYP in various cell and tissue types are vital determinants in tissue-specific toxicity [14,16,19]. Other phase I enzymes such as myeperoxidase (MPO) and other peroxidases (e.g., prostaglandin peroxidase, skin and thyroid peroxidase) can also bioactivate many drugs, in particular those nitrogen-containing drugs via direct oxidations on the nitrogen atom, leading to reactive intermediates or by oxidation at an alternate site in the molecule [11,20,21]. MPO and/or other peroxidases can bioactivate many drugs including phenytoin [22–24], carbamazepine [25], trimethoprim [26], L-dopa [27], clozapine [28–31], fluperlapine [28], olanzapine [30], and procainamide [32] to reactive metabolites/intermediates (e.g., nitroso, nitrogen free radical and iminium species). Diclofenac undergoes *p*-hydroxylation by MPO, resulting in the formation of *p*-aminophenols, two-electron oxidation of which results in the formation of reactive quinoneimines [33].

Conjugation of xenobiotics is often associated with detoxification. However, phase II enzymes [e.g., uridine diphosphate glucuronosyltransferases (UGTs) [34–36] and sulfotransferase (SULT)] [37,38] can catalyze the formation

reactive metabolites of drugs. In particular, three human hepatic UGTs (UGT1A3, 1A9, and 2B7) catalyzed the acyl glucuronidation of a number of carboxylic acid drugs, resulting in electrophilic acyl glucuronides which can covalently bind to endogenous proteins including UGTs themselves [34,35,39–42]. Covalent protein binding of acyl glucuronides by different mechanisms may contribute to drug toxicity and immune responses [35,42,43]. Moreover, numerous compounds are known that are metabolized to chemically reactive metabolites via sulfation (*O*-sulfonation) [37]. The sulfate group is electron-withdrawing and may be cleaved off heterolytically in appropriate molecules, thus leading to the formation of a strongly electrophilic cation which may covalently bind with DNA and proteins [38]. Eleven distinct human SULT isoforms are known, which strongly differ in their tissue distribution and their substrate specificity [44]. Activation by SULTs differs from other activation pathway in its cyclic nature: reaction of a sulfuric acid ester with water usually regenerates the hydroxylated compound, which becomes available for a new cycle of activation.

The liver is the primary target organ for many toxic chemicals, because of its unique metabolism and relationship to the gastrointestinal tract [45,46]. The reactive species formed by hepatic phase I and II enzymes may render nucleophilic attack on these enzymes themselves, causing mechanism-based activation of enzymes, autoantibodies and drug-induced hepatitis [45,47]. The resultant liver injury can be cytotoxic, cholestatic, or mixed, mimicking autoimmune hepatitis or it can evolve to cirrhosis [48]. Monitoring serum alanine aminotransferase (ALT) levels is of unproven effectiveness, but should be considered when there is an increased risk of delayed onset serious hepatitis-like reactions [49]. As most CYPs and UGTs also exist in many extrahepatic organs (e.g., intestine, kidneys, and brain) and they do catalyze drug metabolism [50–53], this may lead to the formation of reactive species and thus cause toxicity in the relevant organs [54].

Reactive species of drugs (e.g., intermediates or metabolites) usually have low electron densities and are capable of reacting with molecular centers of high electron density (i.e., nucleophiles). Target proteins for adduction usually contain strong nucleophilic sites such as cysteine thiols, lysine amines, histidine imidazoles, and protein N-terminal amines which are readily attacked by reactive species [12,55,56]. Some proteins contain less nucleophilic sites including methionine sulfur, arginine guanidinium, tyrosine phenols, serine and threonine hydroxyls, and aspartate and glutamate carboxyls. Plasma proteins [mainly human serum albumin (HSA) and bovine serum albumin (BSA)], hemoglobin (Hb) and various liver proteins are the common targets of reactive species of drugs. HSA is a heterogeneous mixture of mercaptalbumin and nonmercaptalbumin with 35 cysteine residues per mol [57,58] that are easily subjected to nucleophilic attack. The easy sampling of plasma/Hb allows one to monitor in vivo formation of drug–protein adducts [10,59,60]. One protein may be adducted by multiple reactive species, and a reactive species may attack multiple target proteins located within different subcellular fractions, depending on both the proteins attacked and reactive species.

The mechanism for the covalent binding of reactive species to protein molecules remains largely unclear, but nucleophilic substitution and Schiff's base mechanism have been suggested [10,61–65]. Nucleophilic substitution refers to the formation of a new covalent bond from an unshared pair of electrons in the nucleophile, which is considered as the major mechanism for the protein adducts formation. On the other hand, aldehydes formed by bioactivation of drugs can react reversibly with nucleophiles (e.g., amines) to generate Schiff's bases via formation of carbinolamines. For example, the acyl glucuronides of many carboxylic acid drugs can bind covalently to proteins through acylation and/or glycation [34,35,40–42]. Glycation involves intramolecular regroup to react with amino groups of proteins to form inter- and intramolecular crosslinks of proteins, stable end products called advanced Maillard products or advanced end products [34,35,40,41,66].

The formation of drug–protein adducts may be nontoxic, or fatal, depending on the drugs, kinetics of drug–protein adduct formation and degradation, affected proteins and organs, and pathological conditions of the patients [67]. The reactivity of drug intermediates and subcellular localization of major protein targets are important determining factors in the toxicity [9,10]. Selective protein covalent binding by a drug or its metabolite(s) has been associated with target organ toxicity of drugs [68]. However, studies to reveal the role of reactive metabolites and their protein-adducts in the mechanism of drug-induced idiosyncratic reactions are lacking, and thus the underlying mechanisms for the toxicity of protein adducts are largely undefined. The formation of drug–protein adducts can cause cellular and tissue toxicity which may be either intrinsic or idiosyncratic in nature [9,67,69]. Direct and indirect disruption of the bound proteins are critical for cellular functions due to covalent binding of reactive drug metabolites, causing intrinsically cellular damage, death or apoptosis [70]. In contrast, idiosyncratic toxicities are mediated through either a metabolic or immune-mediated mechanism, and appear to have a limited number of protein targets which are usually localized within the subcellular fractions [9,63,71]. The activation of the immune system by drug–protein adducts may cause either a hypersensitivity reaction and/or an autoimmune response [9,63,68,72].

The separation and determination of protein–drug adducts is important in the studies of drug–protein adducts [10,59,73–75]. The last 2 decades have witnessed the development of sophisticated methodologies for the separation and determination of protein adducts. Many of these methods are based on chromatographic [in particular mass spectrometry (MS)] and immunomigration techniques. These currently available analysis techniques for drug–protein adducts have been combined with genomic and proteomic

approaches due to enhanced sensitivity and high throughput capacity, which may allow the identification of the target proteins involved, clinical monitoring of protein adducts in humans as a valid indicator of molecular dosimetry, and elucidation of the molecular mechanisms for the toxicity. The present review highlights the separation technologies for drug–protein adducts, with an emphasis on methodology, advantages and limitations to these techniques, and with a further discussion of the application of these techniques to individual drugs and their target proteins.

2. Practical strategies for the separation and detection of drug–protein adducts

Separation refers to the process of isolating or extracting the target compound from other (possibly structurally related) compounds or contaminants. The efficient separation of drug–protein adducts allow further characterization of the adducts. Scientists have been constantly seeking highly efficient and sensitive methodology for the separation, identification and quantitation of protein–drug adducts since the 1970s. However, the separation and identification of adducted proteins is always difficult and complicated, since protein structures are complex and variable greatly; unmodified proteins are often present in excess; the half-lives of the protein adducts are too short; and there is a multiplicity of protein targets for most electrophiles [9,10,74,76,77]. Currently, there are a number of relatively sensitive and efficient technologies available for the isolation and analysis of drug–protein adducts.

2.1. Conventional approaches

Conventionally, the separation of drug–protein adducts always involve isolation and purification of the drug–protein adducts from tissue/cellular proteins; direct analysis of the purified drug–protein adducts without detachment using chromatographic and immunological methods; detachment of the protein adducts (or adduct–amino acid or adduct–peptide complexes) by chemical methods; separation of the bound drug moiety and protein/peptide/amino acids; and analysis of the detached adduct components by radioisotope assays, chromatographic and/or immunoassays (Fig. 1) [10].

The isolation and purification of the drug–protein adducts from a tissue/cellular protein mixture is always important. Many of these methods are based on chromatographic [e.g., affinity chromatography, ion-exchange chromatography, and high-performance liquid chromatography (HPLC)] and electrophoresis methods [e.g., polyacrylamide gel electrophoresis (SDS–PAGE) and two-dimensional (2D) SDS–PAGE]. If the bound drug moiety is radiolabeled, radioisotope-based assays (e.g., radioactivity counting and autoradiography) can be applied for the analysis of drug–protein adducts (cf. Section 4.4). The purified drug–protein adducts allow the raising of monoclonal or polyclonal antibodies against the

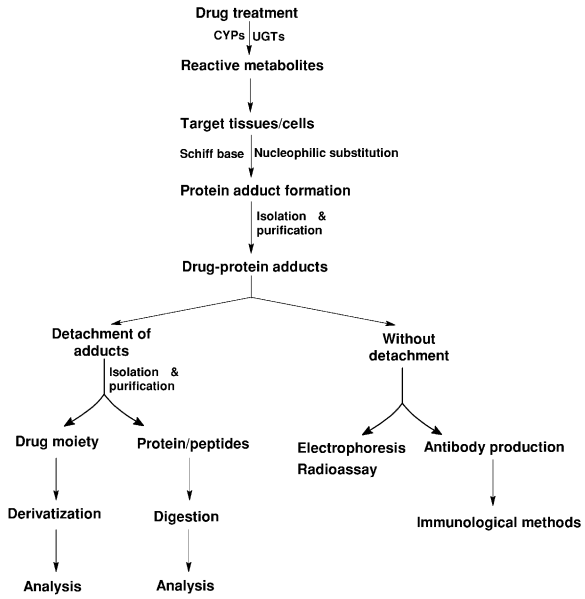

Fig. 1. A conventional strategy for the study of covalent drug–protein adducts.

protein adducts using proper animal models. The raised antibodies are very useful for the detection and quantitation of the drug–protein adducts by immunological techniques [e.g., enzyme-linked immunosorbent assay (ELISA) and Western blotting]. The drug–protein adducts can be detached by chemical or enzymatic methods to release bound drug moiety and proteins. The latter can be further digested by proteases to facilitate analysis. Many released drug moiety can be readily determined by chromatographic methods (e.g., HPLC and LC–MS), whereas derivatization may be required for some other drug moieties.

The conventional approaches are widely used in the study of almost all drug–protein adducts. However, they are labor-intensive and time-consuming—which may be the most labor-intensive work in mechanistic toxicology. For example, it took a few years to develop qualitative and semi-quantitative methods including chromatographic, radiolabeled and immunological techniques in the 1970s and 1980s by which seven protein targets were adducted by the reactive metabolite of APAP, *N*-acetyl-*p*-benzoquinoneimine (NAPIQ) [6,78–81]. These included microsomal, cytosolic, and mitochondrial proteins [9]. With the application of conventional purification and antibody methods and Edman sequencing, approximately 12 hepatic protein targets adducted by the halothane metabolite trifluroacetyl (TFA)-chloride have been identified from 1989 to 1999 [9,82–92]. Although these assays gave valuable data concerning the mechanism of hepatic toxicity caused by APAP and halothane, they have provided very limited sequence information on the target proteins adducted, and thus the identities of the protein targets are usually unknown. Moreover, the antibody-based methods are limited by the availability, quality, and specificity of antibodies to protein adducts,

which greatly hindered the identification of specific protein targets of drugs and their clinical applications [76].

2.2. Novel approaches incorporating proteomic methods

To overcome the difficulties of conventional approaches to identifying drug–protein adducts, an innovative approach has been developed by integrating new MS technologies with proteomic technologies. This high-throughput approach has enabled one to separate, identify unknown protein adducts, and establish the sequence context of specific adducts by offering the opportunity to search for adducts in proteomes containing a large number of proteins with protein adducts and unmodified proteins [76,93]. The combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), in gel enzymatic digestion of proteins separated by 2D gel electrophoresis and searches of molecular mass (M_r) in peptide-mass databases is a powerful and well established method for protein identification in proteomics analysis [76,94–96].

The procedure incorporating proteomic methods always includes the following steps (Fig. 2):

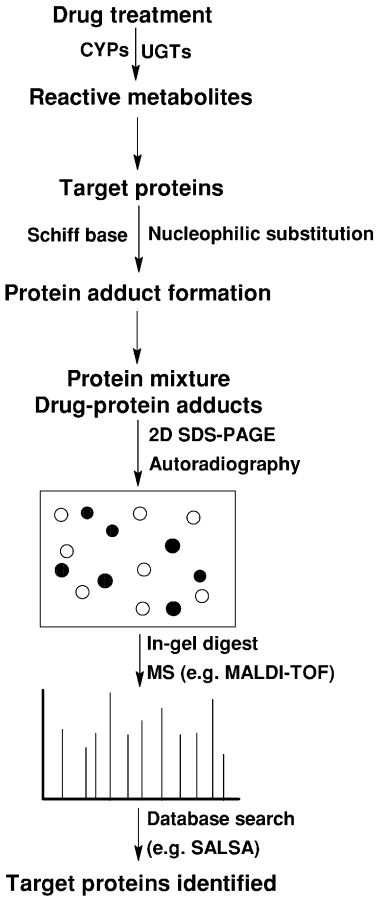

Fig. 2. An innovative strategy incorporating analytical proteomic methods for the study of covalent drug–protein adducts.

- Separation of cellular proteins (adducted and unmodified) by 2D SDS–PAGE.
- In gel digestion of separated protein adducts by enzymes or chemical methods. This step is vital for the quality of the mass data of peptides obtained [96].
- Determination of masses of the resulting peptide mixtures by MS (e.g., MALDI-TOF).
- Searching a protein sequence database.
- Identification of target proteins.

This novel approach has enabled the identification of protein adducted by reactive drug metabolites with high-throughput capacity and high sensitivity [93,97,98]. However, these analyses did not directly identify the protein adducts, although extensive washing was implemented prior to separation of proteins by 2D gel electrophoresis. Instead, they just identified proteins that were present in 2D gel spots containing radiolabeled drugs such as APAP, in which the proteins might be adducted or not. They also have disadvantages of requiring expensive instrumentation and use of radiolabels due to safety issues and limited availability of many drugs.

3. Separation methods for covalent drug–protein adducts

3.1. Chromatography

3.1.1. Affinity chromatography

Affinity chromatography represents one of the most powerful fractionation techniques for purification of macromolecules including modified proteins [99,100]. It has been used to purify efficiently glycated Hb [100]. Different buffers are used to elute unmodified and glycated Hb, and 0.5% acetic acid is used to regenerate the column and to elute the more tightly bound proteins. An affinity chromatographic method has also been used to purify drug–protein adducts. Brown and Gandolfi [92] purified the cytosolic glutathione-*S*-transferase (GST) adducted by the halothane metabolite TFA-chloride from guinea pig liver slices using gel filtration and *S*-hexyl-glutathione affinity chromatography to electrophoretic purity [92]. Before separation, the liver slices were homogenized and subcellular fractions prepared. The cytosolic protein was isolated by ethanol precipitation and washed with trichloroacetic acid to remove unbound metabolites. Protein adducts were quantified using a covalently bound fluorine assay.

Affinity chromatography allows several-fold purification in a single step, usually with high recovery, and is compatible with the use of large amounts of very dilute preparations, as is often the case with some drug–protein adducts. Despite the potential value of affinity chromatography, the use of this methodology is limited by the availability of specific ligands for each protein target. This limitation may be overcome by screening combinatorial libraries screening or computer modeling.

3.1.2. Ion-exchange chromatography

The use of ion-exchange chromatography is based on the theory that each protein has its own unique physiochemical properties such as ion changes [101,102]. Proper mobile phase selection significantly improved high-performance ion-exchange fractionations of proteins [102]. The pH and salt content of the eluent affected chromatographic behavior on both strong and weak ion-exchange columns. Although anion-exchange chromatography has been widely used for protein separation, cation-exchange chromatography is also a useful separation method, as approximately one-third of all proteins reported in the literature have an isoelectric point (p*I*) sufficiently high to be resolved by cation-exchange chromatography [102].

By using strong ion exchangers in combination with HPLC and electrophoresis, drug–protein adducts can be separated from a protein mixture. Anion-exchange chromatography has been combined with HPLC or preparative electrophoresis to purify acetaminophen–protein adducts of different molecular masses [103,104]. One major APAP–protein adduct of M_r 55 000 was isolated using a combination of ion-exchange fast-flow chromatography, hydroxyapatite HPLC and anion-exchange HPLC [103]. Amino acid sequences of eight internal peptides from a trypsin digestion of the M_r 55 000 protein were found to have 97% homology with the deduced amino acid sequence from a cDNA that corresponds to an M_r 56 000 selenium binding protein [103]. Another major cytosolic APAP–protein adduct of M_r 100 000 was purified by using a combination of anion-exchange chromatography and preparative electrophoresis [104]. Before purification, the cytosol was dialyzed with 2 l of 0.01 *M* sodium phosphate buffer (pH 7.2) containing 0.15 *M* NaCl and concentrated using centrifugal ultrafiltration units. Dialyzed cytosol was applied in six fractions at a flow-rate of 5 ml/min to a DEAE-Sepharose fast-flow anion-exchange column (20×1 cm) using a HPLC system that had been equilibrated with 20 m*M* Tris–HCl (pH 7.5). Proteins were eluted using a linear NaCl gradient, and collected fractions were analyzed by SDS–PAGE for their protein content and immunoblotted. The purified M_r 100 000 protein was separated by SDS–PAGE and tested for immunochemical reactivity in a Western blot using anti-*N*-10-formyltetrahydrofolate dehydrogenase. The ion-exchange fraction was enriched with the M_r 100 000 APAP–protein adduct, and after preparative electrophoresis the M_r 100 000 fraction appears to be >97% pure based on Coomassie blue stained gels (Fig. 3). Further sequencing and Western blot analysis indicated that the purified M_r 100 000 acetaminophen–protein adduct was *N*-10-formyltetrahydrofolate dehydrogenase. In another study, an anion-exchange column using *N*-methylpyridinium polymer was used to separate and quantitate mercaptalbumin and nonmercaptalbumin that were covalently bound by captopril [105].

