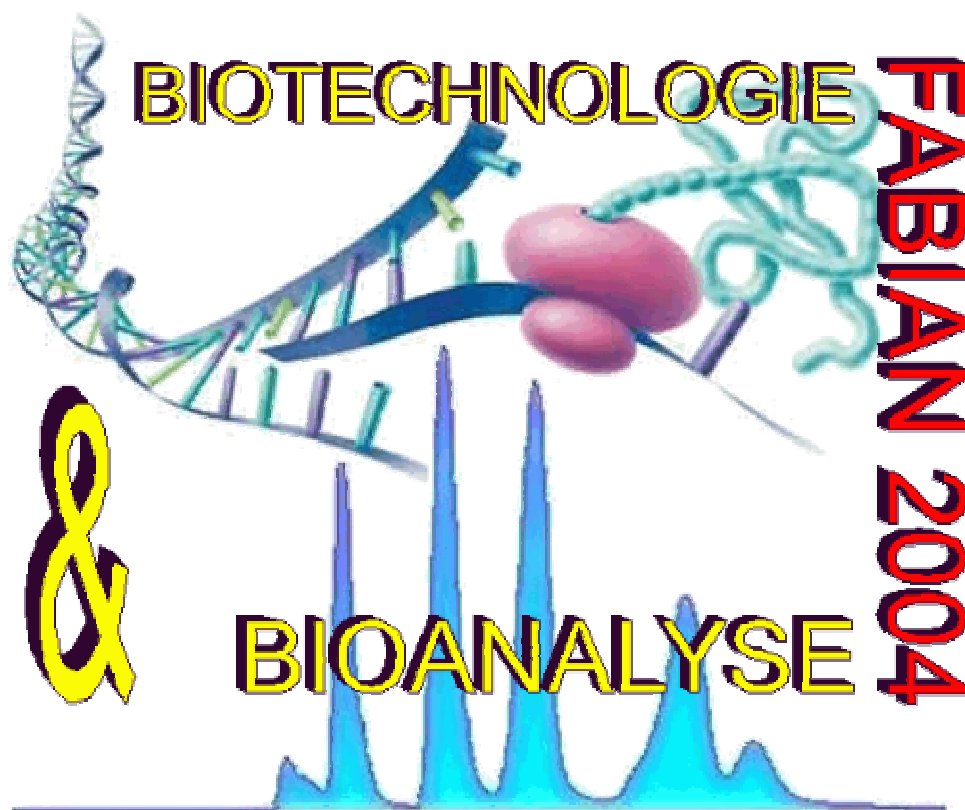


FABIAN SYMPOSIUM, 5 NOVEMBER 2004, GENT (B)
-
THEMABIJEENKOMST "BIOANALYSE EN BIOTECHNOLOGIE"

Locatie: Universiteit Gent



www.bioanalyse.org

Programma

- 09.30 Ontvangst, inschrijven, koffie
Opening
- 10.00 Huub Schellekens (UU/GDL, Utrecht, NL): Why patients make antibodies to therapeutic proteins or can bioanalysis ever beat the immune system in sensitivity ?
- 10.45 Bertjan Ziere (Pharming, Leiden, NL): Pharmacokinetics of recombinant human C1-esterase inhibitor
- 11.15 Tim L. Beumer (PBRGroup, Assen, NL): Antigenicity testing: monitoring the formation of antibodies after administration of macromolecular drugs
- 11.45 Uma Prabhakar (Centocor, Radner, PA, US): Development and validation of bioplex assays for cytokine detection in clinical samples
- 12.30 Lunch, posters
- 14.00 Corné J.M. Stroop (Organon, Oss, NL): The analysis of therapeutic recombinant glycoproteins.
- 14.30 Gideon F. A. Kersten (NVI, Bilthoven, NL): Physicochemical and immunochemical techniques for the assessment of the quality of diphtheria toxoid vaccines
- 15.15 Karel Conrath (GlaxoSmithKline, B): Characterization of vaccine antigens
- 16.00 Sluiting & Borrel / Drink

Nadere informatie

De meest actuele informatie voor, over en van FABIAN vindt u op onze website <http://www.bioanalyse.org>

Bijlagen

- abstracts van lezingen
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Abstracts van Lezingen

WHY PATIENTS MAKE ANTIBODIES TO THERAPEUTIC PROTEINS OR CAN BIOANALYSIS EVER BEAT THE IMMUNE SYSTEM IN SENSITIVITY ?

