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Covalent Protein Modification by Reactive Drug Metabolites Using Online Electrochemistry/Liquid Chromatography/Mass Spectrometry

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We present a rapid and convenient method to perform and evaluate the covalent protein binding of reactive phase I metabolites. The oxidative metabolism of the drugs paracetamol, amodiaquine, and clozapine is simulated in an electrochemical (EC) flow-through cell, which is coupled online to an LC/MS system. Adduct formation of the reactive metabolites with the proteins β-lactoglobulin A and human serum albumin proceeds in a reaction coil between EC cell and injection system of the HPLC system. The formed drug−protein adducts are characterized with online time-of-flight mass spectrometry, and the modification site is localized using FTICR-mass spectrometry. Due to its simple setup, easy handling, and short analysis times, the method provides an interesting tool for the rapid risk assessment of covalent protein binding as well as for the synthesis of covalent drug−protein adducts in high purity and high yield.

The mechanistic details of drug toxicity are still poorly understood. One hypothesis explaining toxic side effects of a drug is the covalent binding of drug metabolites to proteins. Xenobiotics such as drugs are often metabolized by cytochrome P450 enzymes (CYP) to oxidative metabolites. While in most cases drug metabolism is regarded as a detoxification process, sometimes bioactivation to highly reactive species occurs.[1] The majority of reactive metabolites are electrophilic compounds seeking nucleophilic centers as reaction partners,[2] which are omnipresent in proteins in the cellular environment where metabolism proceeds. The covalent modification of proteins can alter their functionality or disrupt regulatory pathways, which can cause cell death,[3] or covalently modified proteins can initiate an immune response.[4] If the modification directly affects the metabolizing CYP enzyme, irreversible inactivation of that enzyme can emerge.[5,6]

First reports about covalent drug−protein adduct formation date back to the 1940s.[7] Since then, the methodology for the detection and identification of drug−protein adducts improved significantly, particularly after introduction of electrospray mass spectrometry.[8] However, the detection and identification of proteins that are covalently modified by a drug or a drug metabolite is still a challenging task since the isolation of a single protein species from a complex mixture such as a microsomal incubation or even an in vivo assay is quite intricate and time-consuming.[9,10] Often, radiolabeled drugs are used for the quantification of covalent protein binding,[11] but thus far, only little information on the nature of the modified proteins can be obtained. Using two-dimensional liquid chromatography/mass spectrometry (LC/MS), covalent adduct formation of metabolites of two model compounds with a CYP enzyme was detected.[12] For more detailed information on the nature of the modified protein, however, isolation and proteolytic digest in combination with mass spectrometric measurements cannot be avoided.[2,9,13−15]

Furthermore, the modification grade of a protein species is generally low, which makes the detection of a modified protein even more difficult. Therefore, the development of a simple method is desirable that allows a rapid assessment of the potential of a drug candidate to covalently modify proteins as well as the characterization of the modified protein including the localization

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of the modification site. As was shown in recent publications, the electrochemical oxidation of drug compounds—simulating the CYP catalyzed oxidative metabolism—can provide valuable information about metabolically labile sites in a molecule, particularly in an online coupling with electrochemistry/liquid chromatography/mass spectrometry (EC/LC/MS).[16−21] The use of a microspray device connected to a mass spectrometer for tagging cysteine residues in synthetic or tryptic peptides[22,23] or in proteins in a nanospray[24] or a microspray electrode[25] with electrogenerated benzoquinone is published as well. However, the reaction products generated in the electrospray interface are obtained only in low yield and cannot be synthesized on a preparative scale for further characterization. Until now, nothing was reported about the use of an EC/(LC)/MS system for the investigation of a drug compound in terms of its tendency to covalently modify proteins. Therefore, we decided to set up an online EC/LC/MS system for assessing the risk of covalent protein binding of drug metabolites. Three model compounds that are known for bioactivation to reactive metabolites were selected for validation of the EC/LC/MS setup. Paracetamol is an analgesic and antipyretic drug that is oxidized by CYP to the reactive *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is known for covalent protein binding thus causing hepatotoxicity.[26,27] For this reason, it was recently decided by the German Federal Institute for Pharmaceuticals and Medical Products to classify paracetamol, which was so far a classical over-the-counter drug, in package sizes of more than 10 g as a prescription drug.[28] The antimalarial agent amodiaquine undergoes bioactivation to a respective quinoneimine (AQQI) and has been withdrawn from malaria prophylaxis due to the occurrence of agranulocytosis and hepatotoxicity,[29] and clozapine which is an antipsychotic agent that is used for the treatment of schizophrenia is metabolized by CYP to a reactive electrophilic nitrenium ion[30] (Figure 1a). The oxidative metabolism of all model compounds as well as the detoxification of the reactive species by addition of glutathione has been successfully

