Pro-Electrophiles

A Previously Overlooked Class of Protein-reactive Extractables?

Extractable and leachable studies on primary packaging systems are well established in the pharmaceutical industry. For extraction studies, guidelines and best practice guides mandate a worst-case approach, taking the active, the formulation and the container closure system into account. The data from the extraction study should enable a meaningful risk assessment about the possible occurrence of impurities leaching from packaging components during the products shelf-life. The guidelines and best practice guides recognise packaging materials as a potential source of impurities and assess their direct toxic or genotoxic effect on the patient. However, an assessment of the protein-reactivity of extractables are not in the scope of standard extraction procedures. Changes to the protein structure caused by compounds acting as a catalyst or by direct covalent reaction, may render the Biopharmaceutical inefficacious or even immunogenic. Previously, we have described a screening assay for packaging material extracts, to identify possible protein-reactive extractables, enabling an assessment including the risk of possible leachable having a direct or indirect effect on the protein structure. However useful this proposed assay is, it only allows an evaluation of the reactivity of extractables during the extraction process. It does not consider extractables, which are not reactive per se, but may be converted to a protein-reactive derivative caused by aging processes, which might occur over the shelf-life of the product. In this article, we propose a modification of the protein-reactivity screening, by adding an electrochemical oxidation step, to not only evaluate extractables, which are reactive during the extraction process, but to also see, if there is a subset of extractables, which oxidised derivatives have protein reactive properties. This modified assay does not only evaluate the reactivity of extractable during the extraction process, but also takes into account, that leaching processes are time dependent and therefore, potential leachable are like the drug product prone to aging dependent degradation or modification, which may have an impact on the reactivity of the compound.

Biopharmaceuticals, drug products with protein- or peptide-based active ingredients, are mainly administered as parenteral solutions. These solutions prepared either prior to application by reconstitution of a lyophilized product with a suitable diluent or are formulated directly as a liquid-for-injection. Parenteral drug products are mainly stored in glass container closure systems sealed with a rubber stopper/septum; the standard vial-stopper configuration. In some cases, the product may be filled directly into syringe, a so-called pre-filled syringe. These consist of a glass barrel, which may be lubricated, a rubber-based plunger and a syringe needle held in place by an adhesive and a capping device. All these components and adhesives are potential sources of leachables, which may migrate into the product over it´s shelf life. To evaluate the risk of leachables migrating into the product, extraction studies are performed. Extractions studies should be designed in a way, that they represent a worst-case scenario in terms of the extractive properties of the extraction media and extraction conditions, mainly temperature and time. Here it is important to note, that a forensic-style evaluation the primary packaging, e.g. by completely dissolving each component under the harshest of conditions, does not give rise to a representative extraction profile. The USP chapters <1663> and <1664> offer guidance on the design, justification and evaluation of data from extractable and leachable studies, but do not propose exact experimental conditions for extraction studies.[1,2] The Product Quality Research Institute (PQRI), however published a widely accepted best practice guides for Orally Inhaled and Nasal (OINDP) as well as for Parenteral and Ophthalmic Drug Products (PODP).[3,4] The guideline covering parenteral formulations, proposes three different extraction media considering aqueous buffered formulations at basic and acidic pH, as well as water with organic solvent to simulate the extractive properties of often highly concentrated protein-based actives and excipients like polysorbates, aiding protein solubility. The guideline also proposes a Safety Concern Threshold (SCT) considering the parenteral and ophthalmic route of administration, under which a daily exposure to an unknown compound is considered to likely have no adverse effect on the patient. The SCT is an extrapolation of no observed effect levels (NOEL) obtained for different substance classes in animal models.[5] For the evaluation of the extraction profiles, the SCT is translated into an analytical evaluation threshold (AET), which takes into account the maximum daily delivered dose of the drug and the extraction volume. Any extractable, which falls short of the concentration limit defined in the AET, are not further evaluated, since they are unlikely to either leach in the product over it´s shelf life or at least are very likely to stay under defined SCT. Compounds in the extract, that are detected above the AET need to be identified and toxicologically assessed to evaluate whether the compound needs to the monitored during a leachable study. The analytical techniques applied must be capable of detecting a single compound, in case of a leachable study, or multiple compounds from an extraction study at the defined AET level. Furthermore, for the evaluation of extracts the analytical techniques must be suitable to detect an array of extractables from inorganic compounds, e.g. metals from glass components, to organic compounds with divers physico-chemical properties, e.g. from rubber components or adhesives.