Huub Schellekens, Universiteit Utrecht, Gemeenschappelijk Dierenlaboratorium

The medical use of proteins has a long history. It started more than a century ago when immune sera from animal origin introduced for the prevention or treatment of infections, followed with the use of insulin of porcine and bovine origin some decades later. These products were immunogenic in patients, sometimes even leading to serious anaphylactic reactions. These side effects were easily explained by the foreign nature of the proteins leading to a classical immune reaction.

The introduction of human derived proteins such as growth hormone and factor VIII also was associated with the induction of antibodies. But these products were mostly given to children with an innate deficiency and therefore a lack of immune tolerance.

With the development of recombinant DNA technology the large-scale production of human homologues like the interferons, growth factors and hormones became feasible resulting in the application in a large number of patients. It was a surprise that these products also induced antibodies, which can not be explained by the lack of immune tolerance.

These antibodies are induced by two mechanisms. There is the classical reaction to foreign proteins as caused by the biopharmaceuticals of bacterial or plant origins such as streptokinase and asparaginase. The other mechanism by which antibodies are induced is based on breaking immune tolerance existing normally to self-antigens. This is the mechanism leading to the antibodies to human homologues like the interferons, IL-2, GM-CSF and EPO. The mechanisms by which tolerance is induced or broken are not completely understood. An important factor way to break tolerance is to present the self antigens in a repetitive way. A periodicity of these antigens as present in aggregates of proteins is apparently very efficient in activating ignorant or anergic B cells that are responsible for tolerance.

In the majority of cases the presence of antibodies has no clinical consequences. The most common biological effect is the loss of efficacy. The most dramatic effect of antibodies occurs if a natural protein with an essential biological activity is neutralised. Such a consequence has been described for Megakaryocyte Derived Growth Factor (MDGF) some years ago. This thrombopoietin like protein induced antibodies neutralising endogenous TPO leading to severe thrombocytopenia in volunteers and cancer patients. This effect is comparable with the EPO-associated PRCA which has been reported in 2002. The cause was a formulation change introduced in 1998. The different possible explanations why this formulation change resulted in immunogenicity will be discussed.

The current physicochemical characterisation methods do not allow to fully predict the biological and clinical properties of biopharmaceuticals. Only clinical studies and careful monitoring of the market can be used to conclusively demonstrate rates of immunogenicity in humans for protein therapeutics.

PHARMACOKINETICS OF RECOMBINANT HUMAN C1-ESTERASE INHIBITOR

Bertjan Ziere (Pharming, Leiden, NL)

<<geen abstract ontvangen>>

ANTIGENICITY TESTING: MONITORING THE FORMATION OF ANTIBODIES AFTER ADMINISTRATION OF MACROMOLECULAR DRUGS

Tim L. Beumer, M. Nemansky en N.C van de Merbel (PBRGroup, Assen, NL)

De laatste jaren worden in toenemende mate macromoleculen ontwikkeld als potentiële nieuwe geneesmiddelen. Onder macromoleculen vallen onder meer eiwitten, DNA, RNA, koolhydraten evenals complexe – slechts gedeeltelijk definieerbare - oplossingen zoals vaccins en surfactanten. Macromoleculen hebben als kenmerk dat ze potentieel in staat zijn om een antigeniteitsreactie op te wekken: dat wil zeggen dat in het lichaam antilichamen gevormd worden tegen het toegediende macromolecuul. Dit is onwenselijk, aangezien antilichamen tegen het geneesmiddel het effect van het geneesmiddel kunnen verminderen of zelfs geheel kunnen neutraliseren. Het is daarom niet verrassend dat registratieautoriteiten, waaronder de FDA, het van belang achten om tijdens de ontwikkelingsfasen van het geneesmiddel te kijken naar potentie van het geneesmiddel om een antigeniteitsreactie op te wekken.