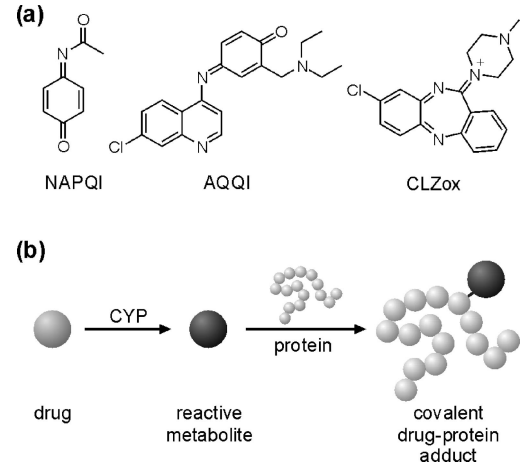

Figure 1. (a) Structures of the reactive metabolites of paracetamol (*N*-acetyl-*p*-quinoneimine, NAPQI), amodiaquine (amodiaquine quinoneimine, AQQI), and clozapine (clozapine nitrenium ion, CLZox). (b) Principle of covalent protein binding of drugs after activation by cytochrome P450 (CYP) enzymes to reactive metabolites.

mimicked in EC/(LC)/MS systems.[31−34] In the present work, a setup for the online synthesis of covalent drug−protein adducts that are subsequently detected and characterized by mass spectrometric techniques is developed.

EXPERIMENTAL SECTION

Reagents and Materials. β-Lactoglobulin A from bovine milk, human serum albumin, and trypsin from bovine pancreas (≥10000 BAEE units/mg of protein) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and were used without further purification. All chemicals and solvents were ordered from Sigma-Aldrich Chemie GmbH, Fluka Chemie GmbH (Buchs, Switzerland), ABCR (Karlsruhe, Germany), or Molekula (Shaftesbury, U.K.) in the highest quality available. Disposable PD-10 Sephadex G-25 M desalting columns were obtained from GE Healthcare (Buckinghamshire, U.K.). Water used for HPLC was purified using a Milli-Q Gradient A 10 system and filtered through a 0.22-μm Millipak 40 (Millipore, Billerica, MA).

Online Electrochemical Generation of Drug−Protein Adducts. A solution containing a 1×10^{-4} M concentration of the desired drug compound in 20 mM aqueous ammonium formate (adjusted to pH 7.4 with ammonia) and acetonitrile (50/50, v/v) was pumped with a flow rate of 10 μL min^{-1} through a commercially available electrochemical flow-through cell (model 5021, conditioning cell, ESA Biosciences Inc., Chelmsford, MA). The potential at the working electrode was controlled by a Coulochem II potentiostat (ESA Biosciences). Paracetamol was oxidized at 600 mV, amodiaquine at 300 mV, and clozapine at 400 mV versus Pd/H_2 in the EC cell. Via a T-piece, a solution containing the protein of interest (2×10^{-5} M) in 8 M urea was added after the EC cell at the same flow rate, and the combined solutions were allowed to react in a reaction coil (Teflon tubing, 0.762-mm inner diameter, 438-mm length) for 10 min. The effluent from the

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reaction coil was collected in a 10-μL injection loop, which was mounted on a 10-port switching valve. By switching this valve, the content of the injection loop was flushed by the HPLC pumps into the LC/MS system.