Anion-exchange chromatography has also been used to investigate the distribution of valproic acid glucuronide

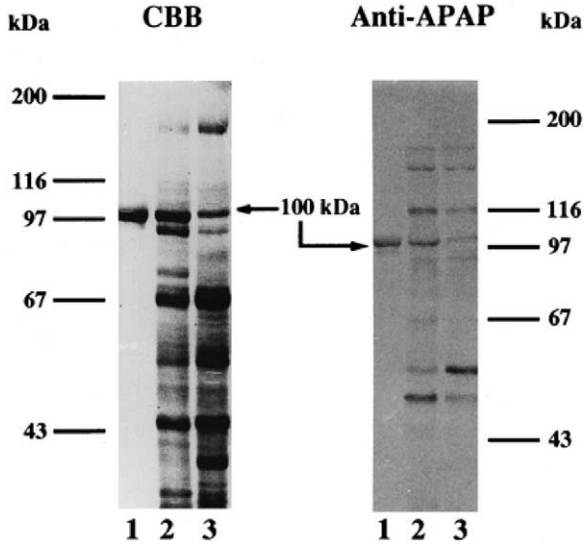

Fig. 3. Purification of the M_r 100 000 cytosolic acetaminophen–protein adduct after SDS–PAGE separation of the proteins. The purification was followed by total protein stain with Coomassie blue (CBB) and immunoblotting with antiacetaminophen (anti-APAP). The individual lanes were: (1) preparative electrophoresis purified APAP M_r 100 000 protein (5 μg), (2) partially purified APAP M_r 100 000 protein by diethylaminoethyl Sepharose anion-exchange chromatography (50 μg) and (3) mouse liver cytosol after 400 mg/kg acetaminophen and killed 2 h after dosing (50 μg). Reprinted from Pumford et al. [81] with permission.

adducts between tubulin and microtubule associated proteins [106]. In this study, bovine brain microtubular protein (comprising 85% tubulin and 15% microtubule associated proteins) was incubated with ^{14}C-labeled isomers of valproic acid glucuronides. Free and bound glucuronides were separated by a DEAE-Sepharose column. The column was then eluted with 25 ml of MEM [50 m*M* 2-(*N*-morpholino)ethanesulfonic acid, 2 m*M* ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.5 m*M* $MgCl_2$, pH 6.6, and containing 1 m*M* ATP] buffer containing 0.2 *M* NaCl to elute microtubule-associated proteins, and a further 25 ml of MEM buffer containing 1.0 *M* NaCl to elute tubulin. The study indicated that of the recovered [^{14}C]-radiolabel, 22% was bound to tubulin and 78% to microtubule-associated proteins [106].

3.1.3. Size-exclusion chromatography

The use of size-exclusion chromatography is based on the theory that each protein has its own unique structure and molecular size, which is suitable for use with proteins, in particularly when biologically active proteins (e.g., enzymes, hormones, and antibodies) are processed. Full recovery of activity after exposure to the chromatography may be achieved, and currently, availability of size-exclusion chromatographic columns is diverse enough to allow fractionation from M_r 10 000–1 000 000 [107]. Extremely basic or hydrophobic proteins may not exhibit true size-exclusion chromatographic character, as the columns tend to have slight hydrophobicity and anionic character.

Thus, a combination of size-exclusion chromatography with other separation methods such as HPLC and electrophoresis will facilitate the protein separation.

Although size-exclusion chromatography has limited use in the study of drug–protein adducts due to the tiny differences in the molecular size between the adducted and unmodified proteins, its combination with other techniques has been used to study proteins adducted by drugs such as L-dopa [108,109] and penicillin [110,111]. With the use of size-exclusion chromatography and other separation methods, covalent bound enzymes such as tyrosine hydroxylase by the reactive quinones of L-dopa can be detected [108,109]. In addition, the covalent conjugation of penicillin or penicilloyl residue with poly-L-lysine can be separated by size-exclusion HPLC [110,111]. These purified conjugates were used in a radio allergo sorbent test for the determination of allergy toward β-lactams [111].

3.1.4. High-performance liquid chromatography

Separations of substances including proteins/peptides using HPLC can be achieved due to the fact that certain substances have different migration rates given a particular column and mobile phase [107]. Thus, the chromatographer can separate substances from each other using HPLC; and the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase. In the late 1970s and 1980s, new methods including reversed-phase liquid chromatography allowed for improved separation between structurally similar substances. Crude tissue extracts may be loaded directly onto the column system and mobilized by gradient elution. Rechromatography under the identical conditions is always an option if further purification is warranted.

HPLC methods have been widely used to isolate and purify proteins adducted by reactive metabolites of drugs [112–117]. For example, after incubation of HSA and excess tolmetin acyl glucuronides in the presence of sodium cyanoborohydride to trap imine intermediates, six tolmetin-containing peptides from trypsin-digested protein were isolated by HPLC [112]. The isolated peptides were further analyzed by LC–MS–MS and found that all six peptides contained tolmetin linked covalently via a glucuronic acid to lysine groups. Major attachment sites on the protein were Lys-195, -199, and -525; minor sites were identified as Lys-137, -351, and -541 [112]. Reversed-phase HPLC was also used to isolate adducts from mouse neutrophils containing Cys-epsilon amino-Lys sulfinamide cross-links and Met-sulfoxide [113], allowing ready identification of these adducted proteins from more complex biological materials by MS technologies.

Preparative HPLC refers to the process of isolation and purification of substances. Important factors are the degree of solute purity and the throughput, which is the amount of substance produced per unit time. This differs from analytical HPLC, where the focus is to obtain information about the sample substance. Preparative HPLC has been used to isolate radiolabeled 5-(4′-hydroxyphenyl)-5-phenylhydantoin and 5-(3′,4′-dihydroxyphenyl)-5-phenylhydantoin and unlabeled 5-(3′,4′-dihydroxyphenyl)-5-phenylhydantoin formed by incubating phenytoin and liver microsomes [114]. The isolated metabolites were then used to adduct target proteins such as various CYPs.

HPLC methods are sensitive technologies for the identification and quantitation of drug–protein adducts. Recently, specific and sensitive HPLC with electrochemical detection has been used to quantify APAP–cysteine in serum proteins from APAP-overdosed patients, and positive linear relationships were found for APAP–cysteine adduct concentration and serum aspartate aminotransferase levels [118]. In this study, the serum protein samples were hydrolyzed by protease and APAP–cysteine released from protein was separated by HPLC and detected by an electrochemical method using tyrosine as an internal standard, without the need to determine the absolute amount of protein hydrolyzed [118].

For the separation and determination of detached drug moieties, HPLC methods are always simple and sensitive technologies for preclinical and clinical applications. Many of the low-molecular-mass drug molecules detached from the protein adducts can be readily quantitated by HPLC. In particular for those proteins adducted by reactive acyl glucuronides of acidic drugs in vitro and/or in vivo, the drug moieties including 5,6-dimethylxanthenone-4 acetic acid, gemfibrozil, ibuprofen, suprofen, diflunisal, probenecid, naproxen were determined after liberation from the respective conjugates via alkaline hydrolysis [41,119–125]. In these studies, the protein mixture containing adducted proteins were precipitated and washed to remove any unbound drugs. The adducted proteins were hydrolysed by a strong base to release the bound drug molecules which were then extracted and analyzed by HPLC.

HPLC in combination with radioassay has been used to separate and detect the covalent binding of acyl glucuronides from carboxylic nonsteroidal anti-inflammatory drugs (NSAIDs) to HSA [126]. Three types of albumin adducts were evidenced. The acyl glucuronide or the drug itself was bound to 0.2–9% of the albumin molecules, depending on the drug, whereas the majority of adducts (23–49% of albumin molecules) retained the glucuronic acid moiety. The possible involvement of specific Lys located in site I of albumin in the formation of these main adducts was demonstrated, using a series of HSA whose specific Lys residues have been chemically modified.

Normal- and reversed-phase HPLC in combination with MS technologies can provide confirmation of structure of the modified protein by drugs. The procedure of this process often involves:

- Fragmentation by proteolysis or chemical cleavage.
- Purification.
- Structural identification and/or sequencing.

For example, the APAP–BSA adduct, isolated from mouse liver microsomal incubations to which the radiolabeled drug and BSA had been added, was cleaved using a combination of specific (cyanogen bromide) and non-specific (acid hydrolysis) procedures, following which the mixture of amino acids obtained was derivatized and the extracted ethoxycarbonyl derivative subjected to analysis using both reversed-phase and normal-phase HPLC techniques [127]. In each HPLC step, one major radioactive amino acid adduct was detected and was identified by MS as the derivative of 3-cystein-*S*-yl-4-hydroxyaniline.

3.2. Electrophoresis

Electrophoresis is a separation technique for ions by their migration in solution under the influence of an electric field. Their rate of migration depends on the strength of the field, the physiochemical properties of the molecules (e.g., net charge, size, shape and ionic strength), and the medium in which the molecules are moving (e.g., buffer ionic strength, viscosity and temperature). As an analytical tool, electrophoresis is simple, rapid and highly sensitive. Macromolecules such as proteins are easily separated by electrophoresis, as their net charge is determined by the pH of the medium in which they are suspended. In a solution with a pH above its p*I*, a protein has a net negative charge and migrates towards the anode in an electrical field. Below its p*I*, the protein is positively charged and migrates towards the cathode. Generally the protein sample is run in a support matrix such as paper, cellulose acetate, starch gel, agarose or polyacrylamide gel. Because dilute agarose gels are generally more rigid and easy to handle than polyacrylamide of the same concentration, agarose is often used to separate large proteins and protein complexes; whereas polyacrylamide is used to separate most proteins that require a small gel pore size for retardation.

3.2.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS as a potent anionic detergent denatures proteins by binding to proteins fairly specifically in a mass ratio of 1.4:1. It is usually necessary to reduce disulfide bridges in proteins with 2-mercaptoethanol or dithiothreitol, before they adopt the random-coil configuration necessary for separation by size. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system. Since protein molecule migration is determined by molecular mass in denaturing SDS–PAGE separations, rather than intrinsic electrical charge of the polypeptide, there is a linear relationship between the logarithm of the M_r of an SDS-denatured polypeptide and its R_F (i.e., the ratio of the distance migrated by the molecule to that migrated by a marker dye-front). SDS–PAGE is often combined with chromatographic and immunological methods for the study of macromolecules including DNA and proteins. Prior separation by SDS–PAGE will allow further detection and identification of modified protein molecules by immunological methods such as immunohistochemical and Western blot analysis and/or MS technologies.

SDS–PAGE is a simple, efficient method for the separation of protein adducts by a number of drugs such as APAP [81,104,128–131], halothane [132], diclofenac [133], sulfamethoxazole [134], mycophenolate mofetil [135,136], and imipramine [137]. For example, SDS–PAGE was employed to separate APAP–protein adducts from in vitro and in vivo studies, and the separated protein adducts were then subjected to immunoblot analysis [81,104,129,131]. SDS–PAGE separation of rat small intestine homogenates and isolated enterocyte subcellular fractions followed by immunoblot analysis with drug-specific antiserum revealed M_r 142 000, 130 000, 110 000, and 55 000 protein adducts of diclofenac [133]. The M_r 142 000 and 130 000 adducts of diclofenac were identified as aminopeptidase N (CD13) and sucrase-isomaltase, respectively, by amino acid sequence analyses and by their reactions with protein-specific antibodies [133]. SDS–PAGE of rat microsomal protein incubated with [^{3}H]imipramine and NADPH showed that the binding was prominent at a molecular mass of approximately 50 000, which was consistent with the CYP2D being a target for the covalent binding [137].

3.2.2. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis is a method for the enhanced separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another [138–140]. This allows the sample to separate over a larger area with increased resolution of each component and further characterization by MS. In the first dimension (isoelectric focusing, a high resolution electrophoretic separation method in which proteins separate on basis of differences in p*I* by having a stable pH gradient in the gel), proteins are separated by their charges; whereas they are separated by molecular mass in the second dimension (SDS–PAGE). Stable pH gradient is produced using a mixture of ampholytes (synthetic polyelectrolytes) in solution. Sample preparation is a key factor in successful 2D gel electrophoresis, and complete solubilization and denaturation of protein sample is often required. Recently, with the maturation of technologies for immobilized pH gradient in the first dimension (isoelectric focusing), its resolution, loading capacity, and especially reproducibility have all been improved significantly. Despite several limitations of the method (e.g., some proteins are poorly represented or lost and use of radiolabels), its ability to separate large numbers of proteins, including their modified forms, ensures that it will continue to be popular in several well-defined areas of proteomics [141].

Two-dimensional gel electrophoresis is a powerful separation method for the separation and identification of protein adducts by APAP and the anti-histamine methapyrilene [93,142–144]. By using a Coomassie Blue R-250 stained 2D

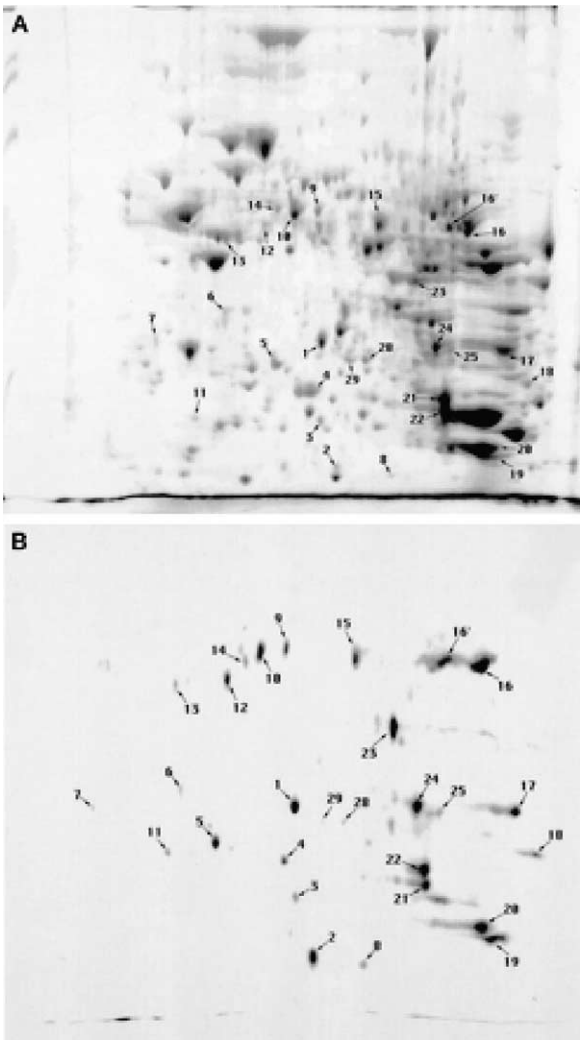

Fig. 4. (A) Coomassie Blue R-250-stained two-dimensional preparative gel of B6C3F1 mouse phenobarbital-induced liver proteins after treatment with radiolabeled acetaminophen (350 mg/kg, intraperitoneal injection). (B) Autoradiogram of the two-dimensional preparative gel. A 2-mg amount of total protein was loaded. Protein samples were focused (*x*-axis, cathode on the right) and then separated by SDS–PAGE (*y*-axis, dye front at the bottom). Reprinted from Qiu et al. [93], with permission.

preparative gel and autoradiographic technique, the mouse phenobarbital-induced liver proteins after treatment with radiolabeled APAP were separated and spotted (Fig. 4). By combining 2D SDS–PAGE with MS technologies, 23 target proteins of APAP in the liver have been separated and identified [93]. Methapyrilene causes hepatotoxicity and has been shown to cause liver carcinoma in chronically treated rats [145]. By using 2D gel electrophoresis, Anderson and co-workers [142,144] were able to distinguish shifts in the p*I* of individual proteins, indicating these proteins were covalently bound by a reactive metabolite of methapyrilene, presumably on lysine groups or other charged amino acids on the proteins [142–144]. Three mitochondrial proteins, the β-subunit of F1 ATPase, heat-shock protein 58, and glucose-regulated protein 75, have been identified by shifts in their p*I* values that formed charge trains of proteins on 2D gels, suggesting sequential increases in covalent adduct forms [142–144].

3.2.3. Capillary electrophoresis (CE)

CE is a sensitive and versatile technique which has emerged over the past 10 years to the forefront of analytical methodology. Separation by CE is carried out in a temperature-controlled capillary tube which is filled with running buffer, and the sample is introduced by dipping one end into the sample and applying an electric field (electrokinetic injection) or by applying gas pressure (pressure injection). Migration through the capillary is driven, directly or indirectly, by an electric field, and analytes are detected as they pass the window at the far end by absorbance or fluorescence. The wall modification methods in CE include covalent modification, adsorbed coatings and polymeric coatings [146]. Conventional CE has been modified to give some variants, including capillary zone electrophoresis (CZE), capillary isoelectric focusing, affinity CE, capillary electrochromatography, micellar electrokinetic capillary chromatography, microchip-based CE, and capillary affinity gel electrophoresis [146–151].

The analysis of DNA adducts has been successfully conducted via the use of capillary HPLC and capillary electrophoretic methods coupled to tandem MS [152,153]. DNA adducts can be analyzed by CE with UV detection [153]. Many adduct nucleotides eluted in a region of the electropherogram free from interferences due to the nonadducted components. Similarly, CE in combination with MS is effective for the analysis of a variety of native and tryptic peptides/proteins such as human plasma proteins, cytochrome *c*, α_1-acid glycoprotein, Hb, bovine serum apotransferrin ($M_r \sim 78\,000$) [154–159]. Modified proteins may be identified by using CE combined with MS technology. For example, C125S mutated interleukin 2 (S-125-IL2) and bovine β-casein have been characterized by an analytical system consisting of CZE coupled to tandem MS via a sheath–flow interface [160]. Following the procedure of proteolytic fragmentation, CZE peptide separation, tandem MS analysis of separated peptides, sequence database search and monitoring of the specific peptides, the modified proteins were characterized as a model of recombinant protein and naturally modified protein, respectively. Protein adducts can also be analyzed by CE coupled with other separation and detection methods. For example, albumin adducts by toluene diisocyanate in humans have been characterized by the use of capillary gas chromatography and MS following separation by ion-exchange chromatography and gel filtration [161]. Since CE as a separation technique has the advantages of short separation times, facile and rapid methods of development and a requirement for very small amounts of analyte (in particular for protein adducts which have always low levels in vivo), it can be predicted that there will be an increasing application of CE in the study of protein adducts by drugs and toxic chemicals.

4. Detection and identification of covalent drug–protein adducts

4.1. Mass spectrometry

A mass spectrometer is an instrument that produces ions and separates them in the gas phase according to their mass-to-charge ratio (*m*/*z*). Mass spectrometry has in the last decade been accepted as a key analytical technique in protein chemistry. It is now the preferred technique for identification of proteins separated by chromatography and one- or two-dimensional SDS–PAGE [162]. Many ionization techniques are available to produce charged molecules in the gas phase, including:

- Electron (impact) ionization (EI).
- Chemical ionization.
- Fast atom bombardment (FAB).
- Plasma desorption.
- Thermospray and particle beam.
- Electrospray (ES).
- MALDI.

ES and MALDI in combination with TOF are the two ionization techniques presently widely used in protein/peptide studies [163,164]. MALDI-MS is shown to be a powerful tool for the elucidation of protein modifications including covalent binding [165]. Low-energy covalent bonds that originate from certain posttranslational modifications dissociate preferentially to produce characteristic mass spectrometric signatures that prove useful for the accurate, confident identification and characterization of such modifications. The method combines the advantageous features of MALDI (i.e., the ability to measure the same sample repeatedly, to measure unfractionated complex mixtures without the need for sample cleaning, and to determine peptide mixtures with sub-pmol sensitivity) with the ease and the speed of the ion trap measurement.

MS is the dominating technique for determination of post-translational modifications in proteins including adducts by drugs [166]. In the case of drug–protein adducts, much of the work with proteins falls into two categories: analysis of intact protein adducts; and more frequently, the analysis of peptides derived from chemically or enzymatically cleaved protein adducts. Thus, molecular mass and amino acid sequence of peptides are readily determined using MS technologies, leading to the identification of target proteins adducted by drugs.

4.1.1. Liquid chromatography–mass spectrometry

The combination of LC and MS is perhaps the most powerful analytical tool available to the analysis of macropmolecules including proteins and nucleic acids. It can provide a wealth of information on individual molecules even when present in complex mixtures, including identification by molecular mass, structure through fragmentation, and quantitation. However, LC–MS is an expensive and complex instrument to handle and maintain, with limited column choices because it cannot be operated with all buffers (e.g., phosphate buffers are unsuitable).