Afhankelijk van de behoefte en de beschikbare informatie en reagentia zijn er verschillende mogelijkheden om de vorming van antilichamen te bestuderen. Als de gevormde antilichamen beschikbaar zijn, hetzij in zuivere vorm, hetzij in klinische monsters, kunnen deze gebruikt worden voor het opzetten van kwantitatieve testen, met behulp van b.v. ELISA. Als zij niet beschikbaar zijn kunnen studiemonsters (semi-kwantitatief) geanalyseerd worden met behulp van titratie experimenten. Tevens kunnen experimenten uitgevoerd worden waarin kwalitatief gekeken wordt naar de soorten en aantallen antilichamen met behulp van elektroforetische technieken zoals SDS-PAGE. In de presentatie zullen deze mogelijkheden besproken worden aan de hand van praktijkervaringen.

DEVELOPMENT AND VALIDATION OF BIOPLEX ASSAYS FOR CYTOKINE DETECTION IN CLINICAL SAMPLES

**Uma Prabhakar, Director, Department of Clinical Pharmacology, Centocor, Radner, PA, US,
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There is increasing evidence suggesting a relationship between cytokine levels and disease pathogenesis, which has led to interest in analyzing multiple cytokines in biological fluids and culture supernatants for various research and clinical studies. The introduction of methodologies allowing simultaneous measurement of inter-related biomarkers/cytokines has further revolutionized this process. In contrast to tissue culture supernatant the measurement of cytokines in serum has proven to be difficult to characterize in multiplexed formats because of the presence of large dynamic concentration ranges of proteins and other interfering factors that are present in this matrix. In the present study, we have used the microsphere-based multiplex method to simultaneously quantitate and compare six analytes, encompassing a representation of the Th1/Th2 cytokine panel (Interleukin [IL]-2, IL-4, IL-5 IFN-g, TNF-a, and IL-10), in both serum and culture supernatants from peripheral blood mononuclear cells (PBMCs). A detailed validation procedure for these determinations is described along with a comparative analysis of the performance of the multiplexed assay in serum and culture supernatant matrices. Our results indicate that precision of the multiplexed assay is comparable in both culture supernatant and serum. However, the accuracy of quantification of cytokines in the serum matrix, but not in culture supernatant may be compromised depending upon the cytokine being analyzed. Therefore, one must use caution when interpreting data from such complex matrices. Nevertheless, this assay format is appropriate to profile cytokines in clinical trial samples.

THE ANALYSIS OF THERAPEUTIC RECOMBINANT GLYCOPROTEINS.

Corné J.M. Stroop, NV Organon, ACD-Analytical Biochemistry, Oss, NL

Protein glycosylation is the post-translational, covalent attachment of carbohydrate chains to proteins. The carbohydrate chains are linked to the side chains of either Asn or Ser residues. They are referred to as N-linked and O-linked glycans, respectively. Glycoproteins are found in all eukaryotes and their importance makes them logical candidates for therapeutic use in humans.

By far the biggest category of recombinant glycoprotein therapeutics are immunoglobulins (such as IgG), commonly known as antibodies. Another well-known example of a glycoprotein therapeutic is erythropoietin, better known as EPO. Even though it has helped many patients, its ability to help athletic performance has gained it notoriety. Yet, in this presentation the focus will be on the analysis of recombinant follicle-stimulating hormone (recFSH). RecFSH stimulates the growth of follicles before fertilization. Therefore, recFSH is suited for treatment of patients suffering from infertility (both male and female). This heterodimer of 32 kDa contains 4 complex-type N-glycans (2 to each subunit).