The general concepts of the guidelines mentioned above regard extractables and leachables as compounds, which may have a direct concentration-dependent genotoxic, toxic or other ways adverse effect in the patient. The evaluation based on SCTs reflects the approach described in guidelines concerning genotoxic and toxic impurities e.g., ICH M7.[6] For protein-based biopharmaceuticals however, an evaluation of extractables purely on a NOEL concept does not consider the potential direct effect an extractable may have on the active itself. This may be a covalent reaction of an extractable with an amino acid side chain

or by acting as a catalyst by e.g., oxidising amino acids prone to oxidation, as already shown for some metal ion leachables.[7]

Apart from this being a potential quality issue, the slights changes to the amino acid sequence can render a protein inefficacious, altering the three-dimensional structure, causing e.g. aggregation,[8] or in a worst-case scenario, triggering an immune response in patients. An immune response can lead to the induction of anti-drug antibodies (ADA) changing the drugs pharmacokinetic properties[9] or, in case the ADA binds to the active site of the drug (so-called neutralising antibody), rendering the drug completely inefficacious in a patient, often for life.[10] Because immunogenic reactions and their severity are highly dependent on the immunogen, the route of administration and the individual genetic makeup of each individual patient, there are no established SCT levels for potentially immunogenic substances available. Le et. al. however, have proposed a limit for protein modifications based on LC-UV peptide mapping data. They suggest that modifications greater than 2% on peptide level should be investigated in regard to immunogenic effects.[11] Furthermore, biopharmaceuticals such as monoclonal antibodies, have a large molecular weight, making it difficult to observe small mass modifications using established standard stability indicating methods. Even the use of sophisticated mass spectrometry applications has its limitations, if screening low level unknown or unexpected modifications. Therefore, a screening assay for protein-reactive extractables is as useful tool to enable a comprehensive risk assessment, based not only on the abundance of each individual compound in the extract in relation to the defined AET, but also indicates which of those compounds might be protein-reactive. The additional information about protein-reactive compounds enables the analyst to either conduct further experiments to verify, if the extractable also reacts with the biopharmaceutical or to aid a screen for such modifications in stored drug product samples.

We have previously described a screening assay for protein-reactive extractables, which can be directly integrated into the extraction process. The screening-assay we proposed uses Glutathione as a surrogate compound. This surrogate is a worst-case scenario when it comes to mimicking the reactivity of amino acids, because in addition to a primary amino group, it also offers a highly reactive free thiol. Other screening assays using peptides or small proteins with no or closed disulfide-bonds as surrogate compounds, arguing that these more closely resemble the drug product. We however believe that Glutathione, as a more reactive compound, is in line with the worst-case evaluations proposed in the guidelines discussed above. Already our assay has proven useful to indicate protein-reactivity of several rubber-oligomers readily found in butyl-rubber extraction studies.[12] But more importantly, the assay helped to identify polysulfides, which are formed by vulcanising agents during the vulcanising process of rubber materials as potential agents capable of modifying disulfide bonds in proteins. These polysulfides usually go undetected in extraction studies, mainly because polymeric substances of undefined chain-length are challenging to analyse, especially so, if they are inorganic. Even though the screening assay we have proposed has shown its use to indicate protein-reactive extractables, it only identifies compounds which are reactive during the extraction experiment. Since leaching processes are time dependent, the extraction studies are performed under accelerated conditions, namely elevated temperature. We recommend 121°C for eight hours. As conditions like this accelerate the extraction process, mimicking the leaching of compounds from the immediate packaging system over the products shelf-life, it does not accelerate aging processes, apart form those which are solely temperature dependent. Therefore, compounds that leach into the product over time are, like the drug substance, prone to aging-dependent degradation, e.g. by oxidation. Therefore, we propose an addition of the established protein-reactivity screening by oxidising extracts to evaluate, if any of extractables designated as non-protein-reactive can be converted into protein-reactive degradation products. For the oxidation, a method described by the work group of Uwe Kaarst,[13] which utilises electrochemical oxidation to simulate cytochrome-mediated activation of drug substances, was adapted. As a proof-of-concept and to establish the oxidation procedure, two commonly found rubber extractables, Dibutyl hydroxytoluene (BHT) and Dimethylethyl hydroxyphenyl (DTHP), a degradation product of IrganoxTM, were used.[14,15] These Hydroxytoluene's are commonly used as antioxidants in rubber formulations and were not indicated as protein-reactive in our Glutathione screening assay. However, these compounds can be converted into Quinone methide (QM) derivatives upon oxidation, which are nucleophilic and therefore should be potentially protein reactive. The addition of an oxidation step to the Glutathione screening assay proposed here, should increase our understanding of the potential fate of compounds leached from the immediate packaging system into the to drug product over it´s shelf-life. The additional information gained from these experiments will enable a more comprehensive risk assessment of the extraction study.