Off-Line Electrochemical Synthesis of Drug−Protein Adducts and Tryptic Digest. The offline electrochemical oxidation was performed as described for the online setup except with a higher flow rate of 50 μL min^{-1} and in a 5-fold higher concentration of the drug compound (5×10^{-4} M). The effluent from the EC cell was collected in a vial containing 1 mL of a 1×10^{-4} M solution of the protein of interest in 8 M urea. After 20 min of collection, corresponding to a total volume of 1 mL of reactive metabolite solution (5-fold molar excess based on the amount of protein), the sample was incubated at 37 °C for 30 min. After the incubation time, the excess reagent was removed on a PD-10 desalting column with pure water as mobile phase according to the supplier's protocol. Reduction of the disulfide bridges was performed at 37 °C for 30 min using a 100 mM stock solution of tris(2-carboxylethyl)phosphine hydrochloride (TCEP) in 0.3 M aqueous ammonia. A 960-mg sample of urea was added to 2 mL of the purified drug−protein adduct and a 9-fold molar excess of TCEP per oxidized *S*-atom in the protein was added from the stock solution. The reduction was terminated by passing the solution again through a PD-10 desalting column. Digestion with trypsin was performed by adding 5 μL of a stock solution of 2.3 mg of trypsin in 1 mL of water to 1 mL of the reduced drug−protein adduct solution containing $\sim 1.6 \times 10^{-5}$ M modified protein and 50 mM ammonium bicarbonate. After 90 min at 37 °C, the digest was terminated by adding 200 μL of ice-cold acetonitrile containing 1% formic acid.

LC/MS Conditions. An HPLC separation was performed in the online EC/LC/MS setup to remove excess reactive metabolite and urea before MS analysis. The LC/MS setup comprised a Shimadzu (Duisburg, Germany) LC system and a micrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an electrospray ionization (ESI) source. For controlling the micrOTOF and data handling, micrOTOFControl 1.1 and DataAnalysis 3.3 (Bruker Daltonics) software were used. The LC system consisted of two LC-10ADVP pumps, a DGC-14A degasser, a SIL-HTA autosampler, a CTO-10AVP column oven, and a SPD-10AVVP UV detector. The separation was performed on a Discovery BIO Wide Pore C5 column (2.1 × 150 mm, 5 μm) from Supelco at 40 °C. The injection volume was 10 μL. A binary gradient with aqueous formic acid (0.1%) and acetonitrile containing 0.1% formic acid at a total flow rate of 300 μL min^{-1} was used. After 2 min of an isocratic step with 5% organic mobile phase, the acetonitrile content was increased to 80% in 10 min and held at 80% for 2 min, and finally, the system was equilibrated for 6 min. On the micrOTOF instrument, full scan spectra (m/z 50−4000) were recorded after HPLC separation using ESI(+)-MS under the following conditions: end plate offset, −500 V; capillary, 4000 V; nebulizer gas (N_2), 0.8 bar; drying gas (N_2), 8.0 L min^{-1}; drying temperature, 200 °C; capillary exit, 150.0 V; skimmer 1, 50.0 V; skimmer 2, 23.0 V; hexapole 1, 23.0 V; hexapole 2, 17.0 V; hexapole rf, 500 V; transfer time, 70.0 μs; prepulse storage, 30.0 μs; detector, −1000 V. Internal calibration was performed using sodium formate clusters at the beginning of each HPLC run.

FTICR-MS Conditions. Nano-ESI-FTICRMS experiments were carried out using the hybrid LTQ FT Fourier transform ion cyclotron resonance hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a 7.0-T actively shielded superconducting magnet and nano-ESI source. Gold-plated nano-ESI pipets (tip i.d. ~5 μm) were from MasCom (Bremen, Germany). The instrument was operated in the positive ionization mode and the mass resolution was set to 50 000 fwhm at m/z 400. Ion transmission into the linear trap and signal intensity were automatically optimized for maximum ion signal of the unmodified peptide T13. The parameters were as follows: source voltage 1.0−1.2 kV, capillary voltage 25 V, capillary temperature 200 °C, and tube-lens voltage 105 V. Accurate mass measurements of peptides were carried out by a zoom scan in a narrow mass window (±10 Da) and displayed mass spectra were averaged from five single spectra. Fragmentation experiments were performed by collisionally induced dissociation (normalized collision energy of 35% with activation $q = 0.25$ and activation time of 30 ms) in the linear ion trap part of the hybrid instrument. Mass analysis of the resulting fragments was done in the FTICR cell, and displayed spectra were averaged from 100 single spectra.