A central characteristic of glycoproteins is the presence of different glycoforms: a single glycosylation site on different copies of the same protein bearing different glycans. This heterogeneity can differ per glycosylation site depending on the different factors that influence biosynthesis. Essentially, the analysis of a glycoprotein is the analysis of a collection of glycoforms. First, several analytical tools are used to analyze a glycoprotein as any other protein, for instance: RP-HPLC, amino acid sequencing, gel electrophoresis, and peptide mapping. In addition, some techniques are used to analyze the glycoforms or the (liberated) glycans, such as isoelectric focusing and glycoprofiling. Additional analytical power has come from the field of proteomics: mass spectrometry. Several of these techniques will be presented and discussed.

PHYSICO-CHEMICAL AND IMMUNOCHEMICAL TECHNIQUES FOR THE ASSESSMENT OF THE QUALITY OF DIPHTHERIA TOXOID VACCINES

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^{c)}Presenting author

The most critical step in the production of diphtheria vaccines is the inactivation of the toxin by formaldehyde. Diphtheria toxoid is produced during this inactivation process through partly unknown, chemical modifications of the toxin. Consequently, diphtheria vaccines are difficult to characterise and the quality of the toxoids is routinely determined with potency and safety tests. This lecture describes the possibility of monitoring the quality in diphtheria vaccine production with a selection of physicochemical and immunochemical tests as an alternative to established in vivo tests. To this end, diphtheria toxin was treated with increasing formaldehyde concentrations resulting in toxoid products varying in potency and residual toxicity. Differences in the quality of the experimental toxoids were also assessed with physicochemical and immunochemical techniques. The results obtained with several of these analyses, including SDS-PAGE, primary amino group determination, fluorescence spectroscopy, circular dichroism and biosensor analysis, showed a clear correlation with the potency and safety tests. A set of criteria is proposed that a diphtheria toxoid must comply with, i.e. an apparent shift of the B-fragment on SDS-PAGE, a reduction of primary amino groups in a diphtheria molecule, an increased resistance to denaturation, an increased circular dichroism signal in the near-UV region and a reduced binding to selected monoclonal antibodies. In principle, a selected set of in vitro analyses can replace the classical in vivo tests to evaluate the quality of diphtheria toxoid vaccines, provided that the validity of these tests is demonstrated in extensive validation studies and regulatory acceptance is obtained.

Karel Conrath, Senior Scientist, R&D ARD, GlaxoSmithKline Biologicals, Belgium

An overview of the technologies used in antigen characterization is presented. These include techniques for the study of primary, secondary, and tertiary-quaternary structure of proteins, glycoproteins, lipoproteins, polysaccharides and conjugates.

The techniques are illustrated with examples from application on Lipo-Ospa (Lyme), gD2t (Herpes simplex), Hiberix (PRRP, TT, PRRP-TT conjugates) and Hepatitis B.

The techniques described start from simple methods as SDS-PAGE, up to sophisticated technologies as Mass spectrometry, Nuclear magnetic resonance spectroscopy and Electron microscopy.

Abstracts van Posters

(overzicht niet compleet; niet alle abstracts tijdig ontvangen)

OPTIMIZATION OF AN IN SOLUTION TRYPTIC DIGEST PROTOCOL FOR USE IN QUANTITATIVE LC/MS OF PROTEINS

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Nowadays, most proteomics applications are purely qualitative or semi-quantitative using ICAT-technologies. However there is a growing need for absolute quantitation of proteins, in particularly due the growing amount of so-called 'protein-medicins' (e.g. Epo and human growth-hormone). Also, in the discovery of new protein-biomarkers there is a growing need for quantitation. Nearly all diagnostic important proteins are now currently measured by immunoassays. Nevertheless, a certain lack of specificity is an important drawback. The use of mass spectrometry coupled to liquid chromatography could solve these problems.

In a new approach, we seek to optimize an alternative technique to quantify proteins with LC-MS. Herefore we enzymatically cleave a given protein, typically with trypsin, and choose a unique set of marker peptides. These peptides, representing the protein of interest are then quantified using LC/MS-MS with an internal standard.