Chemicals

L-Glutathione reduced (Cat.No.: G425); human Angiotensin II (Cat.No.: A9525) and Dibutyl hydroxytoluene (BHT) (Cat.No.: W218405) were purchased from Sigma Aldrich/ Merck KGaA Darmstadt, Germany. Isopropanol (Cat.No.: Nr. AE73.2), Acetone (Cat.No.: KK40.1) and Acetonitrile (Cat.No.: HN40.2) were purchased from Carl Roth GmbH & Co. KG Karlsruhe, Germany. Ammoniumacetate (Cat.No.: 012441) and Formic Acid (Cat.No.: 069141) were purchased from Biosolve B.V. Valkenswaard, Netherlands. Dimethylethyl hydroxyphenyl (DTHP) was purchased from ABCR GmbH, Karlsruhe, Germany.

Methods

Electrochemical Oxidation

The electrochemical oxidation was performed with 500 µL of diluted solutions of BHT and DTHP in a ESA Coulochem II installed with a cell 5021 at a flowrate of 50 µL per minute at 700 mV. For the control samples, the voltage in the coulometric cell was set to 0mV. BHT and DTHP were each diluted to a concentration of 0.5 mM in 50% acetonitrile in water prior to treatment in the coulometric cell. The oxidised BHT and DTHP were each incubated with and equal volume 0.1 mM solution Glutathione at 37°C for 30 min (5:1 ratio of BHT/DTHP to GSH). Angiotensin II diluted to 0.2 mM and treated with and equal volume of BHT and DTHP (1:2.5 ration of Angiotensin to BHT/DTHP).

For the rubber stopper extraction, 1.5 g of a bromo butyl stopper was cut in small pieces and extracted for 8 h at reflux in 5 ml 2-PrOH. The extract was then diluted 1:1 with with 20 mM ammonium acetate in water. 500 µL of the diluted extract was subjected to the coulometric cell and incubated with Glutathione as already described.

LC-MS Analysis

The samples were separated on a Shimadzu Prominence LC20 HPLC System equipped with an Inertsil C8, 100x2.1 mm, 5 um column heated to 50°C. For analysis, 10 µL of samples were in injected into a 300 µL per minute flow. The separation of the samples performed with the following mobiles phased: Eluent A: 0.1% Formic Acid in water and Eluent B: 0.1% Formic Acid in Acetonitrile. The Glutathione treated samples were separated by an HPLC gradient starting at 50% eluent B, which increases to 100% B within 3 minutes and remains at 100% for additional 4 minutes before returning to the starting condition. The Angiotensin II treated samples were separated by an HPLC gradient starting at 10% eluent B which increases to 100% B within 5 minutes and returning to the starting condition after 0.1 minute.

The HPLC flow was directed into an Orbitrap XL system equipped with and ESI ion source. The ion source parameters were set to positive ion mode with a spray voltage of +5kV, tube lens at +90V. The resolution of the mass detector was set to 30000 and the mass range was set to 100 to 1000 amu for the GSH samples treated with BHT and DTHP, 300 to 1500 amu for the Angiotensin samples treated with BHT and DTHP and from 100 to 1500 amu for the GSH samples treated with the rubber extract.