RESULTS AND DISCUSSION

Generating Drug−Protein Adducts in the Online EC/LC/MS System. First experiments were performed using β-lactoglobulin A (β-LGA) as model target protein. Despite its occurrence as the main whey protein in milk, this protein was selected due to its structural homogeneity and relatively small molecular mass (162 amino acids, 18.4 kDa), which allows an easier elucidation of its fate after reaction with reactive metabolites. Paracetamol (APAP), amodiaquine (AQ), and clozapine (CLZ) were selected as model drug compounds that undergo bioactivation by dehydrogenation to reactive electrophilic species (Figure 1a). This bioactivation process is simulated in an EC flow-through cell (Figure 2). The cell is commercially available and comprises a three-electrode arrangement with a porous glassy carbon working electrode, a Pd counter electrode, and a Pd/H_2 reference electrode. The porosity of the working electrode results in a large surface area, which allows for an almost quantitative turnover of the analytes and low maintenance intervals of the EC cell. A solution containing the drug of interest at pH 7.4 was pumped through the EC cell at a constant flow rate of 10 μL/min. The EC potential at the working electrode was selected for each model compound on the basis of previously reported EC oxidation results so that the yield of the respective reactive metabolite was optimal. APAP was thus oxidized to NAPQI at 600 mV versus Pd/H_2, AQ was converted to AQQI at 300 mV versus Pd/H_2, and CLZ was dehydrogenated to CLZox at 400 mV versus Pd/H_2. The outlet of the EC cell was connected via a T-piece with the outlet of a second syringe pump, which delivered the denaturized model protein β-LGA in 8 M urea in a 5-fold lower concentration as the reactive metabolite. The combined flows containing the reactive metabolite and the protein were directed into a reaction coil. For β-LGA, a reaction time of 10 min has proven to be sufficient for a good yield of modified protein. The effluent from the reaction coil was then collected in an injection loop, which was mounted on a 10-port switching valve for injection into the LC/MS system.[33] From the time-of-flight mass spectra of unmodified β-LGA (a) and β-LGA after 10 min of reaction with NAPQI (b), AQQI (c), and CLZox

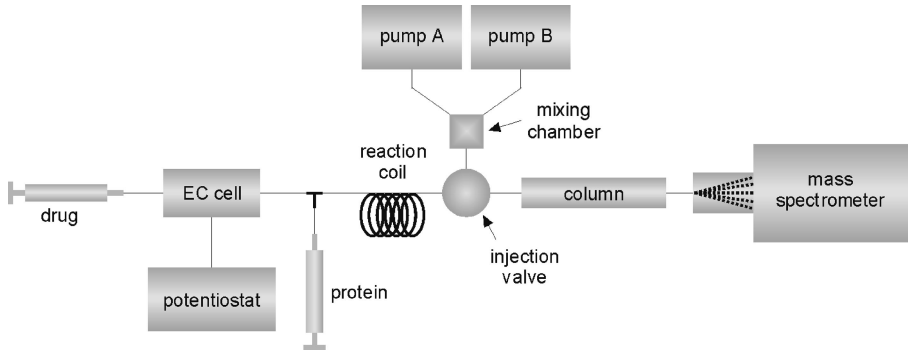

Figure 2. EC/LC/MS setup for the generation and detection of covalent drug−protein adducts. The drug of interest is oxidized in the EC flow-through cell to its oxidative metabolites, which subsequently react online with a protein. HPLC separation is carried out to remove excess reactive metabolites, and the drug−protein adducts are detected with mass spectrometry.