In a first step it is necessary to develop a reproducible in solution trypsin digest procedure. To this end, we evaluated several protocols using Promega and Sigma trypsin. Our protein of interest is Cystatin C, a 146 amino acid protein of 13 kDa with two disulfid bridges between amino acid 99-104 and between amino acid 123-143. Cystatin C is chosen as a model but also for his potential as a marker of kidney failure. We started from the standard Promega in solution trypsin protocol and evaluated the optimum time and duration of the denaturation process. We found 1 h at 65° to be the best conditions. After that DTT vs beta-mercaptoethanol was investigated. A combination of both reducing agents proved to give the best results. Adding iodoacetamide to the protocol for protecting the free SH groups, dramatically increased the sequence recovery. As a last step the optimum protease:protein ratio was investigated. Three different ratios (10:1, 20:1 and 50:1) were compared. In general, the tested ratios didn't effect the sequence coverage.

By optimization of the in solution tryptic digest protocol we reached a 91.92 % sequence recovery. Herefore we denature for one hour at 65° with a solution of 50mM Tris-HCl/ 6M urea/ 5mM DTT/ 10% betaME. After diluting with 50 mM Tris-HCl / 1 mM CaCl₂ to get the urea concentration below 2M we add iodoacetamide to a final concentration of 20 mM. After that trypsin is added in a protease:protein ratio between 1:10 and 1:50.

OPTIMISATION OF LC-MS CONDITIONS FOR A METABOLOMIC APPROACH

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Metabolomics is a rapidly growing area in the 'omics'-science. It endeavours to reliably separate and detect as many metabolites as possible in a single analysis. To this goal, we are developing an LC-MS tool to quantify metabolites in a relative way. Arabidopsis thaliana metabolites of different classes were chosen as representative compounds for evaluation of several chromatographic columns. The main purpose was to create an LC method that has high separation efficiency within an acceptable time. This means that the metabolites must be unravelled as much as possible in a single chromatographic run.

In terms of metabolites, one of the problems one has to deal with is the predominantly polar character of the compounds. In view of the intended LC separation, this has to be taken into consideration when aiming for a reversed phase type of separation. Reversed phase chromatography is considered as it provides high separation efficiency based upon a stable and reproducible stationary phase. A possible alternative would be HILIC chromatography. However, it remains to be proven whether this type of separation mechanism provides the same amount of robustness and application diversity. Recently, specialty reversed phase type materials, particularly suited for the retention of highly polar compounds have become available. Here we report on the use of such a type of material, the Atlantis dC18 column (1mm x 15mm, 3µm, Waters™) for the separation of thirty model metabolites representing a varying degree of polarity. A comparison is drawn up with an Inertsil ODS-3 column (1mm x 15mm, 5µm, LC Packings®) and a Zorbax XDB column (0.5mm x 15mm, 3.5µm, Agilent®). Both of these columns represent the modern types of reversed phase material, suitable for the separation of polar compounds although not specifically introduced for that particular purpose. At the same time, we aimed to use a mobile phase which provided adequate chromatographic separation as well as being compatible with both positive and negative ion electrospray ionisation.

Our chromatography system consisted of a CapLC autosampler equipped with a 10µl injection loop (Waters™), combined with a CapLC pump. A flow of 40µl/minute is used (20µl/minute for the 0.5mm column). During the initial development of a method it became quickly clear that providing retention for the most polar model

compounds e.g. uracil and lysine, was a major issue. Furthermore, we aimed to distribute the compounds covering a broad polarity range (log D (pH3) varying between -7.85 and 6.63) over a reasonable separation duration of 30 minutes. To that end, we have evaluated the columns by testing a number of different gradients (length, steepness and multistep) and comparing the separation efficiency (tR, peak width, AUC, peak capacity...).

To obtain a separation of metabolites of divergent polarity within a narrow time window, a gradient set-up is developed. Taken in account our most polar metabolites, it proved necessary to start with a 100 percent aqueous phase as eluent A. Gradient elution was then performed up to a 90/10 acetonitrile/water mixture (eluent B). Formic acid (0.1%) was chosen as mobile phase additive from a chromatographic as well as a mass spectrometric point of view. Thus it was possible to acquire samples in both positive and negative ion mode without changing eluents.