Results and Discussion

Establishment of Conditions for the Electrochemical Oxidation

The optimal voltage for the electrochemical conversion of Hydroxytoluene based antioxidants in coulometric cells has been previously described by McCabe and Acworth to be between 675 mV and 825 mV. The conversion rate has been reported to be at about 100%.[16] Therefore, we have decided to set to coulometric cell to 700 mV. For the establishment and proof of principle we used standards of antioxidants typically found in rubber formulations as well as in PE and PP-plastic. Whereas PE- and PP-primary packaging materials are commonly not used to store therapeutic protein, butyl-rubber materials are typically used as vial-stoppers or syringe-plungers of many marketed Biopharmaceuticals. Both antioxidants are commonly detected in extraction studies.[14,15] The Hydroxytoluenes Dibutyl hydroxytoluene (BHT) and Dimethylethyl hydroxyphenyl (DTHP), a degradation product of IrganoxTM, convert in the coulometric cell to their quinone methide derivatives, which represent reactive (electrophilic) intermediates (Figure 1).

In the screening assay described here, Glutathione is used as a surrogate for amino acid side chains in biopharmaceuticals. The Glutathione (GSH) added to the electrochemically activated antioxidants should represent potent reaction partners for the quinone-methides (QM), resulting in the respective Glutathione-BHT-QM or -DTHP-QM conjugates (Figure 2).

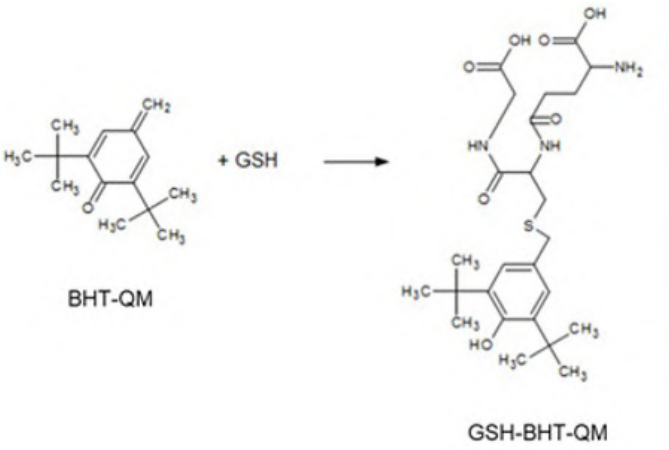


Figure 2: Reaction scheme of Glutathione (GSH) with the oxidised Dibutyl hydroxytoluene quinone method (BHT-QM).

Electrochemically activated BHT and DTHP samples were treated with Glutathione and subsequently analysed by LC-MS. The LC-MS method applied was the same as in the previously described screening assay.[12] The reactivity-assay with Glutathione was performed with BHT and DTHP, which were either subjected to electrochemical activation in a coulometric cell or left untreated. The treated and untreated samples were subsequently analysed by LC-MS and screened for masses corresponding to the Glutathione-quinone-methide conjugates. The Glutathione-BHT-QM conjugate should have a mass of 526.26 amu and the Glutathione-DTHP-QM conjugate of 584.26 respectively.

The experiments highlighted in Figure 3 clearly support the hypothesis, that the unreactive antioxidants BHT and DTHP can be converted into their reactive quinone-methide derivatives, which readily react with the Glutathione surrogate compound offered as a reaction partner.

Reaction of Electrochemically Activated Antioxidants with Angiotensin II

Glutathione with its free thiol is a very reactive compound, therefore it is often argued, that it is not a suitable surrogate to evaluate the protein-reactivity of extractables. It is true, that biopharmaceuticals usually do not contain amino acids with free thiols and that all cysteines in the sequence are paired with another cysteine to form a less reactive disulfide-bond. To

