HPLC-DAD / GC-MS examinations for drug safety of emergency stocks – detection, identification and toxicological qualification of a hitherto new degradation product of pyridostigmine relevant to regulatory legislation.

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During the analysis of stockpiled pyridostigmine bromide tablets, an unknown substance was detected after long-time storage. Subsequent investigations showed that the latter is a degradation product of the active pharmaceutical ingredient (API). The percentage share of this degradation product exceeds the threshold values permitted by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) for new medicinal drugs.

In order to learn about the origin of the unknown substance, stress experiments were conducted with pyridostigmine bromide tablets as well as with the active pharmaceutical ingredient and the matrix. Investigations on the identity and content of the degradation product were made by means of HPLC with diode array detection (HPLC-DAD) and gas chromatography – mass spectrometry (GC-MS).

The structure of the unknown substance was elucidated and confirmed by means of two independent procedures. The substance identified is the degradation product tetramethylurea (TMU, C5H12N2O, CAS No. 632-22-4), which has not been described so far. TMU may develop from pyrodistigmine bromide as a result of hydrolysis, decarboxylation and subsequent formation of acid amide. During the investigations conducted, the TMU content per tablet was found to be between 230 µg and 391 µg, which corresponds to a daily dose of TMU between 690 µg and 1173 µg, with 30 mg of pyridostigmine bromide being administered three times. Current data do not suggest an acute toxic effect in humans for this dose range. Animal studies have shown that in higher doses, TMU exhibits teratogenic properties after oral application. Since in the field of reproduction toxicology, stochastic effects must also be considered, the risk of a teratogenic effect in the dose range relevant for human beings can not be excluded.

A risk-oriented investigation of emergency stocks gives insights on degradation paths and degradation products after long time storage, about which comparable findings are frequently lacking. It reveals data for a scientific based risk estimation. This serves to improve drug safety in the event of a crisis.

Analysis of Proteins with Capillary Gel Electrophoresis – Fabulous Precision

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Capillary Gel Electrophoresis (CGE), also known as Capillary Electrophoresis-Sodium Dodecyl Sulfate (CE-SDS), is established in the pharmaceutical industry replacing SDS-PAGE gel electrophoresis, for example for the purity control of monoclonal antibodies. The method of an application note from Agilent [1] was used and optimized with a protein standard containing myoglobin, carbonic anhydrase, ovalbumin and bovine serum albumin (1-1.5 mg/ml). Separation voltage was -16.5 kV (-30 µA) for 30 min to analyse proteins with a maximal molecular weight (MW) of 70 kDa. It is beneficial to inject hydrodynamically instead of electrokinetically which was employed in earlier works [1-3]. Both techniques were compared in long series runs (n=48). Furthermore, the use of an internal standard was investigated. The RSD% of the migration time was reduced from 0.9% to 0.25%. The RSD% of peak area was improved as well by the use of an internal standard. However, the evaluation by the 100% method avoiding the computation of the injection error into the results demonstrated RSD% for the peak areas typically between 1 and 2%. It was crucial to obtain signal-to-noise ratios greater than 100 for optimum precision. Further, the use of CISS integration software proved to be favourable. The optimized method was used to investigate about its purity a model antibody and the MW of different reduced fragments.

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Protein Quantitation using various Modes of High Performance Liquid Chromatography

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Pharmaceuticals based on proteins (biologicals), such as monoclonal antibodies (mAb), attain more and more relevance since they were established as potent drugs in anticancer therapy or for treatment of autoimmune based diseases. Due to their high efficiency it is essential to have accurate and precise methods for protein quantitation and the detection of protein aggregates, which may lead to adverse effects after application [1]. In order to improve selectivity as well as precision compared to classic protein quantification methods such as the Bradford assay or SDS-PAGE, which do not achieve the necessary specifications of quality control (QC) purposes, High Performance Size Exclusion (HP-SEC) and Anion Exchange Chromatography (SAX) were already introduced as high selective and precise methods (e.g. SEC: < 1.9% and SAX: < 5% RSD % for peak areas inter-day) with low quantitation limits for the model proteins Ovalbumin, Myoglobin and Bovine Serum Albumin [2]. The weak Cation Exchange- (WCX) and the RP-HPLC, both already successfully applied in protein analysis, will be presented as two further possible alternatives for the QC of proteins. Both methods also provide data of high precision (RSD % peak area day-to-day < 2% for RP and < 3.5% for WCX) and low quantitation limits (< 10 µg/ml). Consecutively, the four separation modes will be compared in terms of precision, selectivity, analysis time, effort of sample and mobile phase preparation as well as separating capacity. Moreover the analysis of a monoclonal antibody is included in this study.