LC–MS coupled with ESI and atmospheric pressure chemical ionization (APCI) are the most commonly used atmospheric pressure ionization techniques which employ conditions that give low thermal input and mild ionization. APCI relies on the interaction of the molecule of interest with a reactive ionized reagent species, whereas ionization by ES occurs in a high-voltage field, resulting from an ion evaporation process. During the evaporation process, a spray of droplets is caused by electrostatic dispersion from the liquid ejected from the capillary tip [167,168]. Aided by the heated bath gas (usually nitrogen), the droplets undergo declustering, losing solvent molecules in the process and eventually producing individual ions. Another ionization mechanism is that the desolvation of the droplets leads to an increasing charge density on the droplet surface that will eventually cause a coulombic explosion that leads to individual ions [168]. For macromolecules such as proteins/peptides, ions entering the mass spectrometer usually each have a high number of charges. Because mass spectrometers measure mass-to-charge ratios rather than mass itself, it is possible for high-molecular-mass molecules to carry sufficient numbers of charges to fall within the *m*/*z* range of a quadrupole mass filter, typically *m*/*z* 2500. The two major disadvantages of ESI are that spray formation is adversely affected even by moderate buffer and salt concentrations, and that mixtures of high mass samples can give overlapping charge state distributions that may be difficult to assign to individual components.

LC–MS coupled with ESI and other interfaces is widely used for the direct detection and identification of protein adducts [64,169,170]. The molecular structures of the main resulting products could be sensitively analyzed by MS with prior separation by liquid chromatography, enabling the detection of characteristic binding formations. For example, using LC–MS, the quinoneimine metabolites of amodiaquine were found to adduct GSH readily [170]. Pretreating the cells with GSH and other antioxidants inhibited metabolism-dependent cytotoxicity.

In tandem MS (LC–MS–MS), the ion of interest is selected with the first analyzer (MS-1), collided with inert gas atoms in a collision cell, and the fragments generated by the collision are separated by a second analyzer (MS-2). HPLC–MS–MS with APCI, ESI or MALDI can be used for the identification and quantitation of reactive metabolites and modified peptides/amino acids. For example, HPLC–MS–MS has been employed to identify and measure protein adducts form in vitro by toxic chemicals such as 1,2,3,4-diepoxybutane [171] and benzo[*a*]pyrene [172]. In the case of 1,2,3,4-diepoxybutane, the structural characterization of the Hb adducts formed in vitro with the most reactive 1,3-butadiene metabolite was obtained by LC–MS with ESI analysis of modified tryptic peptides of human Hb chains [171]. The reactive sites of human

Hb by 1,3-butadiene and its hydroxylated derivatives were identified by MS–MS with MALDI. A procedure was set up to quantitate the Hb adducts by isotope dilution mass spectrometry with the use of a deuterated peptide standard. In these analyses the limit of detection can be as low as 50–500 fmol [172]. Thus, these techniques may allow the biomonitoring of occupational exposure to toxic chemical such as butadiene. LC–MS–MS has been employed to identify the GSH adducts by reactive metabolites of diclofenac formed in human liver microsomes [173]. Three adducts, namely, 5-hydroxy-4-(glutathion-*S*-yl)diclofenac, 4′-hydroxy-3′-(glutathion-*S*-yl)diclofenac, and 5-hydroxy-6-(glutathion-*S*-yl)diclofenac, were identified in incubations of diclofenac with human microsomes. LC–MS–MS was used to identify and quantitate the acyl glucuronides and their isomers of several NSAIDs such as zomepirac [174].

For identification of the site of adduct formation in a protein by drugs, LC–MS–MS coupled with ESI or MALDI is a powerful tool which can determine the sequences of the peptides formed by protease hydrolysis. Tandem MS with collision-induced dissociation (CID) is a well-established technique for sequencing proteins/peptides [162,175–177]. This technique involves enzymatic and/or chemical degradation of the protein to a collection of peptides which are then fractionated by 2D gel electrophoresis or HPLC. Each fraction, containing as many as 10–25 peptides, is then analyzed directly, without further purification, by a combination of LC–MS–MS with CID. The ions in one series of fragments differ in mass due to the neutral loss of a single amino acid, and thus CID data can be used to determine the amino acid sequence of peptides. Tandem MS has been used to establish the structure and specific binding sites of covalent protein adducts formed upon incubation of acyl glucuronides of benoxaprofen and tolmetin with HSA in vitro [112,115,116,178]. In these studies, the modified human serum albumins digested with trypsin and separated by HPLC were first detected using LC–MS with ESI (with selected-ion monitoring), and structurally characterized by tandem MS with MALDI in both the post-source decay and high-energy CID modes [115,116]. These studies established that benoxaprofen glucuronide produced covalent protein adducts by binding to Lys-159, while tolmetin acyl glucuronides were found to bind to Lys-199, -195 and -525, and Lys-137, -351, and -541 as minor sites [112,115,116,178]. Other protein adducts by drugs such as sulfonamide [113], zomepirac [178], and tienilic acid [117] have been analyzed by LC–MS–MS with ESI or MALDI.

4.1.2. Gas chromatography–mass spectrometry

GC–MS allows separation of complex mixtures into single components before ionization and mass analysis. This is particularly useful when analyzing relatively low levels of target compounds derived from complex biological matrices. The target analyte must be relatively volatile or must be susceptible to conversion to a volatile derivative to permit GC separation. In general, the derivatized analyte should have an M_r of <1000 in cases where GC–MS can be successfully applied. In special cases, derivatized analytes with an M_r of 1000–2000 can be analyzed. The ionization methods can be EI (rarely used) and chemical ionization (CI) in the positive and negative modes. In general, the highest degree of sensitivity for fluorine-containing derivatives of adducts or adduct–amino acid complexes has been obtained by MS coupled with negative ion chemical ionization; whereas positive-ion CI is always used for accurate determination of the molecular mass of standards. Detection limits are in the range of 0.1–10 fmol for fluorinated derivatives of adducts [10,179,180]. GC–MS is usually done at low resolving power and can be done at high resolving power for target (known) compounds for the purpose of proving compound presence.

For identification and quantitation of protein adducts by GC–MS, prior isolation of isolate the adducts or the modified amino acids from the protein mixture is often required and derivatization may be need to increase the sensitivity, as well as to improve the chromatographic properties of molecules containing several polar groups. GC–MS is a useful tool that has been widely used to analyze protein adducted by toxic chemicals such as perchloroethene, alachlor, benzo[*a*]pyrene, naphthalene and ethylene bis dithiocarbamate pesticide [181–186]. It has been used to detect 2,6-dimethylaniline–hemoglobin adducts from in vitro and in vivo studies [187]. 2,6-Dimethylaniline is a metabolite of lidocaine. It appears that there is rare use of GC–MS in the identification of drug–protein adducts. This may be due to the fact that modified peptides and proteins and certain modified amino acids often have polarity, low volatility and thermal lability.

4.1.3. Capillary electrophoresis–mass spectrometry

CE has become an important complement to HPLC because of its high separation efficiency and ability to separate molecules based on their charge in solution. CE has been directly coupled with MS using various interfaces including ES, FAB, and MALDI [147,152,154,188–191]. The CE–MS is effective for the analysis of native and tryptic peptides and of proteins of high molecular mass such as bovine serum apotransferrin (approximately M_r 78 000). For the analysis of peptides in a tryptic digest, one can vary the pH to effect better separations because the isoelectric points of the peptides vary widely. Adsorption of cationic analytes under acidic buffer conditions is minimized through the use of a non-covalent coated capillary possessing an overall positive charge [154].

CE–MS has been employed to detect and identify DNA adducts with good sensitivity and specificity [152,192–195]. It can detect DNA adduct at levels of four adducts in 10^7 unmodified bases or less [195]. The application of CE–MS to covalent protein modifications has just begun [191]. Increased application of CE–MS to drug–protein adducts is expected. However, the technique has some drawbacks. In the case of the ES interface, problems can arise from high buffer

concentrations, a condition generally required for good CE performance. For CE-FAB, since ionization takes place under high vacuum, pressure-induced flow within the CE capillary can give non-optimal performance. Sample size is also limited to about 2% of the capillary volume if separation efficiency is not to be degraded. By using on-column focusing, the sample load ability onto a CE column can be improved.

4.2. Nuclear magnetic resonance (NMR)

NMR spectroscopy is the use of the NMR phenomenon to study physical, chemical, and biological properties of compounds including small molecules and macromolecules. Simple one-dimensional, or the more complicated 2D techniques is used to study chemical structure [196]. Due to improved specificity and strong ability to explore three-dimensional structure of molecules, these techniques are replacing X-ray crystallography for the determination of protein structure. Recent developments in experimental and computational aspects of NMR spectroscopy have had a significant impact on the accuracy and speed of protein structure determination in solution, particularly with regard to systems of high complexity (such as protein complexes) [197]. NMR has been used to study modified proteins/peptides, covalent substrate–enzyme interactions, and ligand–protein interactions [198–200]. For example, by using ^{1}H- or ^{13}C-NMR, it was found that peptidyl aldehydes were reversible covalent inhibitors of protein tyrosine phosphatases [201]. In order to identify drugs/drug metabolites and macromolecules, various techniques have been used with NMR [202]. HPLC and/or MS have been coupled simultaneously to NMR, giving UV, NMR and mass spectra for each component in a mixture, after on-line efficient separation [202–206].

NMR coupled to HPLC, LC–MS, or CE has been used to investigate protein adducts formed by drugs including phenacetin [207], fluperlapine [31], and clozapine [208]. Phenacetin has been found to covalently bind to proteins via its reactive metabolites, *N*-hydroxy-*p*-phenetidine and *p*-nitrosophenetole, which are formed by multiple CYPs [207,209–211]. Both metabolites of phenacetin conjugate with GSH and DNA rapidly in vitro, as indicated by FAB-MS and ^{1}H-NMR spectroscopy [207]. The binding of reactive phenacetin metabolites to DNA may be responsible for its mutagenicity [207]. An in vitro study indicated that 7-hydroxyfluperlapine (the major metabolite of fluperlapine in humans) was oxidized to reactive iminoquinone species which was trapped by *N*-acetyl-L-cysteine (NAC) as well as GSH [31]. NMR spectra of the NAC adducts indicated that the NAC was bound to the 6 and 9 positions of the aromatic ring [31]. Similar results were found with clozapine using NMR [208].

NMR spectroscopy is also useful for the structural identification of reactive metabolites and exploration of the mechanism for the covalent binding of reactive drug metabolites to proteins. For instance, NMR coupled to HPLC or LC–MS is a powerful tool for confirming the internal acyl migration of reactive acyl glucuronides of drugs such as probenecid [212], ketoprofen [213], naproxen [214], and zomepirac [215]. These isomers bind covalently to plasma proteins. By using radiolabeled phenytoin, it was found that a potentially teratogenic, reactive phenytoin intermediates was produced during in vitro incubations, which bound covalently to microsomal protein or BSA [22]. A free radical intermediate was detected and identified by using NMR spectroscopy [22].

4.3. Immunological methods

Although various chromatographic methods including multistep HPLC and tandem MS are widely used in the study of drug–protein adducts, they are generally limited by expensive instruments, slow sample processing rates, and sometimes requiring sample derivatization. Thus, faster and sensitive immunological methods have been introduced for detecting and measuring protein adducts. These methods are mostly based on the specific interactions between antibodies and antigens [216]. The antibodies are used due to their ability to bind to a wide range of natural and synthetic chemicals, good specificity, good strength of binding, and surviving the separation and signal developing process. Immunological methods are always combined with other techniques such as electrophoresis and chromatographic methods, and amino acid sequencing for isolation and identification in order to achieve optimal analysis. Some separation methods are always needed for all immunoassays to separate bound and unbound fractions, which include adsorption, precipitation, solid-phase antibodies, electrophoresis, gel filtration, and ion exchange. Satoh and co-workers first used antibody-based techniques in the early 1980s to detect adducts in hepatic proteins from animals and humans treated with the volatile anesthetic halothane [132,217]. In the years since, antibody-based methods have been widely used to detect and quantitate proteins adducted by various drugs including APAP, NSAIDs, and immunosuppressants (e.g., mycophenolate mofetil).

4.3.1. Enzyme-linked immunosorbent assay

ELISA is a fundamental tool of immunology for the detection of antigens or antibodies. This test allows for easy visualization of results and can be completed without the additional concern of radioactive materials use. The most commonly used method is noncompetitive indirect solid-phase ELISA in which antigen or antibody is coated on the solid phase. In these assays, samples are incubated and washed, and the enzyme-labeled antibody is then added, and signals are detected [216]. On the other hand, competitive ELISA is used for the detection of antigens, which has great specificity, but less sensitivity. In competitive ELISA assays, enzyme-labeled antibodies are mixed with the sample to compete for binding sites on the first antibody.

ELISA is the first immunoassay developed and used for the detection of drug–protein adducts by Satoh et al. who developed a specific and sensitive ELISA method using peroxidase to identify TFA-hepatocytes treated with halothane [217]. ELISA assay was also employed to analyze protein adducts by drugs including captopril [218,219], APAP [220], and tienilic acid [221]. An uncompetitive ELISA was developed to detect IgG anti-captopril antibody in serum from patients receiving the drug [218], while a competitive ELISA was used to detect captopril–protein adducts in vivo [219]. In the study of immunogenicity of APAP following direct conjugation to carrier protein with horseradish peroxidase, ELISA was used to analyze stimulated immune responses in the mouse by the produced conjugates [220]. A sensitive ELISA assay was developed to detect tienilic acid–protein adducts in human liver microsomes [221].

4.3.2. Immunohistochemistry/immunofluorescence

Immunohistochemical analysis using labeled antibodies against the bound drug and/or protein can reveal the presence of covalently modified proteins in target tissues. The antibodies may be labeled by enzymes, isotope, and more commonly by fluorescent dye. Immunofluorescence uses an antibody labeled with fluorescent dye to detect the presence of antigens (e.g., proteins) on the surface of cells, tissues or circulating serum [216]. It has direct and indirect methods. The direct method uses one antibody labeled with fluorochrome, and thus allow detection of antigen, whereas indirect immunofluorescence uses two antibodies with the second one labeled with fluorochrome which can be visualized under fluorescence microscope. Commonly used fluorochromes include fluorescein isothiocyanate, *R*-phycoerythrin, quantum red, tetramethyl rhodamine, Texas red, phycocyanin, and allophycocyanin.

Immunohistochemical techniques has been used to detect the presence of protein adducted by drugs such as diclofenac, halothane and APAP [39,129,132,133,136,217]. Immunohistochemical analyses using an antinitrotyrosine antibody indicated that nitrotyrosine protein adducts co-localized with the acetaminophen–protein adducts in the centrilobular cells of the mouse liver [129]. The immunogen for this new antiserum was synthesized by coupling 3-nitro-4-hydroxybenzoic acid to keyhole limpet hemocyanin, which resulted in a high titer of an antiserum that recognized bovine serum albumin nitrated with peroxynitrite in a rabbit immunized with this adduct. Immunohistochemical analyses indicated that nitrotyrosine protein adducts were detectable in the centrilobular areas of the liver [129]. Nitration of proteins may occur by peroxynitrite that is generated by the rapid reaction of superoxide with nitric oxide. Both nitric oxide and superoxide may be formed by activated Kupffer cells or by other cells. By using indirect immunofluorescence the TFA–protein adducts were detected in hepatocytes isolated from the rat treated with halothane, which showed a linear and granular pattern on their surface membranes [217].

4.3.3. Western blotting

Western blotting is a gel blotting technique for visualizing a particular subset of protein molecules. It involves multiple steps including [216]:

- Separating the protein molecules by electrophoresis. This is done in a gel which allows the protein molecules to migrate under the influence of the electric field.
- Blotting the protein molecules of interest with a nitrocellulose filter. The molecules stick tightly to the filter and will retain their relative positions when flooded with fluid at the next step.
- Probing the protein molecules by bathing the filter with a solution containing a probe which is a molecule that will combine specifically with the target molecules and allow visualization (e.g., a radioactive, chemiluminescent or fluorescent marker).

Western blotting has been widely used to detect protein adducted by drugs such as APAP [81,104,129,131], diclofenac [133], sulfamethoxazole [134], and mycophenolate mofetil [135,136] and from in vitro and in vivo animal and human studies. For example, by using affinity-purified antisera specific for 3-(cystein-*S*-yl)APAP adducts on immunoblots, more than 15 proteins containing the adducts were detected in the serum and hepatic proteins from mice treated hepatotoxic doses of acetaminophen, following separation by SDS–PAGE [81]. An M_r 55 000 protein was dominant, which was mostly present in the cytosolic fraction. The maximal levels of immunochemically detectable adducts in the M_r 55 000 protein occurred at 1–2 h, with a decrease in intensity 4 h after dosing. Microsomal and mitochondrial proteins were also found to contain 3-(cystein-*S*-yl)APAP adducts with molecular masses from 38 000 to 106 000. Immunoblot analysis of liver homogenates from APAP-treated mice indicated that the major nitrotyrosine protein adducts produced had molecular masses of 36 000, 44 000, and 85 000, with the M_r 85 000 protein having the most intensity [129]. In addition, Western blotting using antiserum specific for *N*-10-formyltetrahydrofolate dehydrogenase and APAP indicated that the M_r 100 000 APAP–protein adduct was *N*-10-formyltetrahydrofolate dehydrogenase, which was consistent with the results obtained by sequencing analysis [104].

4.3.4. Immunoelectrophoresis

When complementary antibodies and antigens are mixed, complexes form and precipitate. Precipitation requires antibody and antigen valences greater than one. Immunoelectrophoresis combines SDS–PAGE separation, diffusion, and immune precipitation of proteins [216]. This technique has been used to study drug–protein adducts. For example, SDS–PAGE-based immunomigration methods have been widely used in the isolation and identification of APAP–cysteine or APAP–nitrotyrosine adducts [81,128–130]. The prior separation will allow detection of APAP–cysteine or APAP–nitrotyrosine adducts by immunological

methods such as immunohistochemical and Western blotting analysis. Immunoelectrophoresis was also used to study imipramine–protein adducts. It has been found that preincubation of rat liver microsomes with imipramine in the presence of NADPH caused a time-dependent loss of bunitrolol 4-hydroxylase activity, indicating that the CYP2D enzyme is inactivated during imipramine metabolism [222], which has also been observed after in vivo administration of imipramine [222,223]. A similar effect was obtained when desipramine, an N-demethylated metabolite of imipramine, was used as an inhibitor, whereas 2-hydroxy-imipramine had no effect on the activity [222,224]. Further study using immunoelectrophoresis technique indicated that the proteins to which [^{3}H]imipramine metabolites covalently bound were immunoprecipitated with the anti-CYP2D antibody [137], indicating that an imipramine reactive metabolite binds covalently to CYP2D, resulting in its inactivation.

In summary, antibody-based immunoassays may be sensitive for the analysis of drug–protein adducts. For example, immunoblot analysis using antiserum that recognized bovine serum albumin nitrated with peroxynitrite indicated that nitrotyrosine present in a bovine serum protein co-incubated with APAP could be easily detected at levels of 20 pmol [129]. The sensitivity can be enhanced when combined with radioisotope techniques. Different immunoassays have variable sensitivity and specificity, depending on the quality of used antibodies. Poor specificity of antiserum will result in cross-reaction with antigens with similar structures. Difficulty in obtaining high quality antiserum has limited the use of immunoassays for the analysis of drug–protein adducts.