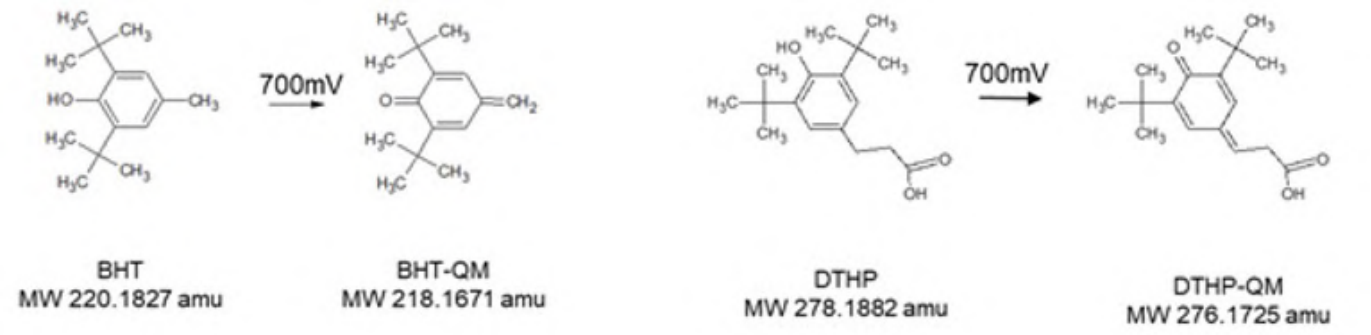


Figure 1: Scheme of the electrochemical conversion of Dibutyl hydroxytoluene (BHT) and Dimethylethyl hydroxyphenyl (DTHP) at 700 mV in a coulometric cell to their respective quinone-methide (QM) derivatives. Below the molecules their masses are given in atomic mass units (amu).

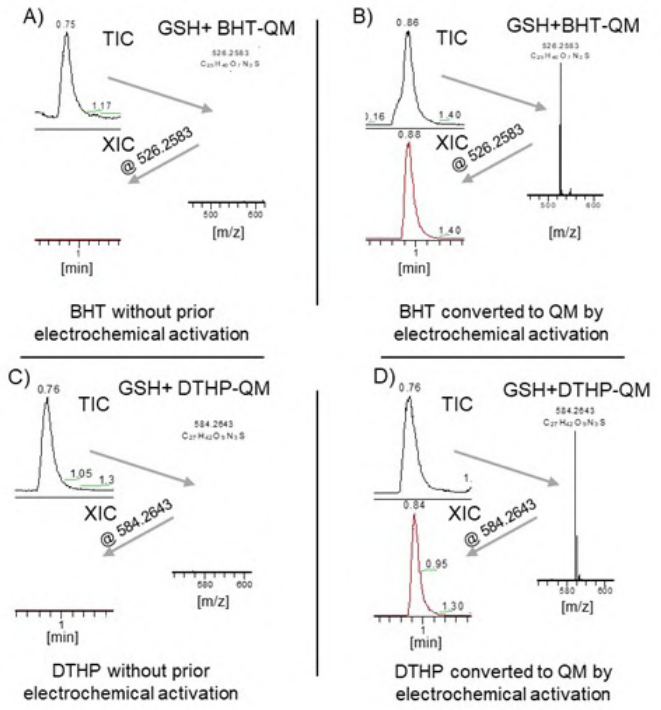


Figure 3: Results from the LC-MS based screening for Glutathione-quinone-methides conjugates. The peaks in Total Ion Chromatrogams (TIC) represent the main component in each experiment. In the experiments with the untreated Hydroxytoluenes Dibutyl hydroxytoluene (BHT) and Dimethylethyl hydroxyphenyl (DTHP), Figures A and C, on ions representing the Glutathione-quinone-methide conjugates could be found in the mass spectra. Whereas in the samples with the electrochemically activated BHT and DTHP, Figures B and C, the corresponding signals, indicating the presents of Glutathione-BHT-QM (526.26 amu) and Glutathione-DTHP-QM (584.26 amu) could be identified in the mass spectra. Further more the Extracted Ion Chromatograms (XIC) of the proposed conjugate masses, indicate real compound eluting from the column.

evaluate, if the quinone-methides generated by electrochemical activation in a coulometric cell, also react with other amino acid side chains, we performed the same experiments using Angiotensin II as a reaction partner. Angiotensin II does not contain any disulfide-bond or cysteines for that matter. Like in the experiments before, BHT and DTHP were with treated in the coulometric cells or left untreated and then added to the reaction partner. In the following only the results for the results from the experiments with BHT as shown as an example. The experiments carried out with DTHP led to the same results (data not shown).

The experiments clearly indicate, that BHT converted to its reactive quinone methide intermediate by electrochemical activation, reacts with Angiotensin II giving rise to its conjugate (Figure 4 B and E). This conjugate can be fragmented by in-source fragmentation to liberate the BHT-QM, further proof of the conjugate (Figure 4 F). As expected, the untreated BHT did not react with Angiotensin II.

The experiments with Angiotensin II firstly underline, that the quinone-methide derivatives of Hydroxytoluene-based antioxidants are very likely protein-reactive. Secondly, that at least for this class of substances, the screening assay using Glutathione as surrogate compound is predictive for studying the potential protein-reactivity of extractables subjected to simulated oxidative aging.