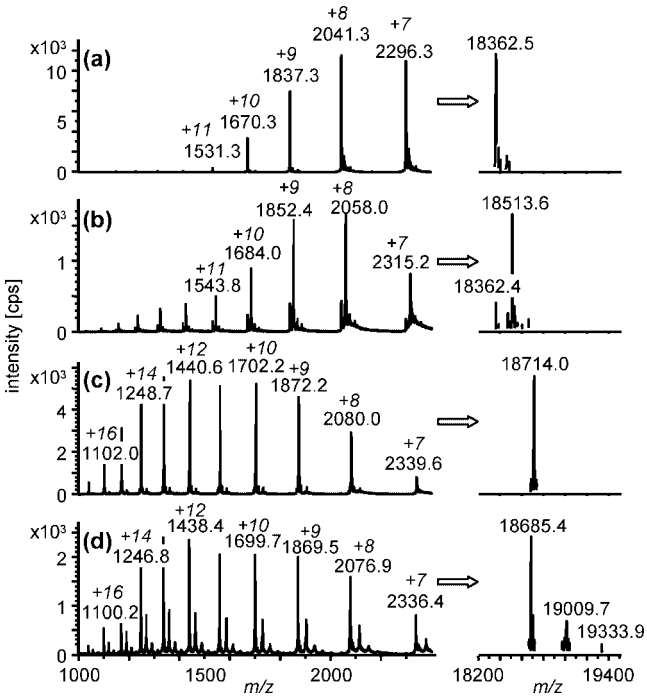

Figure 3. Time-of-flight mass spectra and deconvolution results of (a) unmodified β-LGA, (b) β-LGA after reaction with NAPQI, (c) β-LGA after reaction with AQQI, and (d) β-LGA after reaction with CLZox. The mass spectra of the modified protein were obtained after online reaction of the unmodified protein with electrochemically generated reactive metabolites.

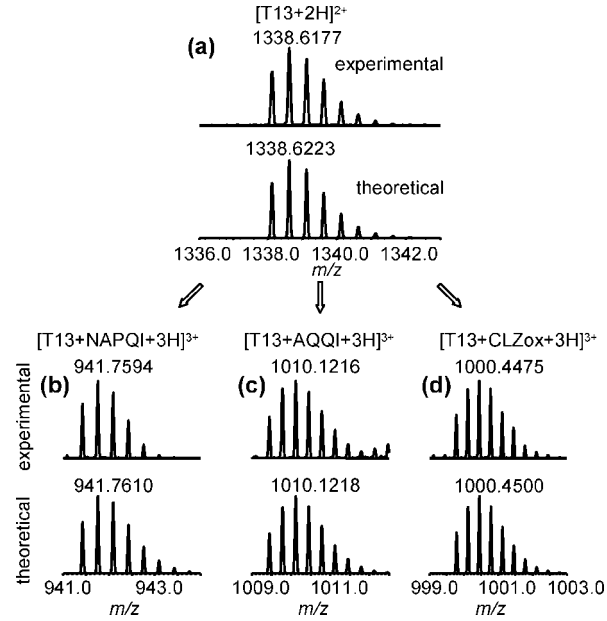

Figure 4. FTICR-MS spectra of the tryptic peptide T13 containing the amino acid residues 102−124 of β-LGA with (a) the $[M + 2H]^{2+}$ of the unmodified protein, (b) the $[M + 3H]^{3+}$ of β-LGA modified with NAPQI, (c) the $[M + 3H]^{3+}$ of β-LGA modified with AQQI, and (d) the $[M + 3H]^{3+}$ of β-LGA modified with CLZox.

(d) (Figure 3), it can clearly be seen that the MS signals are shifted toward higher m/z values after reaction with one of the reactive metabolites, thus proving that a covalent drug−protein adduct formation has occurred. While for the β-LGA−NAPQI adduct, a certain amount of unmodified β-LGA could be observed, the modification grade of β-LGA with AQQI is 100% (no unmodified β-LGA was detectable) and CLZox even seems to generate multiple β-LGA−CLZox adducts. Deconvoluted mass spectra of the modified β-LGA are shown next to the respective raw spectra. Deconvolution of unmodified β-LGA resulted in a molecular mass of the protein of 18 362.5 Da, which corresponds well with the theoretical value of 18 363.1 Da calculated from the amino acid sequence. After reaction with NAPQI, the molecular mass of β-LGA increased to 18 513.6 Da, corresponding to adduct formation with one unit of NAPQI with a monoisotopic mass of 149.1 Da. Modification of β-LGA with AQQI increased the molecular mass of the protein to 18 714.0 Da, which is in good accordance to the attachment of one molecule of AQQI with a monoisotopic mass of 353.1 Da. As could already be seen from the nondeconvoluted mass spectrum, the addition of CLZox with a monoisotopic mass of 325.1 Da even yielded multiple adducts with more than one CLZ molecule, with the monoadduct with a molecular mass of 18 685.5 Da being the most abundant one, next to a bis-adduct with 19009.7 Da and a tris-adduct with 19333.9 Da. It is remarkable that, especially in case of NAPQI and AQQI, and to a certain extent also for CLZox, only a monodrug−protein adduct is formed despite a relatively large excess of reactive metabolite. It thus seems likely that this is due to the protein sequence, probably providing only a single readily available modification site. The electrophilic addition of reactive intermediates often occurs at nucleophilic centers in the protein, being for example the free thiol group of a cysteine. Since β-LGA contains exactly one free cysteine residue that is not bound in disulfide bridges, it is very likely that the modification occurs at this free cysteine residue. In case of CLZox,