4.4. Radioisotope methods

Isotopes of a given element carry different numbers of neutrons, or neutrally charged particles, in their nuclei. Radioisotopes have unstable nuclei, and they disintegrate to form atoms with stable nuclei by the release of subatomic particles and γ rays (akin to X-rays). When an isotope emits an α particle, the resultant daughter product has an atomic number two units less than its parent's atomic number, and an atomic weight four units less than its parent's atomic weight. When an isotope emits a β particle, it decays to a daughter with an atomic number one unit greater and an essentially unchanged atomic weight. Classic radioisotope methods have been widely used in the study of drugs, proteins and their interactions due to their high sensitivity and simplicity [225–227]. Coupling of HPLC and/or MS (with ESI or MALDI) with online or off-line nondestructive radioactivity detection methods have become a powerful useful approach for drug and protein studies [228]. Among these methods, digital autoradiography and flow-cell radioactivity detectors using solid scintillators can be used. Information regarding the identity of radiolabeled metabolites or proteins and data obtained from spectroscopic methods together with database searching could be used during structure elucidation and target protein identification [76].

Radioisotope methods have been extensively used to study the formation of covalent protein adducts formed by a number of drugs including APAP [93,229–232], clozapine [208], diclofenac [136,233], phenytoin [22], imipramine [137], tianeptine [234], procainamide [23,235], and tamoxifen [236]. Extensive washing is often required to remove reversible binding to proteins, and thus the radioactivity of covalent binding can be counted. In vitro studies in 1980s using [^{14}C]APAP indicated that APAP reactive metabolite bound covalently to GSH, Hb, and mouse liver proteins [229,231,232]. In vitro incubation of mouse hepatic microsomes with [^{14}C]APAP demonstrated that 95% of the bound radioactivity was associated with adducts to three intraluminal microsomal proteins: calreticulin and the two forms of thiol: protein disulfide oxidoreductase (Q2 and Q5) [230]. By combining with MALDI-TOF-MS, [^{14}C]APAP was administered to the mouse to investigate the liver target proteins in vivo [93]. [^{14}C]Diclofenac was used to investigate the covalent bound of its acyl glucuronides to rat hepatic microsomal proteins [136,233]. By the use of radiolabeled procainamide [23,235] or clozapine [208], covalent binding of procainamide or clozapine to neutrophils was detected, and the degree of binding correlated with the cells' ability to oxidize procainamide or clozapine [23,235]. Incubation of rat liver microsomes with [^{3}H]imipramine in the presence of NADPH resulted in covalent binding of a [^{3}H]labeled material to microsomal protein [137]. The formation rates of the reactive metabolites covalently bound to protein followed Michaelis–Menten kinetics with a K_m value of 1.1 μ*M* which was close to that for microsomal imipramine 2-hydroxylation [137]. Incubation under air of [^{14}C]tianeptine with a NADPH-generating system and hamster, mouse or rat liver microsomes resulted in the covalent binding of [^{14}C]tianeptine metabolites to microsomal proteins [234]. In addition, radiolabeled phenytoin [22] and tamoxifen [236] were used to investigate their covalent binding to human and rat liver microsomes in vitro [22].

Radioimmunoassay (RIA) is a sensitive technique for the analysis of drugs and macromolecules, and has been used to detect and identify DNA [237,238] and protein adducts [239,240]. Standard curves for RIA are generated by mixing fixed amounts of antibody and tracer with increasing concentrations of inhibitor in a constant volume. With larger amounts of inhibitor, less radioactive material is bound by the antibody. Primary antibody is then precipitated, usually with a secondary antiserum, and radioactivity in the pellet or supernatant is counted. Unknowns are similarly mixed with antibody and tracer, and antigen concentration is determined with the standard curve. For example, RIA and immunoblot were used to detect an autoantibody to protein disulfide isomerase in rats after administration of various hepatotoxic drugs including APAP and D-galactosamine [239]. Although highly sensitive and reproducible, RIA has generally been replaced by ELISA (cf. Section 4.3.1), which do not require the use of radioisotopes and the associated

handling problems. In ELISA, the RIA tracer is replaced by a constant amount of immunogen bound to a microtiter plate.

All these studies using radioisotopes have obtained information on covalent binding of drugs via their reactive metabolites to various proteins. However, these studies using radioisotopes did not provide any structural information on the covalently bound target proteins. The used radioisotopes may render some health risks due to their unfavorable biological effects. Specific antibodies must be developed to each drug–protein adduct or class of protein adducts of interest, but the availability of radioisotopes may be limited for some drugs.

5. Methodology application to individual drugs that bind covalently to proteins

All above-mentioned technologies have been used to separate, detect and identify drug–protein adducts. To address their application to individual drugs that bind covalently to various proteins, a short list of drugs is chosen and discussed briefly. These drugs include APAP, carbamazepine, diclofenac, halothane, tamoxifen, and tienilic acid (Fig. 5, Table 1).

5.1. Acetaminophen

APAP is the principle *para*-aminophenol derivative in clinical use as common over-the-counter analgesic and antipyretic agent. The mechanism of analgesic action has yet to be elucidated, but may be due to inhibition of prostaglandin synthesis by inhibiting weakly COX1/2 both centrally and peripherally [241–243]. APAP is always believed to be relatively safe at therapeutic dose. However, APAP overdose may result in acute, often fatal, centrilobular liver necrosis in humans and animals [244,245]. Less than 0.1% of the estimated 30 million APAP users in the United Kingdom attend hospital with an APAP overdose each year, and approximately 200 people die, most of whom presented late or

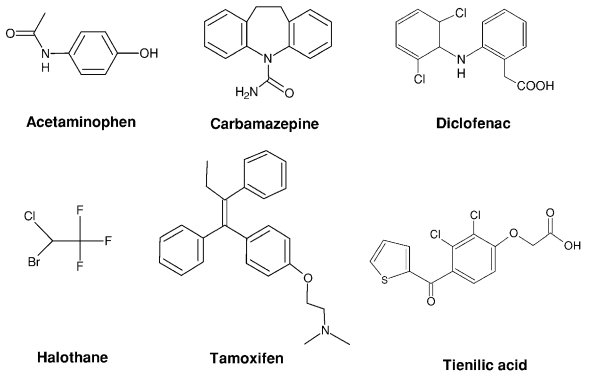

Fig. 5. Chemical structures of acetaminophen, carbamazepine, diclofenac, halothane, tamoxifen, and tienilic acid.

did not receive antidote, *N*-acetylcysteine, within 12 h [246]. The mechanism of APAP hepatotoxicity has yet to be determined, although several mechanisms have been suggested. These include covalent binding to cellular proteins, oxidative stress, apoptosis, disruption of calcium homeostasis, and activation of Kuppfer cells [247,248]. Initiating events in toxicity require biotransformation of APAP to NAPQI by CYP1A2, CYP2E1 and CYP3A4 [7,244], followed by arylation of several important proteins with subsequent alteration of protein structure and function [78,81,247,248]. At therapeutic doses, NAPQI is efficiently detoxified by GSH to form an APAP–glutathione conjugate. However, following toxic doses of APAP,available GSH pools are depleted, allowing excessive reactive metabolite access to liver proteins [8].

Western immunoblotting analysis of hepatic proteins separated by SDS–PAGE indicated that a small number of proteins appeared susceptible to drug adduction [80,81]. Studies using antibodies prepared from different haptens have revealed somewhat different patterns of gel bands that contain putative APAP–protein adducts, with the most extensive modification occurring in a band of proteins around M_r 56 000 [78,80,81,249]. By using conventional approaches (see Section 2.1), a small number of target proteins of APAP have been identified, including selenium-binding protein [103,249], a subunit of glutamine synthetase [250], lamin-A [251], carbamyl phosphate synthetase I [252], glutamate dehydrogenase [253], aldehyde dehydrogenase [254], and *N*-10-formyltetrahydrofolate dehydrogenase [104]. Recently, by using the innovative approaches (see Section 2.2), 23 protein targets for APAP reactive metabolites have been identified [93]. Most of the protein targets identified are of cytosolic origin, but a number of them are from mitochondria as well. Among them a few have been previously identified, including three isomers of selenium-binding proteins and mitochondrial aldehyde dehydrogenase [103,249,254]. However, five additional proteins, which were identified previously by other methods, have not been found by these approaches with proteomic methods, including glutamine synthetase subunit, glutamate dehydrogenase, *N*-10-formyltetrahydrofolate dehydrogenase, lamin-A, and carbamyl phosphate synthetase I [93]. This may be due to the differences in experimental conditions and detection methods between laboratories and animal models, the loss of membrane protein during sample preparation, and easier entry of water-soluble proteins into the gel than hydrophobic membrane proteins [255]. Interestingly, many new target proteins identified by the proteomic methods that are not identified previously by conventional approaches play an important role in the detoxification, mitochondrial homeostasis and protein initiation. These proteins include aryl SULT analog, GST-π, glutathione peroxidase, osteoblast-specific factor 3 (macrophage M_r 23 000 stress protein), mitochondrial ATP synthetase α-subunit, and protein initiation factor 4A [93].

Table 1
Some drugs that binding covalently to proteins

Drug	Reactive species	Activating enzymes[a]	Target proteins[b]	Separation/ detection methods[c]	Possible relevant toxicity	Refs.
Acetaminophen	*N*-Acetyl-*p*-benzoquinoneimine	CYP1A2, 2E1, 3A4	Glutamine synthetase, lamin A, selenium-binding protein, glutamate dehydrogenase, aldehyde dehydrogenase, *N*-10-formyl tetrahydrofolate dehydrogenase, carbamyl phosphate synthetase I, sulfotransferaseanalog, GST-π, glutathione peroxidase, osteoblast- specific factor 3 (macrophage M_r 23 000 stress protein), mitochondrial ATP synthetase α-subunit, and protein initiation factor 4A; Hb	HPLC, LC–MS (ESI, immunoassays (immunoblot, ELISA, immunohistochemistry), radioisotope assays	Hepatic necrosis, renal toxicity	[93,103,104,129,131] [230,231,247,249,250] [253,254,366–369]
Carbamazepine	Carbamazepine 10,11-epoxide, 9-acridine carboxaldehyde, quinines	CYP1A2, 2C, 3A4, MPO	CYP1A2, 2C, 3A4, MPO	HPLC, LC–MS-ESI, immunoblot	Agranulocytosis, aplastic anemia and drug-induced lupus	[25,260–266,370,371]
Diclofenac	Acyl glucuronides, benzoquinone imines, 5-hydroxydiclofenac, 2,2′-dihydroxyazobenzene	CYP2C11, CYP3A4, MPO, UGT2B1 (rat), UGT2B7 (human)	GSH, albumin, CYP2C11, dipeptidyl peptidase IV (CD26), microsomal proteins, intestinal proteins (M_r 55 000–142 000)	HPLC, LC–MS-ESI, radioisotope methods, immunoblot	Hepatotoxicity, bone marrow toxicity, gastrointestinal toxicity?	[33,133,136,173,233] [271–273,278]
Halothane	Trifluroacetyl chloride	CYP2E1 (major), 2A6	Protein disulfide isomerase, isomerase, CYP2E1, carboxylesterase, calreticulin, ERp72, ERp99, UDP-glucose glycoprotein glucosyl-transferase, GST	HPLC, LC–MS, immunoassays (immunoblot, ELISA, immunohistochemistry)	Halothane hepatitis, skin rash, fever	[9,82–92,289,308,372]
Tamoxifen	Tamoxifen, tamoxifen *N*-oxide, *N*-desmethyltamoxifen, tamoxifen *N*-oxide-epoxide, and 3,4-dihydroxytamoxifen	CYP2D6, 3A4	CYP2D6, 3A4	HPLC, LC–MS, radioisotope methods	Carcinogenicity	[314–316,373]
Tienilic acid	Thiophene *S*-oxide	CYP2C9 (major), 2C8, 2C18, 2C19	CYP1A2, 2C9 (residues 365), 2C11, kidney microsomes	SDS–PAGE, immunoassays (immunoblot and ELISA), LC–MS-ESI	Hepatitis	[117,221,323,326–330] [374–379]

[a] CYP, Cytochrome P450; MPO, myeperoxidase; UGT, uridine diphosphate glucuronosyltransferases.
[b] GSH, Glutathione; GST, glutathione-*S*-transferase; Hb, hemoglobin.
[c] ELISA, Enzyme-linked immunosorbent assay; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MALDI, matrix-assisted laser desorption/ionization.

5.2. Carbamazepine

Carbamazepine is an aromatic antiepileptic drug used in the treatment of partial and generalized seizures. However, the use of carbamazepine result in several types of idiosyncratic drug reactions, including cutaneous, hematological, immunological, renal, and hepatic disorders [256–259]. Covalent binding to cellular proteins by reactive metabolites/intermediates have been suggested to be responsible for the hypersensitivity of this drug.

Carbamazepine is converted to reactive metabolites by multiple CYPs [260,261]. CYP2B6 and CYP3A4 are largely responsible for the formation of 3-hydroxycarbamazepine, whereas multiple CYPs (CYP1A2, 2A6, 2B6, 2E1, and 3A4) contributed to 2-hydroxycarbamazepine formation [261]. The resultant reactive metabolites resulted in covalent binding to CYP1A, 2C and 3A in human liver microsomes and yeasts expressing different CYPs [262]. Hydroxycarbamazepine may serve as precursors in the formation of protein adducts. Reactive metabolites/intermediates of carbamazepine such as quinines, 9-acridine carboxaldehyde, iminoquinone metabolite, and arene oxides of carbamazepine have been identified in vitro and in vivo using LC–MS after separation by HPLC [263–267]. Carbamazepine was also activated to reactive metabolites such as 9-acridine carboxaldehyde by MPO in activated neutrophils, and covalent binding of the metabolites to neutrophils was observed [25]. In the bile of rats treated with carbamazepine, carbamazepine 10,11-epoxide and three isometric glutathionyl dihydrohydroxy–carbamazepine adducts were identified by LC–MS [263]. They collectively accounted for 5.8±0.9% of the dose.

5.3. Diclofenac

Diclofenac is an NSAID that is widely used in the treatment of fever, headache and chronic arthritis due to their analgesic, anti-inflammatory and antipyretic activities. It is a potent inhibitor of cyclooxygenase (COX1, the constitutive form; and COX 2, the inducible form in the presence of inflammation), leading to the direct inhibition of prostaglandins and thromboxanes from arachidonic acids [268,269]. Inhibition of COX-2 is considered to be the major mechanism of action, while inhibition of COX-1 is thought to cause some of their toxicities (in particular gastrointestinal toxicity [269]). This has led to the development of new NSAIDs, the selective COX-2 inhibitors, promising minimal NSAID-typical toxicity with full anti-inflammatory efficacy. However, hypersensitivity induced by NSAIDs such as diclofenac is also a problem with their clinical use [270], which has been associated with covalent binding of proteins by their reactive metabolites.

Diclofenac is metabolized to reactive acyl glucuronides by rat UGT2B1 and human UGT2B7 [271]. Acyl glucuronides of diclofenac and their isomers can bind covalently to plasma and cellular proteins in vitro and in vivo, as indicated by the extensive use of separation and detection technologies such as chromatographic and immunological methods [33,136,173,174,233,272,273]. Two mechanisms are suggested for the covalent binding: transacylation and/or Schiff's base mechanism (glycation) [36]. In the direct transacylation mechanism, the glucuronic acid moiety is displaced from the acyl glucuronide by nucleophilic groups such as –OH, –SH or $–NH_2$ located on protein [116,274,275]. In the glycation mechanism, the acyl glucuronide rearrangement isomers, which exist transiently in the open chain form containing a reactive aldehyde group which is reactive and can yield a Schiff's base [36,274–276]. Serum albumin and plasma proteins appear to be the major target proteins of acyl glucuronide of diclofenac based on a variety of in vitro and in vivo studies [33,136,173,174,233,272,273]. However, adduct formation with proteins from other tissues (e.g., liver, kidney, and intestine) have been detected. Such hepatic drug–protein adduct formation could be a causative or initiating factor in hepatotoxicity to diclofenac [277]. Dipeptidyl peptidase IV (CD26) was covalently bound by the acyl glucuronides of diclofenac [278]. It appears that selective protein adduct formation of diclofenac acyl glucuronide is critically dependent on the rat canalicular conjugate export pump [136]. The covalently binding of cellular proteins by acyl glucuronides of diclofenac has been associated with idiosyncratic hepatotoxicity in susceptible patients [33,136,173,174,233,272,273]. The covalent binding of diclofenac to intestinal proteins [133] may partly be responsible for their frequent gastrointestinal toxicity.

In addition to acyl glucuronides, other reactive metabolites/intermediates may be formed by bioactivation and which may be involved in the hepatic and hematological toxicity of diclofenac. It has been shown that diclofenac was metabolized to reactive benzoquinone imines by CYP2C9 and 3A4 in human liver microsomes [173,272] and CYP2C11 in the rat [273]. A highly reactive intermediate(s) inactivating CYP2C11, probably arene-oxide, appears to be generated during the process of diclofenac 4′- and/or 5-hydroxylation [273]. The reactive benzoquinone imines were found to covalently bind to GSH and liver microsomal proteins [173,272]. In vitro and in vivo studies in the rat indicate that a reactive metabolites formed by CYPs inactivated CYP3A2 and other CYPs [279].

5.4. Halothane

Commonly used inhalational halogenated anesthetics such as enflurane, halothane isoflurane are all associated with hepatotoxicity (mild hepatitis, in approximately 20–25% of patients), which is related with the formation of protein adducts in the liver via reactive metabolites. Clinical symptoms of this type of hepatitis often include rash, fever, eosinophilia, white cell infiltration, and liver-kidney microsomal autoantibodies [280–284]. In addition, there is a rare (one in 10 000 patients) form of hepatitis that causes

massive hepatotoxicity and is often fatal. There is a line of evidence indicating that all these inhalational anesthetics have the ability of adduct a number of cellular proteins, which have been separated and identified by chromatographic and immunological methods. All three inhalational anesthetics were activated to TFA-chloride by CYPs [285], which can adduct proteins similarly with cross-reactivity [286–288]. However, more extensive studies on halothane have been done with its protein adducting properties, compared with enflurane and isoflurane, and a number of target proteins have been separated and identified.

Halothane is bioactivated by CYP2E1 and 2A6 to TFA-chloride, bromide and a reactive intermediate that can acetylate liver proteins [90,217,283,289–292]. Covalent binding of the TFA moiety may result in hapten formation leading to the induction of an immune response and ultimately halothane hepatitis. Using patient serum and antibodies that recognize adducted TFA-proteins, many proteins from the liver have been identified to be the targets of TFA. The majority of the reactivity from halothane hepatitis patients is directed against microsomal proteins with M_r values of 54 000, 57 000, 59 000, 63 000, 76 000, 80 000, 82 000, and 100 000 [87,132,217,282,283,293,294]. Both M_r 57 000 and 58 000 adducts were identified as protein disulfide isomerases, by using detergent extraction followed by anion-exchange chromatography, and then further separation by HPLC [82–84,295]. The M_r 59 000 halothane-modified protein was purified using immunoaffinity chromatography utilizing the anti-TFA antibody, and its N-terminal amino acid sequence was homologous with a microsomal carboxylesterase [85,91]. The amino acid sequences from the M_r 63 000 protein adduct had a 98% identity with a reported murine cDNA encoding for a calcium-binding endoplasmic reticulum protein, calreticulin, which is similar to the autoantigen Ro/SS-A in SLE [86].