Electrochemical Activation of a Butyl-rubber Stopper

For proof of principle, a rubber stopper extract was used, because this packaging material is more relevant for Biopharmaceuticals. In the following example a bromated butyl-rubber was extracted by a sealed-vessel extraction procedure with Isopropanol as an extraction medium. The extract was subjected to a coulometric cell and electrochemical oxidation was achieved by applying a voltage of 700mV. As a control, an aliquot of the same extract was placed in the coulometric cell without applying a voltage. Both extracts were subsequently incubated with the Glutathione surrogate compound. The previously described LC-MS method was used to screen for Glutathione conjugates indicative of reactive extractables (see Figure 5).

As previously described the rubber extract contains BHT as a typical extractable. This extractable does not react with the Glutathione surrogate molecule, as we could show previously

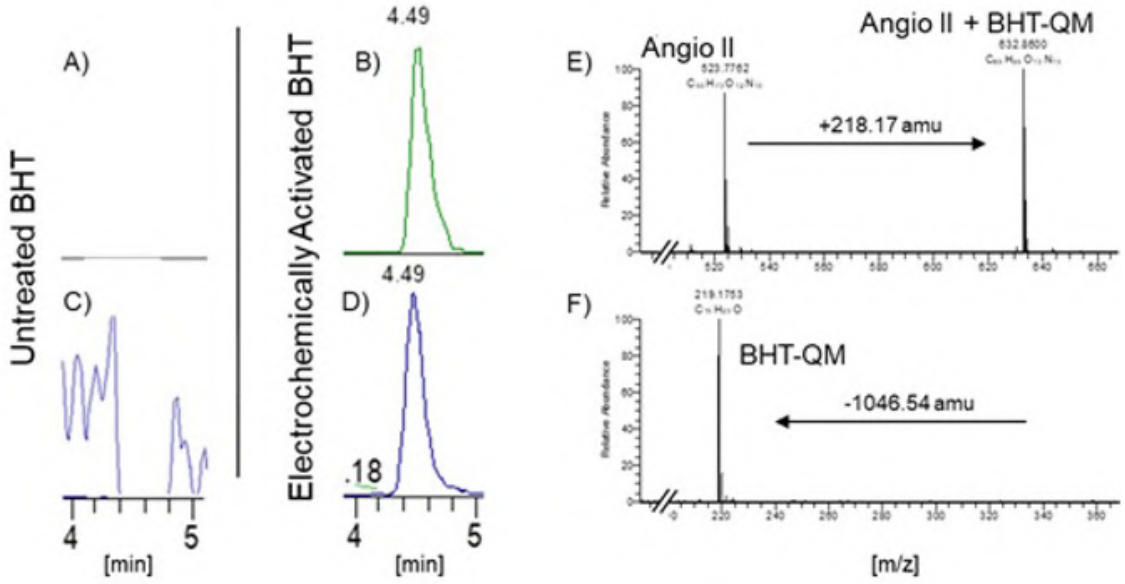


Figure 4: Mass spectrometric analysis of Angiotensin II samples treated with BHT and BHT converted to its quinone-methide derivative. In the sample where Angiotensin was incubated with BHT (Figures A) no signals corresponding to conjugates (1263.72 amu) could be identified. On the other hand, where BHT was converted to its quinone-methide, an extracted ion chromatogram indicative of the Angiotensin II BHT-QM conjugate could be identified at 4.5 minutes (Figures B)). The corresponding mass spectrum features the signal of Angiotensin II at 1045.56 amu und another signal shifted by 218.17 amu, indicative of the conjugate (Figure F). Furthermore, if an in-source fragmentation is performed, the BHT-QM can be liberated from the conjugate, resulting in a signal at 219.18 amu which leads to an extracted ion chromatogram with the same retention time as conjugate (Figure D)).