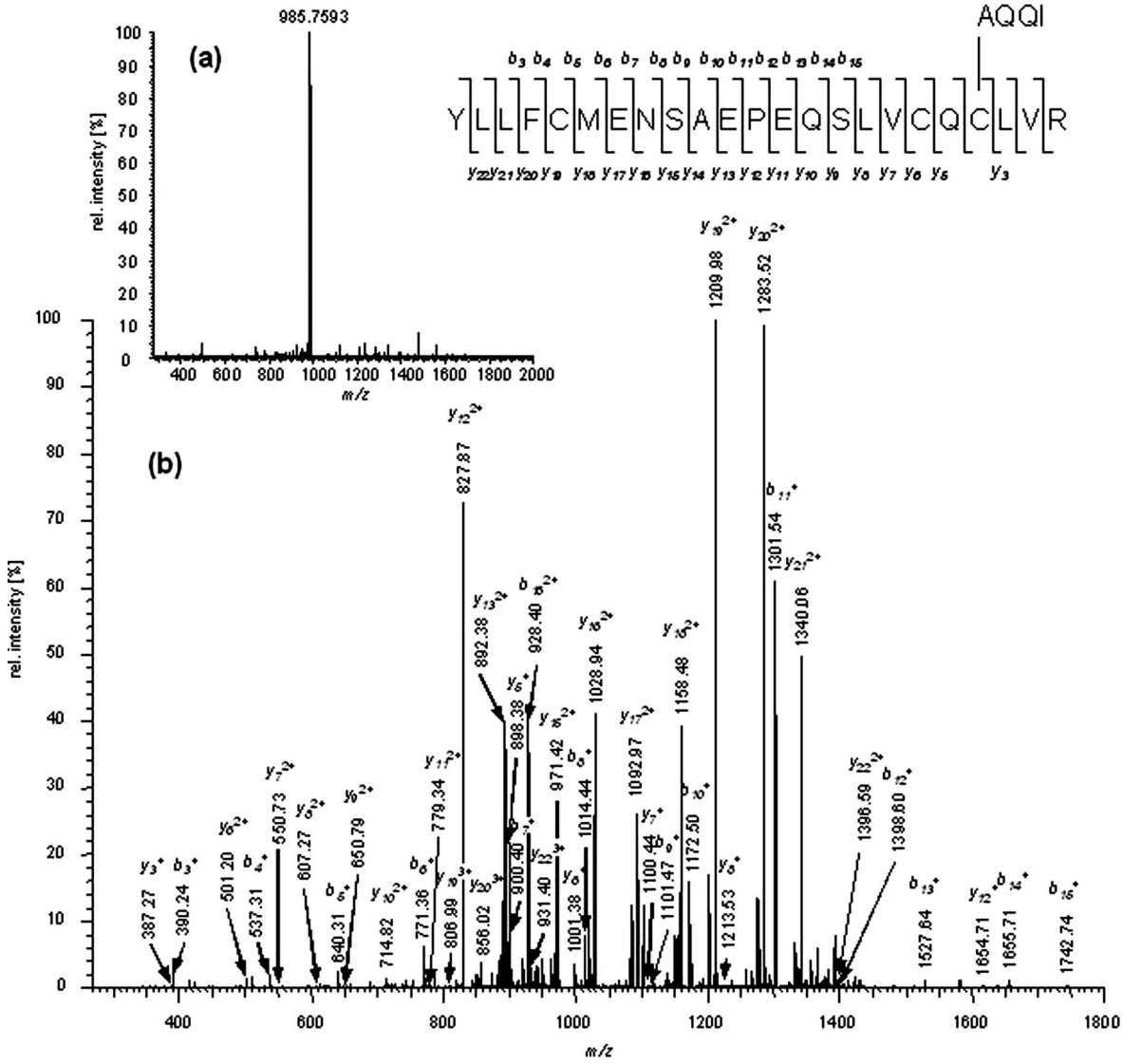

Figure 5. (a) FTICR-MS/MS spectrum of $[T13 + AQQI + 3H]^{3+}$ resulting in loss of the *N*-diethyl group of the AQQI modification. (b) Further fragmentation (MS^3) of the fragment ion with *m*/*z* 985.7593 and assignment of b- and y-ion series to the observed signals.