The M_r 80 000 TFA protein has been found to be ERp72 (an endoplasmic reticulum protein) [87], whereas the M_r 100 000 halothane–protein conjugate was identified asErp99. ERp72 may be a stress protein containing three copies of the active site of protein disulfide isomerase [296,297], which is involved in the degradation of malformed proteins [298]. Erp99 is a conserved glycoprotein of the endoplasmic reticulum,and is also homologous with the M_r 90 000 heat-shock protein, the M_r 94 000 glucose-regulated protein, and endoplasmin [299]. The protein is also a chaperone and binds malformed proteins in the lumen of the endoplasmic reticulum. The M_r 82 000 protein was found to have sequence homology with the glucose-regulated protein GRP78 or BiP [294]. GRP78is a stress-related protein and a molecular chaperone that associates with polypeptides in the endoplasmic reticulum,including immunoglobulin chains. In addition, an M_r 170 000 TFA–protein adduct was immunoprecipitated with antisera specific for the major halothane-modified proteins, and identified as UDP-glucose glycoprotein glucosyl-transferase,a luminal endoplasmic reticulum protein involved in the synthesis of glycoproteins and the maturation of proteins [300].

Some other important proteins/enzymes are also the targets of halothane, as indicated by the detectable serum autoantibodies [89,301–305]. These include autoantibodies against three of the major luminal endoplasmic reticulum proteins [301], purified human liver microsomal carboxylesterase [91], and CYP2E1, the major enzyme that bioactivates halothane to the reactive intermediate [88,305]. The autoantibodies inhibited the activity of CYP2E1 and appeared to be directed against mainly conformational epitopes [305]. Since CYP2E1 became trifluoroacetylated when it oxidatively metabolized halothane, it is possible that the covalently altered form of CYP2E1 may be able to bypass the immunologic tolerance that normally exists against CYP2E1 [305]. A similar mechanism may explain the formation of autoantibodies that have been found against other cellular targets of the reactive metabolite of halothane. Cytosolic proteins such as GST were also found to be targets of the TFA of halothane [306,307]. In guinea pig liver slices exposed to halothane, covalent binding was localized to two cytosolic proteins of M_r 26 000 and 27 000. Using autoradiography, the M_r 27 000 protein was purified and an N-terminal amino acid sequence analysis identified the protein as GST [308]. GST was also found to be a covalent adduct in guinea pigs treated with halothane [92].

5.5. Tamoxifen

Tamoxifen is a nonsteroidal antiestrogen used for adjuvant chemotherapy of all stages of hormone-dependent breast cancer [309–311], and the US Food and Drug Administration also has approved the use of tamoxifen as a chemopreventive agent for women who are at risk for developing breast cancer. However, its use has been associated with a small but significant increase in risk of endometrial cancer [312].

Tamoxifen has been shown to be metabolized to reactive catechol products that have the potential to form protein and DNA adducts [313]. Extensive hepatic metabolism of tamoxifen has been described and the major metabolites formed are tamoxifen-*N*-oxide, *N*-desmethyl-tamoxifen, and 4-OH-tamoxifen. Many studies have shown that metabolic activation of tamoxifen was a prerequisite for the generation of adducts of tamoxifen with DNA and proteins and that the activation was the result of oxidative metabolism of tamoxifen by multiple CYPs. Dehal and Kupfer [314,315] have revealed that drug–protein covalent adduct formation in liver microsomes followed principally from catechol formation due to ortho hydroxylation by CYP3A4 and 2D6. Adduct formation was potentiated by using 4-hydroxytamoxifen as substrate [314], whereas selective chemical inhibitors inhibitory antibodies for CYP3A4 decreased covalent binding of radiolabel tamoxifen in human and rat liver microsomes [236]. Recently, by using recombinant CYPs it has been

found that CYP3A4 was the major enzyme bioactivating tamoxifen to form catechol metabolites that gave rise to tamoxifen–CYP3A4 adducts in vitro, as determined by using SDS–PAGE and fluorography [316–318]. By using baculovirus expressed recombinant human CYP isoforms and LC–MS it has been shown that only CYP3A4 is responsible for the activation of tamoxifen to intermediates that irreversibly bind to exogenous DNA [319]. Although CYP1A1, 2D6, and 3A5 generated catechol metabolite, no covalent protein adduct formation was observed with these isoforms [316]. Conversely, CYP2B6, 2C19, and rat liver microsomes catalyzed drug–protein adduct formation but not catechol formation. Tamoxifen was found to be a mechanism-based inhibitor of CYP2B6 [320]. In addition, tamoxifen was also metabolized to reactive intermediates by endometrial CYPs [321], and α-hydroxytamoxifen was bioactivated by SULT in vitro [322].

5.6. Tienilic acid

Tienilic acid was a formerly used diuretic in the treatment of odema and hypertension, but withdrawn from the market due to severe, sometimes fatal liver injury. The hepatotoxicity is characterized with the presence of anti-LKM2 antibodies reacted immunochemically with CYP2C11 [323–326]. In vitro study has indicated that CYP2C11–tienilic acid adducts are transported to the plasma membrane of rat hepatocytes [326]. However, serum from patients with tienilic acid-induced hepatitis also recognized CYP2C9, but not 2C8 or 2C18 [327]. Ser 365 appears to play a key role in the mechanism-based inactivation of CYP 2C9 by tienilic acid, and OH group of Ser 365 could be the nucleophile forming a covalent bond with an electrophilic metabolite of tienilic acid [328]. Tienilic acid is metabolized to a reactive intermediate(s) by CYP2C9 (major), 2C8, 2C18 and 2C19 that binds covalently to liver microsomal protein [329,330]. This drug is very specific in binding to protein targets, unlike acute hepatotoxins such as APAP which bind to a large number of cellular proteins [303]. These specific protein targets (e.g., CYP) may then interact with the immunesystem, and somehow result in an autoimmune response and the appearance of anti-LKM2 antibodies [324,327,331,332].

6. Method validation in the analysis of drug–protein adducts

6.1. Specificity and sensitivity

Biological samples prepared for analysis of drug–protein adducts subjected to detachment and purification often contain small amounts of impurities. The impurities may interfere with the detection, identification and quantitation, and decreases sensitivity. Removing impurities from the samples is a good way of lowering background noise. This can be obtained by solid-phase extraction such as ion-exchange chromatography [103,104], size-exclusion chromatography [108–111], or affinity chromatography [92]. Adsorption of adducts to glass and other materials will also decrease sensitivity. If the adsorption is significant, correction should be made. Derivatization of hydrophilic groups in the adducts and silanization of glass ware may decrease adsorption and thus improve the sensitivity.

Detection of low levels of adducts, particularly in studies of background exposure in unexposed persons, may be difficult in some cases. For example, low levels of 2,6-dimethylaniline (2,6-DMA)–Hb adducts were observed in human subjects using GC–MS before lidocaine administration [187]. 2,6-DMA may be formed due to prior exposure to other aromatic diamino compounds. However, following administration of lidocaine, all patients have much higher levels of 2,6-DMA–hemoglobin adducts [187]. Most separation and detection methods for drug–protein adducts are based on specific physiochemical properties of the protein adducts themselves. For example, because of the difference in the pI and M_r of protein molecules, 2D gel electrophoresis is a powerful tool for the separation of adducted proteins from unmodified proteins.

HPLC may the most commonly used separation and quantitation technique for drug–protein adducts, in particular, for the detached drug moiety. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, to allow adequate separation and quantitation with acceptable sensitivity. In order to identify and quantitate any drug–protein adducts by HPLC a detector should be selected and is set to optimal detection settings, a separation assay then is developed. Fluorescence detection is sensitive and specific when applied to certain compounds [100,114,119,121,333]. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed.

Chromatographic methods (e.g., HPLC and LC–MS) appear to be more sensitive than immunological assays. For example, Western blot analysis only detected APAP–cysteine adducts in serum when ALT activity exceeded 6000 IU/l [334]. Analyzing these same serum using the HPLC with electrochemical detection, APAP–cycsteine adducts were detectable in serum of patients who overdosed with APAP even though the patients may not have had large increases in serum ALT levels [118]. Other immunological assays such as ELISA and immunochemistry have been developed and used in the analysis of and comparing the relative amounts of protein adducts of drugs such as APAP, these assays are not quantitative [78,80,81,249]. These assays have had limited clinical application since the antibodies are not readily available and the assay was relatively insensitive for the measurement of APAP–cysteine in human serum of APAP overdosed patients [118,334].

Immunological methods may be generally more sensitive than fluorography [335]. However, the specific sensitivity

will vary considerably among different protein adducts due to different affinities and accessibilities of the in vivo adducts to the serum used. It is possible that a protein adduct of low relative abundance could have been previously classified as high due to its high accessibility and affinity for the antibody used. However, such a particular adduct may not be detected by autoradiography due to possible low stoichiometry of modification.

Although LC–MS minimizes sample handling loss and maximizes efficiency of analyses, thus providing higher and improved sensitivity by a factor of >10, conventional LC–MS-ESI is always not sensitive enough for the detection of protein adducts. Therefore, the choice of interfaces coupled with LC–MS is very important for the detection of protein adducts at low levels in biological samples, and thus miniaturized LC–MS-ESI (nano- or capillary-LC with an I.D. of 75–300 μm) may offer more sensitive detection [336], since it has the advantages of reduced background noise and increased response. The use of column switching can also improve sensitivity. With this technique a compound can be analyzed on a miniaturized column after injection of a sample volume which is 10^3 times larger than would otherwise be possible [336].

MALDI is a sensitive technique for the determination of the M_r of peptides, proteins, oligonucleotides, and other compounds of biological origin as well as of small synthetic polymers. The amount of sample needed is very low (pmol or less). The analysis can be performed in the linear mode (high mass, low resolution) up to a m/z of 300 000 (in rare cases) or reflectron mode (lower mass, higher resolution) up to a M_r of 1000. However, the analysis is relatively insensitive to contaminants. Mass accuracy (0.1 to 0.01%) is not as high as for other MS methods. Recent development in delayed extraction TOF allows higher resolving power and mass accuracy. Some structural information for small molecules can be obtained in a "post-source decay" mode, or by collisional activation.

The major limitation in CE is the fact that relatively high concentrations of analyte are required, in other words there is a low concentration limit of detection. This is exacerbated in techniques such as CZE where the stacking effect created by the injection of the sample at an ionic strength much lower than that of the electrolyte is important not only to resolution, but also to sensitivity. This means that not only does the sample normally have to be diluted to achieve this, but also the presence of even moderate amounts of salt can lead to zone broadening and thus compromise the sensitivity. Limits of detection can be improved by increasing the length of the light path by introducing bends in the capillary or using capillaries with a rectangular cross-section. Interfacing between CE and MS has also been developed to improve sensitivity.

6.2. Accuracy and precision

Validating a newly developed analytical procedure is always required, which will confirm whether the procedure is reliable and comparable with established methods. The choice of standards (internal or reference) is of great importance for achieving acceptable precision and accuracy in the determination of drug–protein adducts. MS techniques allow stable isotopically labeled compounds to act as quantitative references in LC separations, providing the highest possible accuracy. A reference standard often consist of the protein/peptide adducts investigated, which are produced by in vitro systems [249,334]. However, the methods for the determination of protein adduct using adducted protein in vitro as a standard are often labor- and time-consuming. Therefore, internal standards corresponding to the detached adduct, modified amino acids or modified peptides are often used. For example, by using HPLC with electrochemical detection, the samples containing protein adducts are dialyzed and then digested with protease, and the APAP–cysteine conjugate is then quantified using tyrosine as an internal standard [118]. This method has good accuracy and precision with improved sensitivity (the lower limit of detection of the assay is approximately 3 pmol/mg of protein), and thus the assay may be useful in the diagnostic evaluation of patients who develop hepatotoxicity following therapeutic misadventures with APAP.

Acyl glucuronides of drugs display limited stability, which is dependent on pH, temperature, nature of the aglycon, and so on [42,43]. They are labile, undergo hydrolysis and pH-dependent intramolecular acyl migration to isomeric conjugates of glucuronic acid. Therefore, careful sample collection, handling, and storage procedures are critical to ensure generation of reliable pharmacologic and toxicologic data during clinical studies. Stabilization of acyl glucuronides can be obtained by lowering pH and temperature and addition of β-glucuronidase inhibitor [41–43]. Acyl glucuronides of drugs can be directly quantified in biologic specimens using chromatographic procedures, and their adducts with plasma or cell proteins can be determined after electrophoretic separation, followed by HPLC, LC–MS, immunoblotting, or ELISA techniques [42,43].

Both albumin and Hb have enzymatic activities [337,338], and thus artefactual formation of adducts during storage of protein samples or during work-up procedures is possible. For instance, the esterase-like property of albumin can convert prodrugs to active drugs in plasma. Optimal procedures for storage of protein adducts have not yet been determined with respect to prevention of oxidative and other chemical changes, but it is generally accepted that the proteins should be stored as precipitated albumin or globulin, as this will stabilize the adducts [10]. The prevention of artefactual formation of adducts is important, in particular when quantitating low levels of protein adducts. On the other hand, the storage of standards in solution may undergo degradation of protein/peptide adducts. In mechanistic studies of the N-alkyl Edman procedure it has been demonstrated that degradation of analytes may occur under alkaline conditions and in the presence of air, hydrolytic and oxidative processes during the detachment reaction [339].

The use of GC–MS often requires stable derivatives of the protein adducts by derivatization of functional groups. However, most protein adducts are always thermolabile, limiting the use of GC–MS in the analysis of protein adducts (cf. Section 4.1.2). Thus, most studies of protein adducts rely on LC–MS techniques which provide higher sensitivity and good reproductivity. It should be noted that the choice of MS interfaces is important to obtain good reproductivity. ESI allows production of molecular ions directly from samples in solution, which is used for small and large M_r macromolecules (peptides, proteins, carbohydrates, and DNA fragments), and lipids. Unlike MALDI, which is pulsed, ESI is a continuous ionization method that is suitable for using as an interface with HPLC or capillary electrophoresis. Multiply charged ions are usually produced. The sample must be soluble, stable in solution, polar, and relatively clean (free of nonvolatile buffers, detergents, and salts). ESI should be considered a complement to MALDI. Now MALDI-TOF-MS has advanced to the point of providing high sensitivity and mass resolution for analysis of unseparated digests as well as providing partial amino acid sequences for components in the low-fmol range from in-gel digestion of protein spots on 2D gel PAGE, particularly after the introduction of delayed extraction technology [94,96,163].

From an analytical point of view, 2D gel PAGE is unmatched in its ability to simultaneously resolve several thousand cellular proteins in a single sample (cf. Section 3.2.2) [77,140,340]. This enables identification of the major proteins in a tissue or subcellular fraction by MS methods. In addition, 2D gel PAGE can be used to compare quantities of proteins in related samples, such as those from exposure to drugs or from mutant and wild type, thus allowing the response of classes of proteins to be determined. Recently, with the maturation of technologies for immobilized pH gradient in the first dimension (isoelectric focusing), its resolution, loading capacity, and especially reproducibility have all been improved significantly [77,140].

7. Conclusions and future perspectives

Over the last few decades, there has been substantial progress in the development of analytical methodologies used in the studies of drug–protein adducts. They mainly include chromatographic, electrophoretic, and immunological methods, allowing the separation and identification of protein adducted by reactive drug metabolites, and the initial establishment of the relevance of covalent binding of drugs to proteins to organ toxicity [9,63,76,244,341]. However, these conventional approaches are always labor-intensive and involve subcellular prefractionation followed by several large-scale chromatographic steps to isolate a single pure protein for Edman amino acid sequence analysis (cf. Section 2.1). They are also of relatively low sensitivity, and poorly suited to dealing with the numbers of modified proteins involved. Recently, novel approaches by combining 2D SDS–PAGE with MS technologies have been developed and used in the analysis of drug–protein adducts (cf. Section 2.2). In the study of target proteins of APAP, total hepatic proteins from mice treated with radiolabeled APAP were separated by 2D SDS–PAGE, and spots containing radiolabel in gel was digested by trypsin followed by MS analysis [93]. Post-source decay of peptide adduct molecule ions was used to determine sequence information for peptides derived from the proteins. The obtained peptide masses and sequence information, together with protein database searching, led to the identification of 23 target proteins, including the two proteins previously identified by conventional methods over the past 10 years [93]. Since this novel approach incorporating analytical proteomic techniques has been recently validated, it is highly recommended. It is expected that the underlying mechanisms for the drug toxicity due to covalent binding to target proteins may be elucidated, as the whole suite of target proteins can be identified with the use of integrated proteomic approach.

The development of efficient and sensitive analytical methods for the separation, identification, quantification of drug–protein adducts have important clinical and toxicological implications [10,73–75]. These techniques allow the identification of drug–protein adducts and exploration of the mechanisms involved in the adduct-induced toxicity. The latter is largely unclear, but it is based on the so-called hapten hypothesis, which requires drug bioactivation, covalent binding to proteins, followed by uptake, antigen processing and a polyclonal immune response [69,70,342]. The recently proposed “danger hypothesis” by Pirmohamed and co-workers [342,343] can be considered to be additive to the hapten hypothesis, which hypothesizes that the immune system only responds to danger signals. Thus, stimulation of an immune response to a drug–protein adduct (signal 1) requires the presence of co-stimulatory signals and cytokines (signals 2 and 3), which propagate and determine the type of immune response. For example, in APAP toxicity, covalent binding of NAPQI to proteins (signal 1) may induce the production of nitric oxide (signal 2) due to the activation of Kupffer cells, which then scavenges superoxide to produce peroxynitrite (signal 3), which then causes protein nitration and tissue injury [45]. The nature of the danger signal is poorly defined, as some drug–protein adducts are not toxic in vivo. Further studies are required to elucidate how drug–protein adducts interact with cellular signal networks, leading to organ toxicity.

The identification of target proteins for drug reactive metabolites may provide an insight into the mechanisms for drug-induced toxicity. For example, patients treated with certain drugs may develop drug-induced hepatitis, which is characterized by the presence of autoantibodies against various CYPs [324,327,331,332,344,345]. The common individual drugs associated with drug-induced SLE are procainamide, dihydralazine, isoniazid [346], methyldopa, penicillamine, quinidine, sulfasalazine, some anticonvulsants, beta-blockers, and sulfonamides [347]. The drug-induced

SLE is characterized by arthralgia, myalgia, pleurisy, rashes and fever in association with antinuclear antibodies in the serum. The pathogenesis is unknown, but in many cases is thought to be due to covalent binding of reactive metabolites to histones or DNA, rendering them immunogenic [347,348]. Moreover, the drugs that are bioactivated by leucocyte MPO have been associated with drug-induced agranulocytosis and vasculitis [20,349]. Thyroid peroxidase can also oxidize some drugs to reactive metabolites, and this may be responsible for the thyroid autoimmunity observed in connection with some hypersensitivity reactions [350]. Peroxidases have also been observed in the skin and in platelets, and their presence may be responsible for the high incidence of skin reactions in the hypersensitivity response and the occurrence of immune-mediated thrombocytopenia, respectively [20]. However, the covalent binding of drug to important cellular proteins may be beneficial. For example, the covalent binding of reactive drug metabolites to albumin may prevent other important cellular proteins from the nucleophilic attack. In addition, the covalent binding of tubulin and microtubule associated proteins can be a important mechanism of the anticancer activity of some drugs [351].

The development of efficient and sensitive analytical methods for the separation, identification, quantification of drug–protein adducts will enable use to do clinical monitoring of drug exposure and risk assessment, and establish dose–toxicity relationships. For example, a simple and sensitive HPLC with electrochemical detection assay procedure has been validated and applied to the determination of NAPQI–cysteine adducts in the plasma of patients with APAP overdose [118,334,352]. This assay may provide a useful tool for the systematic study of APAP toxicity and for the diagnostic evaluation in patients who develop hepatotoxicity following toxic exposures or therapeutic misadventures with APAP. In addition, an immunoblot assay was used to monitor plasma adduct formation of acyl glucuronide of mycophenolic acid in pediatric renal transplant recipients [135]. The plasma adduct formation may serve as a marker for extended exposure of mycophenolic acid acyl glucuronide that has immunosuppressive and pro-inflammatory activity.