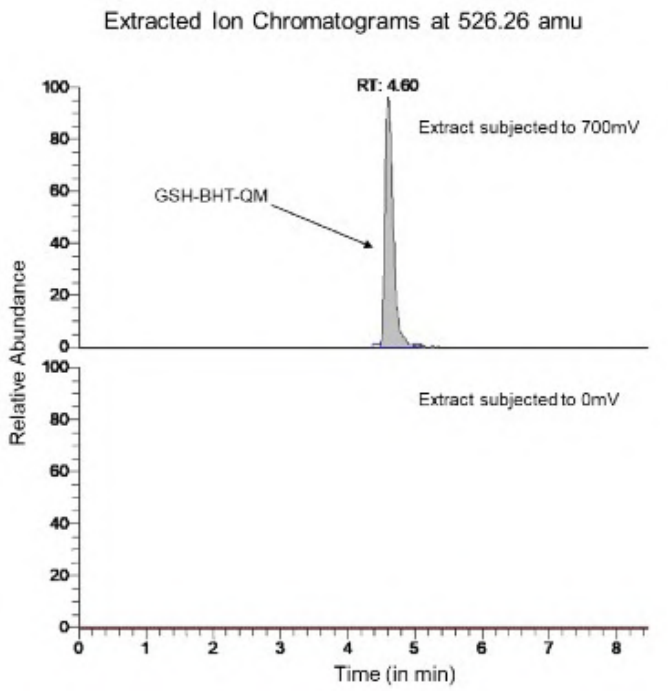


Figure 5: Extracted ion chromatograms (XIC) of 526.26 amu the mass representative for the Glutathione-BHT quinone methide (GSH-BHT-QM) conjugate. The upper XIC displays the trace obtained form the extract subjected to 700mV. The lower trace represents the control sample where no voltage was applied.

with our screening assay for electrophilic extractables.[12] Only if the rubber extract is subjected electrochemical oxidation by means of applying a voltage in a coulometric cell, a Glutathione conjugate with BHT-QM can be observed (see Figure 5). This clearly shows that the approach of electrochemical oxidation can be successfully applied to real packaging material extracts to simulate aging-dependent oxidation of extractables.

Summary

The previously described screening assays to evaluate the protein-reactivity of compounds in packaging material extracts[12] have been shown to give useful additional information to conventional extraction studies. The information from theses reactivity screenings enables a more comprehensive risk assessment of the extraction study. Whereas a conventional extraction study only allows to evaluate the risk of individual compounds based on their abundance in respect to a predefined AET, a reactivity screening, highlights compounds, which are difficult to monitor, once reacted with the biopharmaceutical drug substance. Knowledge about these compounds enables the investigator to perform further experiments, e.g. to screen for packaging alternatives which yield less protein reactive extractables, to evaluate the real risk of a drug substance to be influenced by a protein-reactive compound, to employ additional methods the monitor potential reaction products in stability- or leachable studies, or to come to the conclusion, that there are no further actions required. Whatever the outcome of a risk assessment based on a reactivity screening might be, it is needless to say, that a potential risk can only be evaluated, if it is known.

Based on the idea to broaden the foundation of the risk-based evaluation of extraction studies, we propose to include an evaluation of aging-associated reactions of extractables. The reactivity screening assays already proposed, only evaluate the reactivity of extractables during the relatively short extraction process, usual several hours or up to a day.[11,17] Therefore, extraction studies can be viewed as an accelerated leachable study, however they are definitely not set up as accelerated stability studies of the leached compounds. This excludes an evaluation of the fate of potential leachabes during the shelf-life of the product. There is a possibility, that some of the commonly found non-reactive extractables might degrade over time to form protein-reactive compounds. Therefore, a risk assessment based on an extraction study with reactivity screening will fall short to evaluate potential aging-associated degradation of leachables over the self-life of the product.