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Nachweis nicht deklarierter Arzneistoffe in Verdachtsproben mittels HPLC/DAD und Absicherung über ESI-MS

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Die Arzneimitteluntersuchungsstelle im LIGA.NRW untersucht im behördlichen Auftrag Arzneimittelproben des legalen und illegalen Arzneimittelhandels. Dem OMCL (Official Medicines Control Laboratory) werden von Zoll-, Polizei- und Arzneimittelüberwachungsbehörden illegal gehandelte Arzneimittel zur Untersuchung als Verdachtsproben vorgelegt. Darunter befinden sich häufig Doping- , Potenz und Schlankheitsmittel, Haarwuchspräparate und als rein pflanzlich deklarierte Nahrungsergänzungsmittel verschiedenster Indikationen). Zur rechtlichen Einstufung der Präparate und zur ggf. erforderlichen gerichtlichen Verfolgung von Straftaten sind der analytische Nachweis der verwendeten Wirkstoffe und meistens auch eine Gehaltsbestimmung erforderlich. Erste Hinweise auf mögliche pharmakologisch aktive Substanzen ergeben sich nach Einsatz einer HPLC/DAD Sceeningmethode. Die eindeutige Identifizierung, Absicherung oder Strukturaufklärung erfolgt dann mittels HLPC-MS.

Current status of hyphenated low and high resolution mass spectrometry in clinical and forensic toxicology as well as in drug metabolism

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The reliability of analytical methods helps to ensure the quality of analytical data needed for correct interpretation of analytical findings in clinical and forensic toxicology thus helping to avoid wrong treatment of the patient or analytical data being contested in court. The analytical strategy mostly includes screening, confirmation and identification followed by quantification of relevant compounds and pharmacokinetics-based interpretation of the results. Mass spectrometry coupled to gas chromatography (GC-MS) or liquid chromatography (LC-MS) is the gold standard in this field because of its universality, reliability, high sensitivity and specificity. Besides GC-MS more and more LC-MS techniques are used for target and comprehensive screening, library-assisted identification, and validated quantification of drugs, poisons and their metabolites in blood, urine or alternative matrices. Concepts and procedures using LC-MS techniques in the areas of toxicology and drug monitoring with special focus on multi-analyte procedures as well as in studying the metabolism of drugs of abuse will be presented and discussed [1-6]. The presentation will close with a short discussion of the potential of high resolution mass analyzers and future perspectives of MS in these fields.

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Illegal Medicines – a Challenge for OMCLs and Mass Spectrometry

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Medicines are counterfeited much more than all other goods - approx. 15% of world market versus 7%. This criminal activity is quickened by the free global trade and especially by the misuse of internet. This presentation shows the role and function of an OMCL for protecting public health from the serious risks of organized pharma crime. Key aspects will be the used analytical technologies, especially mass spectroscopy coupled with chromatographic separation techniques and some case studies on actual illegal medicines and counterfeits.

A novel Method for Absolute Quantification and Stoichiometry Determination of Protein Complexes

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Novel Aspect: SRM-based absolute quantification allows determination of stoichiometry and cell copy numbers of major protein complexes.

In eukaryotes, a cascade of events during cell division ensures the correct segregation of chromosomes to prevent aneuploidy. First, duplicated chromosomes are physically tethered together by a multi-subunit complex called cohesin. This process referred to as sister chromatid cohesion is established during replication in S phase and is maintained until mitosis.

We use SRM based absolute quantification to determine copy numbers and stoichiometries of these complexes throughout the cell cycle. Using our previously described EtEP method we generated an equimolar mixture of more than 100 isotopically labeled reference peptides to monitor about 30 proteins. Complex stoichiometries are determined from affinity purified protein complexes and copy numbers are measured from total cell extracts, both obtained from HeLa cells enriched in different cell cycle stages by double thymidine block and release experiments.

Molecular insights into the aging of elastic fibers

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Depending on its physiological function and the anatomical requirements of vital tissue, elastin forms different types of structures with a characteristic organization of cross-linked elastic fibers inside the extracellular matrix. Damaged fibers, which may occur as a consequence of processes such as enzyme dysregulation, pathological conditions and aging, result in a loss of elasticity. Furthermore, the release of elastin-derived peptides with biological effects may influence various cell activities including cell adhesion, proliferation and apoptosis. To understand the structural changes of elastin during these processes, it is necessary to gain insight into the morphological and molecular constitution of the native protein.
Due to its insolubility, mature elastin is hardly accessible for most studies. To date, various procedures for extracting matrix compounds from tissue and subsequently obtaining elastin have been described, of which most use relatively harsh experimental conditions. Elastin isolated by such methods is pre-damaged and thus unsuitable for further investigations.
We developed a method that facilitates the isolation of highly purified and intact elastin from small single punch biopsies. These biopsies were derived from cartilage, skin, foreskin and aorta of individuals aged between 4 and 90 years. Furthermore, few samples were obtained from the skin of patients having a genetic disorder called Williams syndrome. All elastin samples were then hydrolyzed using an elastase and the solubilized peptides were analyzed using a highly reproducible analytical approach based on nanoflow liquid chromatography coupled on-line to a high resolution mass spectrometer with nanoelectrospray ionization. The LC/MS data was then processed using a sophisticated software tool developed in-house for the extraction and normalization of all molecular species from the 3-D dataset. Comparisons were carried out by subsequent multivariate statistical analyses such as PCA.
Results of this study show clear tissue-, age- and disease-related differences in the peptide patterns of elastins. Such differences occur as complete absence of certain peptide species as well as distinct quantitative variations. For identification purposes of such peptides selected samples were subjected to tandem mass spectrometric measurements and the resulting fragment spectra were processed using a combined de novo and database sequencing approach. The identification of such markers enables the investigation of elastinolytic abilities of proteases to degrade elastin at particular residues and, thus, allow conclusions to be drawn about molecular characteristics of different elastins. Moreover, it makes it possible to identify potential changes and modifications of elastin, for instance in aged or UV-exposed skin or in tissue affected by diseases of the ECM.