Unlike the studies of DNA adducts where there has been a well-established relationship between the covalent binding of chemical carcinogens to DNA and ensuing genotoxicity [72,277,353,354], it continues to be a challenge to elucidate the role of covalent binding of drugs via their reactive metabolites in the organ toxicity. It is well known that not all possible covalent modifications of proteins cause toxicities [355], particularly when metabolites,such as those of APAP, bind to so many proteins. For example, urate oxidase (a peroxisomal enzyme that catalyzes the oxidation of uric acid to allantoin in most mammals) was one of the major targets for NAPQI [93].However, this enzyme is lost during primate evolution. Because both humans and mice develop centrilobular liver necrosis with similar per kg doses of APAP, it is unlikely that covalent binding of urate oxidase plays a significant role in APAP toxicity in mice. Therefore, to elucidate the role of covalent binding in this toxicity, it will be essential to single out those protein adducts relevant to APAP toxicity. Drug binding covalently to a protein can lead to complex effects through the initiation of a series of cellular events. Therefore, implications of modifications of these proteins are indeed profound and certainly cannot be answered until the effect of formation of these adducts, not only on functions of these proteins directly modified but also on other related cellular functions, are characterized. The percentage of protein modified may be more important than the absolute amount of modification and the abundance of mammalian proteins varies from a few to hundreds of millions copies per cell, thus it is likely that some low abundance protein adducts not detected by currently available approaches may play important roles in organ toxicity.

The development of sensitive analytical methods for the analysis drug–protein adducts may play an important role in drug development [356]. It is currently impossible to accurately predict which new drugs will be associated with the formation of protein adducts via reactive metabolites, leading possible idiosyncratic drug reactions. However, by screening drug candidates for the formation of reactive metabolites and establishing structure–activity relationships it is possible to halt the development of drugs that adduct proteins and cause idiosyncratic hypersensitivity reactions at early stages [357,358]. A ligand–protein inverse docking approach has been used for computer-automated search of a protein cavity database to identify protein targets [356]. Application of computer approach can potentially facilitate the prediction of toxicity and side effect of a drug or drug lead. It appears that the chemical properties of a drug critical to covalent binding and idiosyncratic drug toxicity include formation of reactive metabolites; metabolism by CYPs; preponderance of CYP inducers, and occurrence of clinically significant pharmacokinetic interactions with co-administered drugs [358]. Although difficulties have been encountered, it is likely to predict the biological reactivity of drugs on the basis of its physico-chemical properties and constructed quantitative structure–activity relationships [359–364]. Moreover, the application of genomic and proteomic approaches to the study of drug–protein adducts has the potential to lead to a more effective screen due to their high throughput capacity. Microarray technologies have been used to study gene expression following treatment of liver toxins [365]. They may be a useful and highly sensitive technique for safety screening of drug candidates and for the classification of drugs that bind covalently to cellular proteins.

8. Nomenclature

ALT	Alanine aminotransferase
Anti-LKM2	Anti-liver kidney microsome
APAP	Acetaminophen

APCI	Atmospheric pressure chemical ionization
BSA	Bovine serum albumin
CBB	Coomassie blue
CE	Capillary electrophoresis
CE–MS	Capillary electrophoresis–mass spectrometry
CID	Collision-induced dissociation
COX	Cyclooxygenase
CYP	Cytochrome P450
CZE	Capillary zone electrophoresis
2,6-DMA	2,6-Dimethylaniline
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
ES	Electrospray
FAB	Fast atom bombardment
GC–MS	Gas chromatography–mass spectrometry
GSH	Glutathione
GST	Glutathione-*S*-transferase
Hb	Hemoglobin
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
LC–MS	Liquid chromatography–mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
M_r	Molecular mass
MPO	Myeperoxidase
NAC	*N*-Acetyl-L-cysteine
NAPQI	*N*-Acetyl-*p*-benzoquinoneimine
NAT	*N*-Acetyltransferase
NMR	Nuclear magnetic resonance
NSAIDs	Nonsteroid antiinflammatory drugs
p*I*	Isoelectric point
R_F	Ratio of the distance migrated by the molecule to that migrated by a marker dye-front
RIA	Radioimmunoassay
SLE	Systemic lupus erythematosus
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SULT	Sulfotransferase
TFA	Trifluroacetyl
UGT	Uridine diphosphate glucuronosyltransferase

Acknowledgements

The author appreciates the support of the National University of Singapore Academic Research Fund.

References

[1] U. Groth, H.G. Neumann, Chem. Biol. Interact. 4 (1972) 409.
[2] L.R. Pohl, H. Satoh, D.D. Christ, J.G. Kenna, Annu. Rev. Pharmacol. Toxicol. 28 (1988) 367.
[3] E.C. Miller, J.A. Miller, Cancer 47 (1981) 2327.
[4] S. Osterman-Golkar, L. Ehrenberg, D. Segerback, I. Hallstrom, Mut. Res. 34 (1976) 1.
[5] C.J. Calleman, L. Ehrenberg, B. Jansson, S. Osterman-Golkar, D. Segerback, K. Svensson, C.A. Wachtmeister, J. Environ. Pathol. Toxicol. 2 (1978) 427.
[6] D.J. Jollow, J.R. Mitchell, W.Z. Potter, D.C. Davis, J.R. Gillette, B.B. Brodie, J. Pharmacol. Exp. Ther. 187 (1973) 195.
[7] W.Z. Potter, D.C. Davis, J.R. Mitchell, D.J. Jollow, J.R. Gillette, B.B. Brodie, J. Pharmacol. Exp. Ther. 187 (1973) 203.
[8] W.Z. Potter, S.S. Thorgeirsson, D.J. Jollow, J.R. Mitchell, Pharmacology 12 (1974) 129.
[9] N.R. Pumford, N.C. Halmes, Annu. Rev. Pharmacol. Toxicol. 37 (1997) 91.
[10] M. Tornqvist, C. Fred, J. Haglund, H. Helleberg, B. Paulsson, P. Rydberg, J. Chromatogr. B 778 (2002) 279.
[11] A.S. Kalgutkar, D.K. Dalvie, J.P. O'Donnell, T.J. Taylor, D.C. Sahakian, Curr. Drug Metab. 3 (2002) 378.
[12] F.P. Guengerich, D.C. Liebler, Crit. Rev. Toxicol. 14 (1985) 259.
[13] O. Pelkonen, H. Raunio, Environ. Health Perspect. 105 (1997) 767.
[14] O. Pelkonen, Drug Metab. Rev. 34 (2002) 37.
[15] S. Rendic, F.J. Di Carlo, Drug Metab. Rev. 29 (1997) 413.
[16] S. Rendic, Drug Metab. Rev. 34 (2002) 83.
[17] P. Hlavica, Drug Metab. Rev. 34 (2002) 451.
[18] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui, F.P. Guengerich, J. Pharmacol. Exp. Ther. 270 (1994) 414.
[19] O. Pelkonen, A.R. Boobis, P. Kremers, M. Ingelman-Sundberg, A. Rane, in: G.M. Pacifici, O. Pelkonen (Eds.), Interindividual Variability in Human Drug Metabolism, Taylor and Francis, London, 2001, p. 269.
[20] J. Uetrecht, Crit. Rev. Toxicol. 20 (1990) 213.
[21] P.J. O'Brien, Chem. Biol. Interact. 129 (2000) 113.
[22] S. Kubow, P.G. Wells, Mol. Pharmacol. 35 (1989) 504.
[23] J. Uetrecht, N. Zahid, Chem. Res. Toxicol. 1 (1988) 148.
[24] D.C. Mays, L.J. Pawluk, G. Apseloff, W.B. Davis, Z.W. She, A.L. Sagone, N. Gerber, Biochem. Pharmacol. 50 (1995) 367.
[25] S.M. Furst, J.P. Uetrecht, Biochem. Pharmacol. 45 (1993) 1267.
[26] W.G. Lai, N. Zahid, J.P. Uetrecht, J. Pharmacol. Exp. Ther. 291 (1999) 292.
[27] A.J. Nappi, E. Vass, J. Biol. Chem. 276 (2001) 11214.
[28] J. Uetrecht, N. Zahid, A. Tehim, J.M. Fu, S. Rakhit, Chem. Biol. Interact. 104 (1997) 117.
[29] D.P. Williams, M. Pirmohamed, D.J. Naisbitt, J.L. Maggs, B.K. Park, J. Pharmacol. Exp. Ther. 283 (1997) 1375.
[30] I. Gardner, J.S. Leeder, T. Chin, N. Zahid, J.P. Uetrecht, Mol. Pharmacol. 53 (1998) 999.
[31] W.G. Lai, I. Gardner, N. Zahid, J.P. Uetrecht, Drug Metab. Dispos. 28 (2000) 255.
[32] J.P. Uetrecht, N. Zahid, Chem. Res. Toxicol. 4 (1991) 218.
[33] G. Miyamoto, N. Zahid, J.P. Uetrecht, Chem. Res. Toxicol. 10 (1997) 414.
[34] B.C. Sallustio, L. Sabordo, A.M. Evans, R.L. Nation, Curr. Drug Metab. 1 (2000) 163.
[35] J.K. Ritter, Chem. Biol. Interact. 129 (2000) 171.
[36] H. Spahn-Langguth, M. Dahms, A. Hermening, Adv. Exp. Med. Biol. 387 (1996) 313.
[37] H. Glatt, Chem. Biol. Interact. 129 (2000) 141.
[38] H. Glatt, H. Boeing, C.E. Engelke, L. Ma, A. Kuhlow, U. Pabel, D. Pomplun, W. Teubner, W. Meinl, Mutat. Res. 482 (2001) 27.
[39] N. Terrier, E. Benoit, C. Senay, F. Lapicque, A. Radominska-Pandya, J. Magdalou, S. Fournel-gigleux, Mol. Pharmacol. 56 (1999) 226.
[40] U.A. Boelsterli, Curr. Drug Metab. 14 (2002) 439.
[41] S.F. Zhou, J.W. Paxton, M.D. Tingle, P. Kestell, M.B. Jameson, P.I. Thomson, B.C. Baguley, Xenobiotica 31 (2001) 277.
[42] M. Shipkova, V.W. Armstrong, M. Oellerich, E. Wieland, Ther. Drug Monit. 25 (2003) 1.
[43] H.K. Kroemer, U. Klotz, Clin. Pharmacokinet. 23 (1992) 292.

[44] H. Glatt, C.E. Engelke, U. Pabel, W. Teubner, A.L. Jones, M.W. Coughtrie, U. Andrae, C.N. Falany, W. Meinl, Toxicol. Lett. 112–113 (2000) 341.
[45] H. Jaeschke, G.J. Gores, A.I. Cederbaum, J.A. Hinson, D. Pessayre, J.J. Lemasters, Toxicol. Sci. 65 (2002) 166.
[46] S. Buratti, J.E. Lavine, Curr. Opin. Pediatr. 14 (2002) 601.
[47] Z.D. Goodman, Clin. Liver Dis. 6 (2002) 381.
[48] H.J. Zimmerman, Clin. Liver Dis. 4 (2000) 73.
[49] N. Kaplowitz, Drug Safety 24 (2001) 483.
[50] D.R. Krishna, U. Klotz, Clin. Pharmacokinet. 26 (1994) 144.
[51] B. Burchell, C.H. Brierley, G. Monaghan, D.J. Clarke, Adv. Pharmacol. 42 (1998) 335.
[52] J.W. Lohr, G.R. Willsky, M.A. Acara, Pharmacol. Rev. 50 (1998) 107.
[53] M.B. Fisher, M.F. Paine, T.J. Strelevitz, S.A. Wrighton, Drug Metab. Rev. 33 (2001) 273.
[54] X. Ding, L.S. Kaminsky, Annu. Rev. Pharmacol. Toxicol. 43 (2003) 149.
[55] F.P. Guengerich, H. Cai, W.W. Johnson, A. Parikh, Adv. Exp. Med. Biol. 500 (2001) 639.
[56] A. Casini, A. Scozzafava, C.T. Supuran, Environ. Health Perspect. 110 (2002) 801.
[57] M. Sogami, S. Nagoka, S. Era, M. Honda, K. Noguchi, Int. J. Pept. Protein Res. 24 (1984) 96.
[58] H.A. Petersen, J.F. Foster, J. Biol. Chem. 240 (1965) 3858.
[59] A. Shimizu, T. Nakanishi, M. Kishikawa, A. Miyazaki, J. Chromatogr. B 776 (2002) 15.
[60] M. Tornqvist, J. Mowrer, S. Jensen, L. Ehrenberg, Anal. Biochem. 154 (1986) 255.
[61] P.L. Skipper, S.R. Tannenbaum, Prog. Clin. Biol. Res. 340C (1990) 301.
[62] P.L. Skipper, Chem. Res. Toxicol. 9 (1996) 918.
[63] N.R. Pumford, N.C. Halmes, J.A. Hinson, Drug Metab. Rev. 29 (1997) 39.
[64] P. Reichardt, A. Schreiber, G. Wichmann, G. Metzner, J. Efer, F. Raabe, Environ. Toxicol. 18 (2003) 29.
[65] A. Heine, G. DeSantis, J.G. Luz, M. Mitchell, C.H. Wong, I.A. Wilson, Science 294 (2001) 369.
[66] M.B. Yim, H.S. Yim, C. Lee, S.O. Kang, P.B. Chock, Ann. N. Y. Acad. Sci. 928 (2001) 48.
[67] S. Chitturi, J. George, Semin. Liver Dis. 22 (2002) 169.
[68] S.D. Cohen, N.R. Pumford, E.A. Khairallah, K. Boekelheide, L.R. Pohl, H.R. Amouzadeh, J.A. Hinson, Toxicol. Appl. Pharmacol. 143 (1997) 1.
[69] D.J. Naisbitt, D.P. Williams, M. Pirmohamed, N.R. Kitteringham, B.K. Park, Curr. Opin. Allergy Clin. Immunol. 1 (2001) 317.
[70] C. Ju, J.P. Uetrecht, Curr. Drug Metab. 3 (2002) 367.
[71] P.M. Dansette, E. Bonierbale, C. Minoletti, P.H. Beaune, D. Pessayre, D. Mansuy, Eur. J. Drug Metab. Pharmacokinet. 23 (1998) 443.
[72] U.A. Boelsterli, Drug Metab. Rev. 25 (1993) 395.
[73] H. Greim, Adv. Exp. Med. Biol. 500 (2001) 715.
[74] M.C. Poirier, R.M. Santella, A. Weston, Carcinogenesis 21 (2000) 353.
[75] R.T. van Welie, R.G. van Dijck, N.P. Vermeulen, N.J. van Sittert, Crit. Rev. Toxicol. 22 (1992) 271.
[76] D.C. Liebler, Environ. Health Perspect. 110 (2002) 3.
[77] M. Hamdan, M. Galvani, P.G. Righetti, Mass Spectrom. Rev. 20 (2001) 121.
[78] J.B. Bartolone, K. Sparks, S.D. Cohen, E.A. Khairallah, Biochem. Pharmacol. 36 (1987) 1193.
[79] D.W. Roberts, N.R. Pumford, D.W. Potter, R.W. Benson, J.A. Hinson, J. Pharmacol. Exp. Ther. 241 (1987) 727.
[80] N.R. Pumford, J.A. Hinson, D.W. Potter, K.L. Rowland, R.W. Benson, D.W. Roberts, J. Pharmacol. Exp. Ther. 248 (1989) 190.
[81] N.R. Pumford, J.A. Hinson, R.W. Benson, D.W. Roberts, Toxicol. Appl. Pharmacol. 104 (1990) 521.
[82] J.L. Martin, J.G. Kenna, B.M. Martin, D. Thomassen, G.F. Reed, L.R. Pohl, Hepatology 18 (1993) 858.
[83] J.L. Martin, N.R. Pumford, A.C. LaRosa, B.M. Martin, H.M. Gonzaga, M.A. Beaven, L.R. Pohl, Biochem. Biophys. Res. Commun. 178 (1991) 679.
[84] M. Bourdi, D. Demady, J.L. Martin, S.K. Jabbour, B.M. Martin, J.W. George, L.R. Pohl, Arch. Biochem. Biophys. 323 (1995) 397.
[85] H. Satoh, B.M. Martin, A.H. Schulick, D.D. Christ, J.G. Kenna, L.R. Pohl, Proc. Natl. Acad. Sci. USA 86 (1989) 322.
[86] L.E. Butler, D. Thomassen, J.L. Martin, B.M. Martin, J.G. Kenna, L.R. Pohl, Chem. Res. Toxicol. 5 (1992) 406.
[87] N.R. Pumford, B.M. Martin, D. Thomassen, J.A. Burris, J.G. Kenna, J.L. Martin, L.R. Pohl, Chem. Res. Toxicol. 6 (1993) 609.
[88] E. Eliasson, J.G. Kenna, Mol. Pharmacol. 50 (1996) 573.
[89] D.B. Njoku, R.S. Greenberg, M. Bourdi, C.B. Borkowf, E.M. Dake, J.L. Martin, L.R. Pohl, Anesth. Analg. 94 (2002) 243.
[90] D.K. Spracklin, D.C. Hankins, J.M. Fisher, K.E. Thummel, E.D. Kharasch, J. Pharmacol. Exp. Ther. 281 (1997) 400.
[91] G.C. Smith, J.G. Kenna, D.J. Harrison, D. Tew, C.R. Wolf, Lancet 342 (1993) 963.
[92] A.P. Brown, A.J. Gandolfi, Toxicology 89 (1994) 35.
[93] Y.C. Qiu, L.Z. Benet, A.L. Burlingame, J. Biol. Chem. 273 (1998) 17940.
[94] V. Soskic, J. Godovac-Zimmermann, Proteomics 1 (2001) 1364.
[95] C.H. Wheeler, S.L. Berry, M.R. Wilkins, J.M. Corbett, K. Ou, A.A. Gooley, I. Humphery-Smith, K.L. Williams, M.J. Dunn, Electrophoresis 17 (1996) 580.
[96] H. Katayama, T. Nagasu, Y. Oda, Rapid Commun. Mass Spectrom. 15 (2001) 1416.
[97] H. Badghisi, D.C. Liebler, Chem. Res. Toxicol. 15 (2002) 799.
[98] K.L. O'Connell, J.T. Stults, Electrophoresis 18 (1997) 349.
[99] D.S. Hage, J. Austin, J. Chromatogr. B 739 (2000) 39.
[100] I. Miksik, Z. Deyl, J. Chromatogr. B 699 (1997) 311.
[101] S. Gupta, E. Pfannkoch, F.E. Regnier, Anal. Biochem. 128 (1983) 196.
[102] W. Kopaciewicz, F.E. Regnier, Anal. Biochem. 133 (1983) 251.
[103] N.R. Pumford, B.M. Martin, J.A. Hinson, Biochem. Biophys. Res. Commun. 182 (1992) 1348.
[104] N.R. Pumford, N.C. Halmes, B.M. Martin, R.J. Cook, C. Wagner, J.A. Hinson, J. Pharmacol. Exp. Ther. 280 (1997) 501.
[105] R. Narazaki, K. Harada, A. Sugii, M. Otagiri, J. Pharm. Sci. 86 (1997) 215.
[106] G.R. Cannell, M.J. Bailey, R.G. Dickinson, Life Sci. 71 (2002) 2633.
[107] F.E. Regnier, Methods Enzymol. 91 (1983) .
[108] Y. Xu, A.H. Stokes, R.J. Roskoski, K.E. Vrana, J. Neurosci. Res. 54 (1998) 691.
[109] D.M. Kuhn, R.E.J. Arthur, D.M. Thomas, L.A. Elferink, J. Neurochem. 73 (1999) 1309.
[110] E. Caneva, P. Di Gennaro, F. Farina, M. Orlandi, B. Rindone, P. Falagiani, Bioconjug. Chem. 4 (1993) 309.
[111] E. Bolzacchini, V. Consonni, P. Gramatica, S. Meinardi, M. Orlandi, B. Rindone, R. Todeschini, M. Blanca, C. Mayorga, Bioconjug. Chem. 10 (1999) 332.
[112] A. Ding, J.C. Ojingwa, A.F. McDonagh, A.L. Burlingame, L.Z. Benet, Proc. Natl. Acad. Sci. USA 90 (1993) 3797.
[113] M.J. Raftery, C.L. Geczy, J. Am. Soc. Mass Spectrom. 13 (2002) 709.
[114] A.J. Munns, J.J. De Voss, W.D. Hooper, R.G. Dickinson, E.M. Gillam, Chem. Res. Toxicol. 10 (1997) 1049.
[115] Y. Qiu, A.L. Burlingame, L.Z. Benet, Drug Metab. Dispos. 26 (1998) 246.
[116] A. Ding, P. Zia-Amirhosseini, A.F. McDonagh, A.L. Burlingame, L.Z. Benet, Drug Metab. Dispos. 23 (1995) 369.
[117] L.L. Koenigs, R.M. Peter, A.P. Hunter, R.L. Haining, A.E. Rettie, T. Friedberg, M.P. Pritchard, M. Shou, T.H. Rushmore, W.F. Trager, Biochemistry 38 (1999) 2312.