the electrophilic nitrenium ion seems to be more reactive than NAPQI or AQQI, thus attacking also other nucleophilic centers.

Off-Line Analysis of the Drug−Protein Adducts. To obtain more information about the modification site in the protein, a tryptic digest of the modified proteins after reduction of disulfide bonds was performed. Therefore, the modified proteins were synthesized in an off-line approach according to the online assay, except that the effluent from the EC cell was collected in a vial containing a β-LGA solution, which was incubated at 37 °C after termination of the collection. Urea and excess reactive metabolite were removed on a PD-10 desalting column, and disulfide bridges were reduced using TCEP before tryptic digest. Trypsin is a proteolytic enzyme that cleaves proteins specifically at the C-terminal site of lysine and arginine into smaller peptides. In case of a covalently modified protein, one or more of these peptides carry the modification and these peptides have to be analyzed in more detail. Particular attention was paid to the peptide bearing the only free cysteine thiol group in the protein, which is the peptide containing the amino acid residues 102−124 (YLLFC-MENSAEPEQSLVCQ**C**LVR, peptide T13). Cysteine 121 is the amino acid residue of interest. The nonmodified peptide T13 has a monoisotopic neutral mass of 2674.2263 Da and was detected using positive nanoelectrospray Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) as $[M + 2H]^{2+}$ in the tryptic digest of unmodified β-LGA (Figure 4a). If this peptide is modified by one unit of either NAPQI, AQQI, or CLZox, the monoisotopic neutral mass increases by the molecular weight of the modification. All these modified peptides were detected as $[M + 3H]^{3+}$ in the respective tryptic digests of the modified β-LGA, and the measured mass spectra are in good agreement with the calculated spectra (Figure 4b−d).

The site of the modification could thus be restricted to one of the amino acids in peptide T13. To further narrow the location, the respective peptides were fragmented by collisionally induced dissociation in the linear quadrupole ion trap of the hybrid MS. The resulting fragment ions were then analyzed in the FTICR cell for accurate mass information at high resolution. The fragmentation pathway of peptide T13, which is modified with AQQI (T13 + AQQI) should be discussed representatively for all modified peptides. In the MS/MS spectrum of this peptide (Figure 5a), one main fragmentation product of T13 + AQQI was observed at *m*/*z* 985.7593, which corresponds to a loss of $C_4H_{11}N$. Obviously,

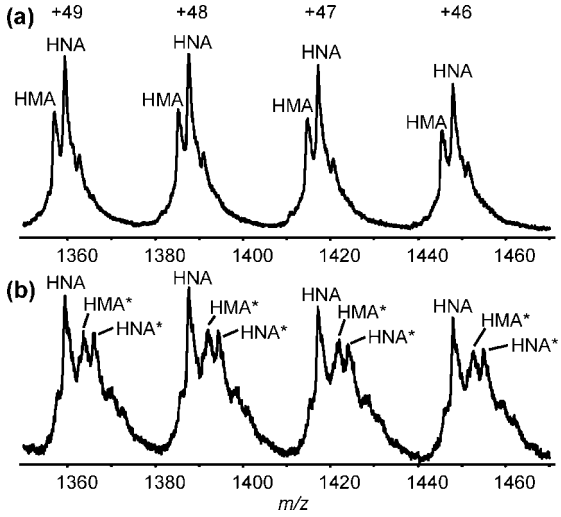

Figure 6. (a) Section of a time-of-flight mass spectrum of commercially available human serum albumin. Mercaptalbumin (HMA, with a free thiol group), and nonmercaptalbumin (HNA, with the thiol group in a mixed disulfide bond with cysteine) detected with a deconvoluted molecular mass of 66 437.6 (calculated 66 437.5 Da) and 66 557.8 Da (calculated 66 556.6 Da), respectively. (b) Section of a time-of-flight mass spectrum of commercially available human serum albumin after reaction with CLZox. Both HMA and HNA were modified by CLZox to HMA* with a deconvoluted molecular mass of 66 768.0 Da (calculated 66 762.3 Da) and HNA* with a deconvoluted molecular mass of 66 879.4 Da (calculated 66 881.4 Da), respectively.