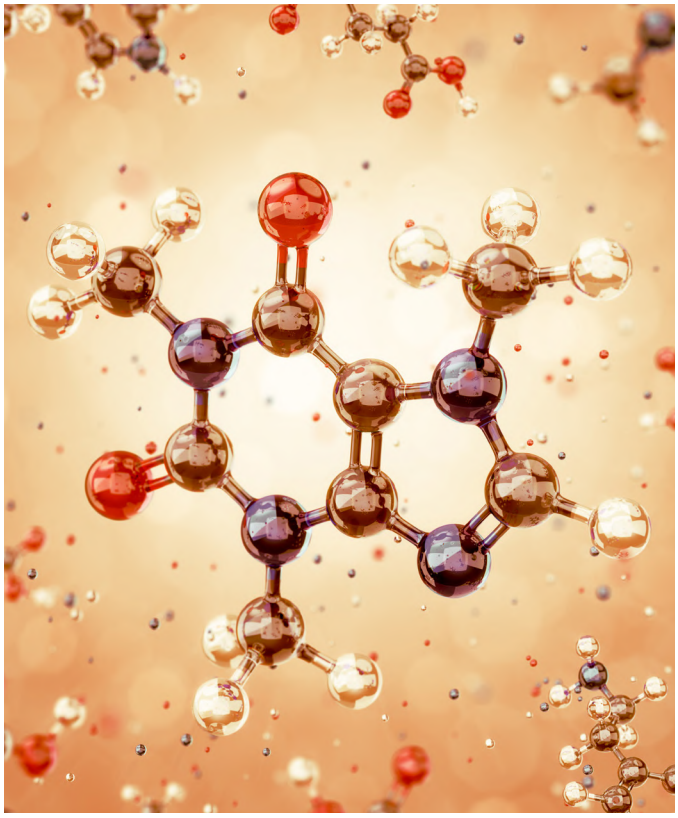
One of the common aging-related reactions monitored during stability studies are oxidations, which affect not only the drug substance and excipients, but all substances in the drug product, including leachabes. We have established an electrochemical oxidation using a coulometric cell. This approach has been previously successfully applied to study CPY-mediated oxidative conversion of small molecule drug substances.[13,18] To establish the oxidative aging of packaging material extracts, two commonly found rubber extractables were used. Dibutyl hydroxytoluene (BHT) and Dimethylethyl hydroxyphenyl (DTHP), a degradation product of IrganoxTM, are typical antioxidants used in rubber formulations.[19,20] These Hydroxytoluene based antioxidants, which are not protein reactive are known to be converted into electrophilic quinone-methide intermediates (Figure 1).[21] In this study these two antioxidants have been subjected to electrochemical oxidation, to evaluate if this approach can be applied to simulate aging-dependent oxidation. The experiments shown here clearly indicate that BHT and DTHP can be oxidized to their respective quinone-methide derivatives, which expectedly react, like all previous tested electrophilic extractables, with the Glutathione surrogate compound (Figure 3). To address the argument, that Glutathione is a too reactive surrogate to study protein-reactivity, the same experiments were repeated with Angiotensin II as a surrogate. Angiotensin II is a polypeptide with eight amino acids, none of which have a reactive free thiol and therefore features amino acid side chains that are more representative of common therapeutics proteins. The results of these experiments confirm, that BHT (Figure 4) and DTHP (data not shown) can be oxidized to yield electrophilic quinone-methide derivatives, which are capable of reacting with less-reactive amino acid side chains. Which of these amino acids are involved in the reaction with the quinone-methides still needs to be investigated.

A proof of principle experiment with an extract obtained from a bromated butyl rubber stopper, commonly used to seal glass vials containing Biopharmaceuticals, could show that the procedure of electrochemical oxidation can be applied to real packaging material extracts. The BHT extractable, as expected, does not react with Glutathione as could be shown previously.[12] However, if the extract is subjected to electrochemical oxidation, BHT is converted to its reactive quinone methide derivative which readily reacts with Glutathione (Figure 5).

The reactivity screening of packaging material extracts after electrochemical oxidation, is a valuable addition to the previously described reactivity assay. Whereas, the commonly used assays are successful in highlighting electrophilic

extractables as protein reactive, they do not account for the potential fate of leachables over the shelf-life of the drug product. During storage, aging-processes, e.g. oxidation may convert previously unreactive leachables into protein-reactive degradation products as could be shown here for the Hydroxytoluene-based antioxidants. The finding, that Hydroxytoluenes can be converted into electrophilic quinone-methides by oxidation is not particular surprising. However, the routine application of this approach to extraction studies of different packaging materials, might enable the identification of a previously overlooked extractable, as it was the case for polysulfides and their possible interaction with disulfide discovered previously.[12]

Do any of the already identified protein reactive extractables or possible reactive degradation products of leachables pose any real danger to patient safety in terms of eliciting an immunogenic response by modified biopharmaceuticals? At least the great majority of biopharmaceuticals marketed to date in standard immediate packaging systems suggest there is not. But for the sake of a thorough risk assessment, it worthwhile to make the effort, because we are only able to assess risks that are aware of!



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