[118] K.L. Muldrew, L.P. James, L. Coop, S.S. McCullough, H.P. Hendrickson, J.A. Hinson, P.R. Mayeux, Drug Metab. Dispos. 30 (2002) 446.
[119] A. Hermening, A.K. Grafe, G. Baktir, E. Mutschler, H. Spahn-Langguth, J. Chromatogr. B 741 (2000) 129.
[120] M. Iwaki, T. Ogiso, S. Inagawa, K. Kakehi, J. Pharm. Sci. 88 (1999) 52.
[121] S.F. Zhou, P. Kestell, M.D. Tingle, J.W. Paxton, J. Chromatogr. B 757 (2001) 343.
[122] M. Castillo, Y.W.F. Lam, M.A. Dooley, E. Stahl, P.C. Smith, Clin. Pharmacol. Ther. 57 (1995) 636.
[123] M. Castillo, P.C. Smith, Drug Metab. Dispos. 23 (1995) 566.
[124] P.C. Smith, J.H. Liu, Xenobiotica 23 (1993) 337.
[125] G.E. McKinnon, R.G. Dickinson, Res. Commun. Chem. Pathol. Pharmacol. 66 (1989) 339.
[126] H. Georges, I. Jarecki, P. Netter, J. Magdalou, F. Lapicque, Life Sci. 65 (1999) PL151.
[127] K.J. Hoffmann, A.J. Streeter, D.B. Axworthy, T.A. Baillie, Chem. Biol. Interact. 53 (1985) 155.
[128] J.A. Hinson, D.W. Roberts, N.C. Halmes, J.D. Gibson, N.R. Pumford, Adv. Exp. Med. Biol. 387 (1996) 47.
[129] J.A. Hinson, S.L. Michael, S.G. Ault, N.R. Pumford, Toxicol. Sci. 53 (2000) 467.
[130] A.M. Matthews, D.W. Roberts, J.A. Hinson, N.R. Pumford, Drug Metab. Dispos. 24 (1996) 1192.
[131] D.J. Hoivik, J.E. Manautou, A. Tveit, D.C. Mankowski, E.A. Khairallah, S.D. Cohen, Fundam. Appl. Toxicol. 32 (1996) 79.
[132] H. Satoh, J.R. Gillette, H.W. Davies, R.D. Schulick, L.R. Pohl, Mol. Pharmacol. 28 (1985) 468.
[133] J.A. Ware, M.L. Graf, B.M. Martin, L.R. Lustberg, L.R. Pohl, Chem. Res. Toxicol. 11 (1998) 164.
[134] A.E. Cribb, C.E. Nuss, D.W. Alberts, D.B. Lamphere, D.M. Grant, S.J. Grossman, S.P. Spielberg, Chem. Res. Toxicol. 9 (1996) 500.
[135] M. Shipkova, V.W. Armstrong, L. Weber, P.D. Niedmann, E. Wieland, J. Haley, B. Tonshoff, M. Oellerich, Ther. Drug Monit. 24 (2002) 390.
[136] S. Seitz, A. Kretzrommel, R.P.J.O. Elferink, U.A. Boelsterli, Chem. Res. Toxicol. 11 (1998) 513.
[137] Y. Masubuchi, S. Igarashi, T. Suzuki, T. Horie, S. Narimatsu, J. Pharmacol. Exp. Ther. 279 (1996) 724.
[138] A. Görg, C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber, W. Weiss, Electrophoresis 21 (2000) 1037.
[139] A. Görg, W. Postel, S. Gunther, Electrophoresis 9 (1998) 531.
[140] K.S. Lilley, A. Razzaq, P. Dupree, Curr. Opin. Chem. Biol. 6 (2002) 46.
[141] S.E. Ong, A. Pandey, Biomol. Eng. 18 (2001) 195.
[142] N.L. Anderson, D.C. Copple, R.A. Bendele, G.S. Probst, F.C. Richardson, Fundam. Appl. Toxicol. 18 (1992) 570.
[143] M.L. Cunningham, L.L. Pippin, N.L. Anderson, M.L. Wenk, Toxicol. Appl. Pharmacol. 131 (1995) 216.
[144] F.C. Richardson, S.C. Strom, D.M. Copple, R.A. Bendele, G.S. Probst, N.L. Anderson, Electrophoresis 14 (1993) 157.
[145] M. Ohshima, J.M. Ward, L.M. Brennan, D.A. Creasia, J. Natl. Cancer Inst. 72 (1984) 759.
[146] C.Y. Liu, Electrophoresis 22 (2001) 612.
[147] C. Gelfi, A. Vigano, M. Ripamonti, P.G. Righetti, R. Sebastiano, A. Citterio, Anal. Chem. 73 (2001) 3862.
[148] C.L. Colyer, S.D. Mangru, D.J. Harrison, J. Chromatogr. A 781 (1997) 271.
[149] Y. Ishihama, T. Miwa, N. Asakawa, Electrophoresis 23 (2002) 951.
[150] H.S. Kim, J. Austin, D.S. Hage, Electrophoresis 23 (2002) 956.
[151] P.A. Wijnen, M.P. Van Diejen-Visser, Eur. J. Clin. Chem. Clin. Biochem. 34 (1996) 535.
[152] W.A. Apruzzese, P. Vouros, J. Chromatogr. A 794 (1998) 97.
[153] C.B. Norwood, E. Jackim, S. Cheer, Anal. Biochem. 213 (1993) 194.
[154] P. Thibault, C. Paris, S. Pleasance, Rapid Commun. Mass Spectrom. 5 (1991) 484.
[155] P. Cao, M. Moini, J. Am. Soc. Mass Spectrom. 9 (1998) 1081.
[156] J.H. Wahl, D.C. Gale, R.D. Smith, J. Chromatogr. A 659 (1994) 217.
[157] P.G. Righetti, C. Gelfi, A. Bossi, E. Olivieri, L. Castelletti, B. Verzola, A.V. Stoyanov, Electrophoresis 21 (2000) 4046.
[158] K. Sei, M. Nakano, M. Kinoshita, T. Masuko, K. Kakehim, J. Chromatogr. A 958 (2002) 273.
[159] M. Sugano, H. Hidaka, K. Yamauchi, T. Nakabayashi, Y. Higuchi, K. Fujita, N. Okumura, Y. Ushiyama, M. Tozuka, T. Katsuyama, Electrophoresis 21 (2000) 3016.
[160] T. Liu, X.X. Shao, R. Zeng, Q.C. Xia, J. Chromatogr. A 855 (1999) 695.
[161] P. Lind, M. Dalene, V. Lindstrom, A. Grubb, G. Skarping, Analyst 122 (1997) 151.
[162] K. Biemann, Annu. Rev. Biochem. 61 (1992) 977.
[163] P. Roepstorff, EXS 88 (2000) 81.
[164] M.M. Siegel, Curr. Top. Med. Chem. 2 (2002) 13.
[165] J. Qin, B.T. Chait, Anal. Chem. 69 (1997) 4002.
[166] K. Biemann, Protein Sci. 4 (1995) 1920.
[167] J.V. Iribame, B.A. Thomson, J. Chem. Phys. 64 (1976) 2287.
[168] P. Kebarle, J. Mass Spectrom. 35 (2000) 804.
[169] L.K. Lightning, W.F. Trager, Methods Enzymol. 357 (2002) 296.
[170] D.J. Naisbitt, D.P. Williams, P.M. O'Neill, J.L. Maggs, D.J. Willock, M. Pirmohamed, B.K. Park, Chem. Res. Toxicol. 11 (1998) 1586.
[171] A. Basile, P. Ferranti, G. Pocsfalvi, G. Mamone, N. Miraglia, S. Caira, L. Ambrosi, L. Soleo, N. Sannolo, A. Malorni, Rapid Commun. Mass Spectrom. 15 (2001) 527.
[172] H. Helleberg, M. Tornqvist, Rapid Commun. Mass Spectrom. 14 (2000) 1644.
[173] W. Tang, R.A. Stearns, R.W. Wang, S.H. Chiu, T.A. Baillie, Chem. Res. Toxicol. 12 (1999) 192.
[174] S. Bolze, N. Bromet, C. Gay-Feutry, F. Massiere, R. Boulieu, T. Hulot, Drug Metab. Dispos. 30 (2002) 404.
[175] K. Biemann, J. Am. Soc. Mass Spectrom. 13 (2002) 1254.
[176] D.F. Hunt, J.R.r. Yates, J. Shabanowitz, S. Winston, C.R. Hauer, Proc. Natl. Acad. Sci. USA 83 (1986) 6233.
[177] M.S. Bolgar, S.J. Gaskell, Biochem. Soc. Trans. 23 (1995) 907.
[178] P. Zia-Amirhosseini, A. Ding, A.L. Burlingame, A.F. McDonagh, L.Z. Benet, Biochem. J. 311 (1995) 431.
[179] M. Tornqvist, Methods Enzymol. 231 (1994) 650.
[180] S.S. Hecht, S.G. Carmella, S.E. Murphy, Methods Enzymol. 231 (1994) 657.
[181] R. Pastorelli, R. Allevi, S. Romagnano, G. Meli, R. Fanelli, L. Airoldi, Arch. Toxicol. 69 (1995) 306.
[182] A. Pahler, J. Parker, W. Dekant, Toxicol. Sci. 48 (1999) 5.
[183] G. Boysen, S.S. Hecht, Mutat. Res. 543 (2003) 17.
[184] S. Waidyanatha, M.A. Troester, A.B. Lindstrom, S.M. Rappaport, Chem. Biol. Interact. 141 (2002) 189.
[185] W. Volkel, A. Pahler, W. Dekant, J. Chromatogr. A 847 (1999) 35.
[186] G.R. Lambert, W.T. Padgett, M.H. George, K.T. Kitchin, S. Nesnow, Anal. Biochem. 268 (1999) 289.
[187] M.S. Bryant, H.F. Simmons, R.E. Harrell, J.A. Hinson, Carcinogenesis 15 (1994) 2287.
[188] R.D. Smith, D.R. Goodett, J.H. Wahl, J.P. Landess (Eds.), CRC Handbook of Capillary Electrophoresis (1993) 177CRC PressBoca Raton, FL.
[189] R.M. Caprioli, W.T. Moore, M. Martin, K.B. Wilson, S. Moring, J. Chromatogr. 480 (1989) 247.
[190] A. Zamfir, D.G. Seidler, H. Kresse, J. Peter-Katalinic, Rapid Commun. Mass Spectrom. 16 (2002) 2015.
[191] P.T. Jedrzejewski, W.D. Lehmann, Anal. Chem. 69 (1997) 294.
[192] C.L. Andrews, P. Vouros, A. Harsch, J. Chromatogr. A 856 (1999) 515.
[193] L.A. Marzilli, C. Koertje, P. Vouros, Methods Mol. Biol. 162 (2001) 395.

[194] J. Ding, P. Vouros, J. Chromatogr. A 887 (2000) 103.
[195] J.P. Barry, C. Norwood, P. Vouros, Anal. Chem. 68 (1996) 1432.
[196] M. Pellecchia, D.S. Sem, K. Wuthrich, Nat. Rev. Drug Discov. 1 (2002) 211.
[197] G.M. Clore, C.D. Schwieters, Curr. Opin. Struct. Biol. 12 (2002) 146.
[198] M.C. Summers, Prog. Clin. Biol. Res. 183 (1985) 39.
[199] D.J. Craik, J.A. Wilce, Methods Mol. Biol. 60 (1997) 195.
[200] D.L. Purich, Methods Enzymol. 354 (2002) 1.
[201] H. Fu, J. Park, D. Pei, Biochemistry 41 (2002) 10700.
[202] I.D. Wilson, J. Chromatogr. A 892 (2000) 315.
[203] K.I. Burton, J.R. Everett, M.J. Newman, F.S. Pullen, D.S. Richards, A.G. Swanson, J. Pharm. Biomed. Anal. 15 (1997) 1903.
[204] G.J. Dear, R.S. Plumb, B.C. Sweatman, J. Ayrton, J.C. Lindon, J.K. Nicholson, I.M. Ismail, J. Chromatogr. B 748 (2000) 281.
[205] R.M. Holt, M.J. Newman, F.S. Pullen, D.S. Richards, A.G. Swanson, J. Mass Spectrom. 32 (1997) 64.
[206] J.C. Lindon, J.K. Nicholson, I.D. Wilson, J. Chromatogr. B 748 (2000) 233.
[207] G.J. Mulder, F.F. Kadlubar, J.B. Mays, J.A. Hinson, Mol. Pharmacol. 26 (1984) 342.
[208] Z.C. Liu, J.P. Uetrecht, J. Pharmacol. Exp. Ther. 275 (1995) 1476.
[209] J.A. Hinson, J.B. Mays, J. Pharmacol. Exp. Ther. 238 (1986) 106.
[210] J.A. Hinson, J.B. Mays, Adv. Exp. Med. Biol. 197 (1986) 691.
[211] K. Kobayashi, M. Nakajima, K. Oshima, N. Shimada, T. Yokoi, K. Chiba, Drug Metab. Dispos. 27 (1999) 860.
[212] K. Akira, T. Uchijima, T. Hashimoto, Chem. Res. Toxicol. 15 (2002) 765.
[213] K. Akira, T. Taira, H. Hasegawa, C. Sakuma, Y. Shinohara, Drug Metab. Dispos. 26 (1998) 457.
[214] O. Corcoran, R.W. Mortensen, S.H. Hansen, J. Troke, J.K. Nicholson, Chem. Res. Toxicol. 14 (2001) 1363.
[215] J. Hasegawa, P.C. Smith, L.Z. Benet, Drug Metab. Dispos. 10 (1982) 469.
[216] D. Wild, The Immunoassay Handbook, Nature Publishing Group, New York, 2001.
[217] H. Satoh, Y. Fukuda, D.K. Anderson, V.J. Ferrans, J.R. Gillette, L.R. Pohl, J. Pharmacol. Exp. Ther. 233 (1985) 857.
[218] J.W. Coleman, J.H. Yeung, D.H. Roberts, A.M. Breckenridge, B.K. Park, Br. J. Clin. Pharmacol. 22 (1986) 161.
[219] R. Narazaki, H. Watanabe, T. Maruyama, A. Suenaga, M. Otagiri, Arch. Toxicol. 72 (1998) 203.
[220] J. Chesham, G.E. Davies, Clin. Exp. Immunol. 61 (1985) 224.
[221] E. Bonierbale, P. Valadon, C. Pons, B. Desfosses, P.M. Dansette, D. Mansuy, Chem. Res. Toxicol. 12 (1999) 286.
[222] Y. Masubuchi, C. Takahashii, N. Fujio, T. Horie, T. Suzuki, S. Imaoka, Y. Funae, S. Narimatsu, Drug Metab. Dispos. 23 (1995) 999.
[223] W.A. Daniel, A. Haduch, J. Wojcikowski, J. Pharm. Pharmacol. 54 (2002) 1545.
[224] C.M. McNeil, M. Murray, Biochem. Pharmacol. 51 (1996) 15.
[225] M.T. Bush, J.D. Alvin, Ann. N. Y. Acad. Sci. 26 (1973) 36.
[226] J. Oravcova, B. Bohs, W. Lindner, J. Chromatogr. B 677 (1996) 1.
[227] R.E. Gibson, H.D. Burns, T.G. Hamill, W.S. Eng, B.E. Francis, C. Ryan, Curr. Pharm. Res. 6 (2000) 973.
[228] G. Morovjan, B. Dalmadi-Kiss, I. Klebovich, E. Mincsovics, J. Chromatogr. Sci. 40 (2002) 603.
[229] A. Wendel, P. Cikryt, Res. Commun. Chem. Pathol. Pharmacol. 33 (1981) 463.
[230] L. Zhou, B.A. McKenzie, E.D.J.r. Eccleston, S.P. Srivastava, N. Chen, R.R. Erickson, J.L. Holtzman, Chem. Res. Toxicol. 9 (1996) 1176.
[231] D.B. Axworthy, K.J. Hoffmann, A.J. Streeter, C.J. Calleman, G.A. Pascoe, T.A. Baillie, Chem. Biol. Interact. 68 (1988) 99.
[232] K.J. Hoffmann, A.J. Streeter, D.B. Axworthy, T.A. Baillie, Mol. Pharmacol. 27 (1985) 566.
[233] A. Kretz-Rommel, U.A. Boelsterli, Drug Metab. Dispos. 22 (1994) 956.
[234] P. Letteron, V. Descatoire, M. Tinel, P. Maurel, G. Labbe, J. Loeper, D. Larrey, E. Freneaux, D. Pessayre, Biochem. Pharmacol. 38 (1989) 3241.
[235] J. Uetrecht, B. Sokoluk, Drug Metab. Dispos. 20 (1992) 120.
[236] C. Mani, R. Pearce, A. Parkinson, D. Kupfer, Carcinogenesis 15 (1994) 2715.
[237] M.C. Poirier, A. Weston, S. Gupta-Burt, E. Reed, Basic Life Sci. 53 (1990) 1.
[238] R.M. Santella, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 733.
[239] S. Nagayama, T. Yokoi, Y. Kawaguchi, T. Kamataki, J. Toxicol. Sci. 19 (1994) 155.
[240] F.Z. Sheabar, J.D. Groopman, G.S. Qian, G.N. Wogan, Carcinogenesis 14 (1993) 1203.
[241] K. Brune, Med. Toxicol. 1 (1986) 1.
[242] R. Botting, J. Physiol. Pharmacol. 51 (2000) 609.
[243] R.M. Botting, Clin. Infect. Dis. 31 (2000) S202.
[244] J.A. Hinson, N.R. Pumford, D.W. Roberts, Drug Metab. Rev. 27 (1995) 72.
[245] J.A. Hinson, L.R. Pohl, T.J. Monks, J.R. Gillette, Life Sci. 29 (1981) 107.
[246] A. Jones, Am. J. Ther. 9 (2002) 245.
[247] J.G.M. Bessems, N.P.E. Vermeulen, Crit. Rev. Toxicol. 31 (2001) 55.
[248] S.D. Cohen, E.A. Khairallah, Drug Metab. Rev. 29 (1997) 59.
[249] J.B. Bartolone, R.B. Birge, S.J. Bulera, M.K. Bruno, E.V. Nishanian, S.D. Cohen, E.A. Khairallah, Toxicol. Appl. Pharmacol. 113 (1992) 19.
[250] S.J. Bulera, R.B. Birge, S.D. Cohen, E.A. Khairallah, Toxicol. Appl. Pharmacol. 134 (1995) 313.
[251] M. Hong, S.D. Cohen, E.A. Khairallah, Toxicologist 427 (1994) .
[252] S. Gupta, L.K. Rogers, C.V. Smith, Toxicologist 15 (1995) 153.
[253] N.C. Halmes, J.A. Hinson, B.M. Martin, N.R. Pumford, Chem. Res. Toxicol. 9 (1996) 541.
[254] J.S. Landin, S.D. Cohen, E.A. Khairallah, Toxicol. Appl. Pharmacol. 141 (1996) 299.
[255] C. Adessi, C. Miege, C. Albrieux, T. Rabilloud, Electrophoresis 18 (1997) 127.
[256] N.H. Shear, S.P. Spielberg, J. Clin. Invest. 82 (1988) 1826.
[257] S.P. Spielberg, G.B. Gordon, D.A. Blake, E.D. Mellits, D.S. Bross, J. Pharmacol. Exp. Ther. 217 (1981) 386.
[258] C.C. Vittorio, J.J. Muglia, Arch. Intern. Med. 155 (1995) 2285.
[259] J.S. Leeder, Epilepsia 39 (1998) S8.
[260] Y. Masubuchi, T. Nakano, A. Ose, T. Horie, Arch. Toxicol. 75 (2001) 538.
[261] R.E. Pearce, G.R. Vakkalagadda, J.S. Leeder, Drug Metab. Dispos. 30 (2002) 1170.
[262] P. Wolkenstein, C. Tan, S. Lecoeur, J. Wechsler, N. Garcia-Martin, D. Charue, M. Bagot, P. Beaune, Chem. Biol. Interact. 113 (1998) 39.
[263] S. Madden, J.L. Maggs, B.K. Park, Drug Metab. Dispos. 24 (1996) 469.
[264] J.H. Lillibridge, B.M. Amore, J.T. Slattery, T.F. Kalhorn, S.D. Nelson, R.H. Finnell, G.D. Bennett, Drug Metab. Dispos. 24 (1996) 509.
[265] S.M. Furst, P. Sukhai, R.A. McClelland, J.P. Uetrecht, Drug Metab. Dispos. 23 (1995) 590.
[266] M. Pirmohamed, N.R. Kitteringham, T.M. Guenthner, A.M. Breckenridge, B.K. Park, Biochem. Pharmacol. 43 (1992) 1675.
[267] C. Ju, J.P. Uetrecht, J. Pharmacol. Exp. Ther. 288 (1999) 51.
[268] F. Camu, C. Vanlersberghe, Best Pract. Res. Clin. Anaesthesiol. 16 (2002) 475.
[269] D.O. Stichtenoth, J.C. Frolich, Drugs 63 (2003) 33.
[270] J.A. Namazy, R.A. Simon, Ann. Allergy Asthma Immunol. 89 (2002) 542.