instead of fragmentation of the amino acid sequence, the AQQI modification is the main site where fragmentation occurs, and the AQQI residue is cleaved under loss of the *N*-diethyl functionality in the MS/MS experiment, which provides further evidence for the presence of AQQI in this peptide. This resulting ion with m/z 985.7593 was fragmented further in an MS^3 experiment, and this time, fragmentation of the amino acid chain was observed (Figure 5b). The b-ion and particularly the y-ion series are covered well, and since both y_3 without AQQI modification and y_5 with AQQI modification could be detected, the modification site could be assigned to Cys121, which is the free cysteine in the native protein.

Applicability of the Online EC/LC/MS Method to Serum Albumins. The newly developed method should be applied not only to the whey protein β-LGA but also to human plasma proteins of relevance in drug metabolism studies such as human serum albumin (HSA). HSA is the most abundant plasma protein and is synthesized in the liver, where extensive drug metabolism occurs. Therefore, it is a potential target protein for reactive drug metabolites. Adduct formation of CLZox with HSA was examined with the EC/LC/MS setup according to the experiments with β-LGA. Figure 6 shows a section of the mass spectrum of HSA before and after reaction with CLZox. The unmodified protein is microheterogeneous (Figure 6a), which is mostly due to partial blockage of the only free cysteine thiol functionality via a mixed disulfide bond with small bioavailable thiols such as cysteine or glutathione.[35] After reaction with CLZox, adducts of both HSA with the free thiol group (mercaptalbumin, HMA) and the HSA variant in which the free cysteine is blocked with another cysteine residue (nonmercaptalbumin, HNA) were detected (Figure 6b). The reactive CLZox thus seems to exhibit a reactivity toward other nucleophilic functional groups in the amino acid side chains, as for example the additional amino group in lysine or the hydroxyl group of serine, as was already observed for β-LGA, where adducts of more than one activated drug molecule were detected. Even though the adduct yield of HSA with CLZox was not as high as for β-LGA, it is evident that the online EC/LC/MS method also has high potential in the risk assessment of covalent protein binding, for example, to the biologically relevant plasma protein HSA.

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CONCLUSIONS

We present a simple and rapid online method for the generation and identification of drug−protein adducts. In an EC flow-through cell, the oxidative phase I metabolism of drugs is mimicked by an electrochemical oxidation. The LC/MS equipment used is currently standard instrumentation in drug metabolism laboratories. Upgrading an LC/MS system by an EC cell is thus easily accomplished, which makes the method interesting for all researchers being active in the field of drug metabolism.

The experiments demonstrate that our method offers interesting features in the risk assessment of covalent protein binding of drug metabolites. Rapid information on the possibility of covalent protein binding may be obtained, and in combination with the off-line approach for the characterization, the modification site can be localized. If reactive metabolites mainly react with free thiol groups, endogenous detoxification pathways as, for example, the detoxification by addition of glutathione may lower the risk of undesired side effects; but if protein modification also occurs at other functional groups, the risk of covalent binding to other macromolecules such as DNA, which has more serious consequences as covalent protein binding regarding the toxicity, should also be considered. For such considerations, our method composes an excellent basis since it provides rapid and reliable information with minimum technical effort and analysis time.

In terms of complexity, our method is superior to conventionally used techniques. All standard procedures, such as microsomal incubations or in vivo experiments, yield only a small percentage of modified protein, which has to be isolated from a complex biological matrix for identification and characterization, while our method yields very clean modified proteins with a high degree of modification. This saves time, labor, and money, and it facilitates the subsequent determination of the position of the modification site.

To sum up, our simple and elegant method offers two new possibilities: the risk assessment of covalent protein binding of a new chemical entity and the rapid and clean synthesis of covalently modified proteins.

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