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About Biological Tissues, Mass Spectrometry and Imaging

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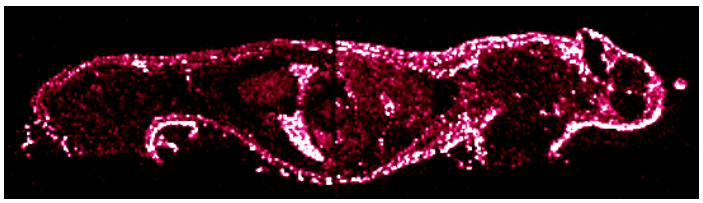
When mass spectrometric imaging started emerging from academic labs, the pharmaceutical research community rapidly developed interest in this promising new technology. Expectations ranged from discovery of new drug targets, evaluation of compound metabolism to finding of biomarkers. During the development of the technology, many aspects were tested in a number of different labs and impressive results were reported in literature. This lecture discusses the successful (and failed) applications, their value to pharmaceutical research and currently unmet needs.



This examples shows the compound distribution in a dosed rat, measured label-free by MALDI mass spectrometric imaging.

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The web site http://maldi.ms maintained by MS imaging experts contains background information relevant to this topic.

BioEquality: A Platform for the Comprehensive Analysis of Data from Stability Studies and Market Approval of Biosimilars

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Since the first approval and release of a biotechnologically produced recombinant protein in the nineteen eighties it is now estimated, that by the year 2016 eight of the top ten drugs marketed world wide will be a Biopharmaceutical [1]. Adding to this trend is the fact that by the end of 2015 Biopharmaceuticals with a revenue of 64 billion US Dollars will lose their patent protection, paving the way for generic Biopharmaceuticals the so-called Biosimilars [2].

The complexity of protein structures poses new challenges for scientists involved in early development through to production and market approval. Smallest modifications in this structure caused by slightest changes in production or during the shelf life of the product, can lead to a loss of efficacy or serious immunogenic reactions in patients. In addition to the classical analytical methods, which monitor the overall quality of the drug product, new analytical tools need to be employed, which are able to monitor modifications in the primary structure of a Biopharmaceutical.

High resolution mass spectrometry has proven to be such a tool, which delivers information about the primary structure of a protein including all its wanted and unwanted modifications. The highly complex mass spectrometric data can be analysed by specialised software solutions, which give an indication of the protein structure. One software package purpose-built for this task is the MassMap software designed by Prof. Dr. Wozny.

However the comparison of data obtained from proteins of different production batches or even a Biosimilar to the Originator is still very complex and hence time consuming.

The software designed in the BioEquality project will provide a platform for the storage and comparison of data sets created by MassMap. This will enable an analyst to monitor the primary structures of proteins during development, manufacturing and large scale stability studies. The BioEquality software is the first software package which offers its user a complete history of the Biopharmaceutical from early development right through to QC samples from market batches.

The BioEquality software will have an even greater impact on the analysis of Biosimilars. The regulatory agencies demand that the manufacturer displays the equivalence of Biosimilar to the Originator before granting market approval [3,4]. The developed software is designed to aid this process helping to reduce the high development costs for Biosimilars.

In addition to the mass spectral data the software will also integrate information from other methods, e.g., chromatographic LC and CE data, water content, osmolality ect. With this additional information researchers can correlate changes in the primary protein structure to, e.g, an altered water content in the drug product. In this way BioEquality will aid researchers to efficiently pinpoint factors that influence the efficacy of the drug product.

The BioEquality software is the first software solution that integrates structural information of the pharmaceutical active protein from mass spectrometry with test results from other methods that describe the overall quality of the drug product.

This integrative software tool will help to develop safer Biopharmaceuticals and Biosimilars, while at the same time reducing the overall development costs.