[271] C. King, W. Tang, J. Ngui, T. Tephly, M. Braun, Toxicol. Sci. 61 (2001) 49.
[272] S. Shen, M.R. Marchick, M.R. Davis, G.A. Doss, L.R. Pohl, Chem. Res. Toxicol. 12 (1999) 214.
[273] Y. Masubuchi, A. Ose, T. Horie, Drug Metab. Dispos. 29 (2001) 1190.
[274] A.M. Williams, R.G. Dickinson, Biochem. Pharmacol. 47 (1994) 457.
[275] A.M. Williams, S. Worrall, J. Dejersey, R.G. Dickinson, Biochem. Pharmacol. 49 (1995) 209.
[276] M. Wang, R.G. Dickinson, Drug Metab. Dispos. 26 (1998) 98.
[277] U.A. Boelsterli, H.J. Zimmerman, A. Kretz-Rommel, Crit. Rev. Toxicol. 25 (1995) 207.
[278] S.J. Hargus, B.M. Martin, J.W. George, L.R. Pohl, Chem. Res. Toxicol. 8 (1995) 993.
[279] Y. Masubuchi, E. Masuda, T. Horie, Gastroenterology 122 (2002) 774.
[280] D. Vergani, D. Tsantoulas, A.L. Eddleston, M. Davis, R. Williams, Lancet 2 (1978) 801.
[281] D. Vergani, V.G. Mieli, A. Alberti, J. Neuberger, A.L. Eddleston, M. Davis, R. Williams, New Engl. J. Med. 303 (1980) 66.
[282] J.G. Kenna, J. Neuberger, R. Williams, J. Pharmacol. Exp. Ther. 242 (1987) 733.
[283] J.G. Kenna, H. Satoh, D.D. Christ, L.R. Pohl, J. Pharmacol. Exp. Ther. 245 (1988) 1103.
[284] J.G. Kenna, J. Neuberger, R. Williams, Hepatology 8 (1988) 1635.
[285] D. Njoku, M.J. Laster, D.H. Gong, E.I.n.d. Eger, G.F. Reed, J.L. Martin, Anesth. Analg. 84 (1997) 173.
[286] D.D. Christ, J.G. Kenna, W. Kammerer, H. Satoh, L.R. Pohl, Anesthesiology 69 (1988) 833.
[287] D.D. Christ, H. Satoh, J.G. Kenna, L.R. Pohl, Drug Metab. Dispos. 16 (1988) 135.
[288] J.B. Clarke, C. Thomas, M. Chen, K.L. Hastings, A.J. Gandolfi, Int. Arch. Allergy Immunol. 108 (1995) 24.
[289] A. Madan, A. Parkinson, Drug Metab. Dispos. 24 (1996) 1307.
[290] J. Geneve, D. Larrey, P. Letteron, V. Descatoire, M. Tinel, G. Amouyal, D. Pessayre, Biochem. Pharmacol. 36 (1987) 323.
[291] E.D. Kharasch, D.C. Hankins, K. Fenstamaker, K. Cox, Eur. J. Clin. Pharmacol. 55 (2000) 853.
[292] D.E. Feierman, Z. Melnikov, Alcohol. Clin. Exp. Res. 21 (1997) 1606.
[293] J.G. Kenna, J.L. Martin, H. Satoh, L.R. Pohl, Drug Metab. Dispos. 18 (1990) 788.
[294] J.C. Davila, N.R. Pumford, B.M. Martin, D. Thomassen, J.L. Martin, Toxicologist 11 (1991) 268.
[295] L.R. Pohl, D. Thomassen, N.R. Pumford, L.E. Butler, H. Satoh, Adv. Exp. Med. Biol. 283 (1990) 111.
[296] A.J. Dorner, L.C. Wasley, P. Raney, S. Haugejorden, M. Green, R.J. Kaufman, J. Biol. Chem. 265 (1990) 22029.
[297] R.A. Mazzarella, M. Srinivasan, S.M. Haugejorden, M. Green, J. Biol. Chem. 265 (1990) 1094.
[298] R. Urade, Y. Takenaka, M. Kito, J. Biol. Chem. 268 (1993) 22004.
[299] R.A. Mazzarella, M. Green, J. Biol. Chem. 262 (1987) 8875.
[300] H.R. Amouzadeh, M. Bourdi, J.L. Martin, B.M. Martin, L.R. Pohl, Chem. Res. Toxicol. 10 (1997) 59.
[301] L.R. Pohl, N.R. Pumford, J.L. Martin, Eur. J. Haematol. 57 (1996) 98.
[302] N.R. Kitteringham, J.G. Kenna, B.K. Park, Br. J. Clin. Pharmacol. 40 (1995) 379.
[303] P. Obermayer-Straub, C.P. Strassburg, M.P. Manns, Can. J. Gastroenterol. 14 (2000) 429.
[304] E. Eliasson, I. Gardner, H. Hume-Smith, I. de Waziers, P. Beaune, J.G. Kenna, Chem. Biol. Interact. 116 (1998) 123.
[305] M. Bourdi, W. Chen, R.M. Peter, J.L. Martin, J.T.M. Buters, S.D. Nelson, L.R. Pohl, Chem. Res. Toxicol. 9 (1996) 1159.
[306] J.W. Harris, L.R. Pohl, J.L. Martin, M.W. Anders, Proc. Natl. Acad. Sci. USA 88 (1991) 1407.
[307] J.W. Harris, J.P. Jones, J.L. Martin, A.C. LaRosa, M.J. Olson, L.R. Pohl, M.W. Anders, Chem. Res. Toxicol. 5 (1992) 720.
[308] A.P. Brown, K.L. Hastings, A.J. Gandolfi, D.C. Liebler, K. Brendel, Toxicology 73 (1992) 281.
[309] B. Fisher, J.P. Constantino, D.L. Wickerham, C.K. Redmond, M. Kavanah, W.M. Cronin, V. Vogel, A. Robidoux, N. Dimitrov, J. Atkins, M. Daly, S. Wieand, E. Tan-Chiu, L. Ford, N. Wolmark, J. Natl. Cancer Inst. 90 (1998) 1371.
[310] B.J.A. Furr, V.C. Jordan, Pharmacol. Ther. 25 (1984) 127.
[311] J.R. Benson, V. Pitsinis, Curr. Opin. Obstet. Gynecol. 15 (2003) 13.
[312] B. Fisher, J.P. Costantino, C.K. Redmond, E.R. Fisher, D.L. Wickerham, W.M. Cronin, J. Natl. Cancer Inst. 86 (1994) 527.
[313] W. Davis, S. Venitt, D.H. Phillips, Carcinogenesis 19 (1998) 861.
[314] S.S. Dehal, D. Kupfer, Cancer Res. 56 (1996) 1283.
[315] S.S. Dehal, D. Kupfer, Drug Metab. Dispos. 27 (1999) 681.
[316] L.M. Notley, C.J. De Wolf, R.M. Wunsch, R.G. Lancaster, E.M. Gillam, Chem. Res. Toxicol. 15 (2002) 614.
[317] H.K. Crewe, L.M. Notley, R.M. Wunsch, M.S. Lennard, E.M. Gillam, Drug Metab. Dispos. 30 (2002) 869.
[318] H.K. Crewe, S.W. Ellis, M.S. Lennard, G.T. Tucker, Biochem. Pharmacol. 53 (1997) 171.
[319] D.J. Boocock, K. Brown, A.H. Gibbs, E. Sanchez, K.W. Turteltaub, I.N. White, Carcinogenesis 23 (2002) 1897.
[320] C. Sridar, U.M. Kent, L.M. Notley, E.M. Gillam, P.F. Hollenberg, J. Pharmacol. Exp. Ther. 301 (2002) 945.
[321] J. Hukkanen, M. Mantyla, L. Kangas, P. Wirta, J. Hakkola, P. Paakki, S. Evisalmi, O. Pelkonen, H. Raunio, Pharmacol. Toxicol. 82 (1998) 93.
[322] G. Chen, S. Yin, S. Maiti, X. Shao, J. Biochem. Mol. Toxicol. 16 (2002) 279.
[323] J.C. Homberg, C. Andre, N. Abuaf, Clin. Exp. Immunol. 55 (1984) 561.
[324] P. Beaune, P.M. Dansette, D. Mansuy, L. Kiffel, M. Finck, C. Amar, J.P. Leroux, J.C. Homberg, Proc. Natl. Acad. Sci. USA 84 (1987) 551.
[325] S. Lecoeur, C. Andre, P.H. Beaune, Mol. Pharmacol. 50 (1996) 326.
[326] M.A. Robin, M. Maratrat, M. Le Roy, F.P. Le Breton, E. Bonierbale, P. Dansette, F. Ballet, D. Mansuy, D. Pessayre, J. Clin. Invest. 98 (1996) 1471.
[327] S. Lecoeur, E. Bonierbale, D. Challine, J.C. Gautier, P. Valadon, P.M. Dansette, R. Catinot, F. Ballet, D. Mansuy, P.H. Beaune, Chem. Res. Toxicol. 7 (1994) 434.
[328] A. Melet, N. Assrir, P. Jean, M. Pilar Lopez-Garcia, C. Marques-Soares, M. Jaouen, P.M. Dansette, M.A. Sari, D. Mansuy, Arch. Biochem. Biophys. 409 (2003) 80.
[329] P.M. Dansette, C. Amar, C. Smith, C. Pons, D. Mansuy, Biochem. Pharmacol. 39 (1990) 911.
[330] M. Belghazi, P. Jean, S. Poli, J.M. Schmitter, D. Mansuy, P.M. Dansette, Adv. Exp. Med. Biol. 500 (2001) 139.
[331] P. Beaune, D. Pessayre, P. Dansette, D. Mansuy, M. Manns, Adv. Pharmacol. 30 (1994) 199.
[332] J. Neuberger, R. Williams, Gut 30 (1989) 515.
[333] M. Dahms, H. Spahnlangguth, Pharmazie 51 (1996) 874.
[334] L.P. James, H.C. Farrar, J.E. Sullivan, T.G. Givens, G.L. Kearns, G.S. Wasserman, P.D. Walson, J.A. Hinson, N.R. Pumford, J. Clin. Pharmacol. 41 (2001) 846.
[335] R.A. Laskey, Radioisotope Detection in Fluorography and Intensifying Screens (1984) AmershamArlington Heights, IL.
[336] J. Abian, A.J. Oosterkamp, E. Gelpi, J. Mass Spectrom. 34 (1999) 244.
[337] P. Ponka, Am. J. Med. Sci. 318 (1999) 241.
[338] U. Kragh-Hansen, V.T. Chuang, M. Otagiri, Biol. Pharm. Bull. 25 (2002) 695.
[339] P. Rydberg, B. Lüning, C.A. Wachtmeister, L. Eriksson, M. Törnqvist, Chem. Res. Toxicol. 15 (2002) 570.
[340] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[341] S.D. Nelson, Drug Metab. Rev. 27 (1995) 147.

[342] M. Pirmohamed, D.J. Naisbitt, F. Gordon, B.K. Park, Toxicology 181–182 (2002) 55.
[343] D.J. Naisbitt, M. Pirmohamed, B.K. Park, Curr. Allergy Asthma Rep. 3 (2003) 222.
[344] Y. Masubuchi, T. Horie, Drug Metab. Dispos. 26 (1998) 338.
[345] Y. Masubuchi, T. Horie, Chem. Res. Toxicol. 12 (1999) 1028.
[346] M. Salazar-Paramo, R.L. Rubin, I. Garcia-De La Torre, Ann. Rheum. Dis. 51 (1992) 1085.
[347] E.J. Price, P.J. Venables, Drug Safety 12 (1995) 283.
[348] X. Jiang, G. Khursigara, R.L. Rubin, Science 266 (1994) 810.
[349] K. Suzuki, Int. J. Hematol. 74 (2001) 134.
[350] B.R. Smith, Scand. J. Clin. Lab. Invest. 235 (2001) 45.
[351] B. Shan, J.C. Medina, E. Santha, W.P. Frankmoelle, T.C. Chou, R.M. Learned, M.R. Narbut, D. Stott, P. Wu, J.C. Jaen, T. Rosen, P.B. Timmermans, H. Beckmann, Proc. Natl. Acad. Sci. USA 96 (1999) 5686.
[352] T. Rivera-Penera, R. Gugig, J. Davis, S. McDiarmid, J. Vargas, P. Rosenthal, W. Berquist, M.B. Heyman, M.E. Ament, J. Pediatr. 130 (1997) 300.
[353] J.A. Miller, Cancer Lett. 83 (1994) 9.
[354] D.B. Couch, Drug Chem. Toxicol. 19 (1996) 133.
[355] J.L. Holtzman, Drug Metab. Rev. 27 (1995) 277.
[356] Y.Z. Chen, C.Y. Ung, J. Mol. Graph. Model. 20 (2001) 199.
[357] J. Uetrecht, Curr. Opin. Drug Discov. Dev. 4 (2001) 55.
[358] A.P. Li, Chem. Biol. Interact. 142 (2002) 7.
[359] B.J. Blaauboer, Toxicol. Lett. 138 (2003) 161.
[360] M.D. Barratt, Cell Biol. Toxicol. 16 (2000) 1.
[361] J.E. Ridings, M.D. Barratt, R. Cary, C.G. Earnshaw, C.E. Eggington, M.K. Ellis, P.N. Judson, J.J. Langowski, C.A. Marchant, M.P. Payne, W.P. Watson, T.D. Yih, Toxicology 106 (1996) 267.
[362] M.D. Barratt, R.A. Rodford, Curr. Opin. Chem. Biol. 5 (2001) 383.
[363] M.D. Barratt, Environ. Health Perspect. 106 (1998) 459.
[364] A. Vedani, M. Dobler, Prog. Drug Res. 55 (2000) 105.
[365] J.F. Waring, R.A. Jolly, R. Ciurlionis, P.Y. Lum, J.T. Praestgaard, D.C. Morfitt, B. Buratto, C. Roberts, E. Schadt, R.G. Ulrich, Toxicol. Appl. Pharmacol. 175 (2001) 28.
[366] S. Gupta, L.K. Rogers, S.K. Taylor, C.V. Smith, Toxicol. Appl. Pharmacol. 146 (1997) 317.
[367] H. Min, B. Shane, E.L. Stokstad, Biochim. Biophys. Acta 967 (1998) 348.
[368] S.S. Lee, J.T. Buters, T. Pineau, P. Fernandez-Salguero, F.J. Gonzalez, J. Biol. Chem. 271 (1996) 12063.
[369] L. Zhou, R.R. Erickson, J.P. Hardwick, S.S. Park, S.A. Wrighton, J.L. Holtzman, J. Pharmacol. Exp. Ther. 281 (1997) 785.
[370] S.M. Furst, J.P. Uetrecht, Int. J. Immunopharmacol. 17 (1995) 445.
[371] M. Pirmohamed, N.R. Kitteringham, A.M. Breckenridge, B.K. Park, Br. J. Clin. Pharmacol. 33 (1992) 183.
[372] S.M. Furst, D. Luedke, A.J. Gandolfi, Toxicology 120 (1997) 119.
[373] I.N. White, F. De Matteis, A.H. Gibbs, C.K. Lim, C.R. Wolf, C. Henderson, L.L. Smith, Biochem. Pharmacol. 49 (1995) 1035.
[374] N.T. Ha-Duong, S. Dijols, A.C. Macherey, J.A. Goldstein, P.M. Dansette, D. Mansuy, Biochemistry 40 (2001) 12112.
[375] D. Mansuy, J. Hepatol. 26 (1997) 22.
[376] P. Jean, P. Lopez-Garcia, P. Dansette, D. Mansuy, J.L. Goldstein, Eur. J. Biochem. 241 (1996) 797.
[377] M.P. Lopez-Garcia, P.M. Dansette, D. Mansuy, Biochemistry 33 (1994) 166.
[378] M. Bourdi, D. Larrey, J. Nataf, J. Bernuau, D. Pessayre, M. Iwasaki, F.P. Guengerich, P.H. Beaune, J. Clin. Invest. 85 (1990) 1967.
[379] C. Pons, P.M. Dansette, C. Amar, M. Jaouen, C.R. Wolf, J. Pharmacol. Exp. Ther. 259 (1991) 1328.