Detection was performed using a QTOF micro instrument (WatersTM) equipped with a Lockspray in both positive and negative ion mode (ESI+/-). Optimal Q-TOF parameters were investigated for each compound, resulting in a consensus of acquisition parameters for all metabolites available. All samples were acquired in both positive and negative ion mode, resulting in a separation device according to ionisation efficiency.

We conclude that for our application, the performance was very similar for the Atlantis dC18 and Inertsil ODS-3 columns. Those columns were insensitive to dewetting problems and showed very reproducible retention [mean CV% (relative retention time): Atlantis dC18 3.16±8.79; Inertsil ODS-3 3.08±9.06]. The Zorbax XDB column showed less dewetting resistance and thus less reproducibility [mean CV% (relative retention time): 11.25±13.92]. At the end, we chose for the Atlantis dC18 column because some compounds only showed up using this column. We succeeded in achieving a good separation of polar and apolar compounds in a broad polarity range/limited time gradient. It nevertheless has to be stressed that complete separation was never the ultimate goal. Indeed, this separation will now be extended towards biological (plant) extracts resulting inevitably in major overlap of the many metabolite peaks, even while we succeeded in making as much as possible use of the whole polarity range within a 30 minutes separation. Single MS detection using high resolution data will alleviate identification uncertainty of coeluting metabolites.

EVALUATION OF THE METABOLOMIC TOOL: MALDI-MS FOR THE ANALYSIS OF SMALL MOLECULES

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The most recent 'omics' technology applied to marker discovery is metabolomics, which can be defined as the study of the collection of small molecule (<1000 Da) metabolites in biofluids to elucidate differences in population groups due to genetic modification, disease state and environmental stress.

Spectroscopic techniques such as H-NMR have been applied in a number of studies but also complementary MS technology, especially exact-mass LC-MS, has been introduced. LC-MS does not provide complete metabolomic coverage. In particular, very polar compounds, such as simple sugars and many amino acids, elute in the void of the column. Another area of improvement is the cycle time of the LC-MS analysis and interpretation of results. Because approaches to quantitative small molecule MALDI-MS have been reviewed recently, its inherent robustness, low sample consumption, no preceding separation and high sensitivity, we want to evaluate this technique based on MALDI as ionisation technique and accurate mass analysis, in our metabolomics study. By using this method in combination with multivariate techniques, we aim to distinguish groups based on their differences on metabolite level.

The object of this study was the exploration of the operational parameters concerning the application of MALDI-ionisation technique for small molecules on a Q-TOF mass spectrometer and the evaluation of its potential relative quantitative character.

After an optimized effective calibration with a PEG 400-600-Nal-mix, we performed an evaluation of different parameters with the help of clearly measurable drug compounds. The optimized conditions e.g. laser firing rate = 10, speed = 3, collision energy = 4, MS profile and positive ionisation mode remained intact for the tests of different matrices (2 mg CCA, 2 mg DHB, 3.95 mg pyridinium-CCA salt, 4.1 mg methylimidazole-CCA salt, 9.27 mg tributylamine-CCA salt/ml 50/50 acetonitrile/water and 0,1% TFA) at variable matrix-compound ratios (2, 5 and 20 mg matrix/ml and 5, 10 and 25 ng analyte/ml). Based on the preceding results, we optimized the analysis of metabolites (ng-mg/ml) on a MALDI-Q-TOF. Because these results were disappointing for metabolites, we tested other small molecules in negative mode, with different structure and by using variable matrices.

In the light of given experiences with a MALDI-TOF (M@LDI-instrument) we also performed an investigation (n=5) into the influence of the laser energy on the ionisation of small molecules with different matrices by changing the lens position of the nitrogen laser against the source block, thus adapting laser fluency.

During the initial development of the MALDI-ionisation technique it became quickly clear that the analysis of some small pharmaceutical drugs is possible with a MALDI-Q-TOF by using 2 mg/ml CCA as matrix, but no quantitative results are observed. Not even a roughly increasing tendency for a growing amount of analyte, nor better ionisation by increasing matrix quantity is perceptible.

Moreover, a clear signal for metabolites was never really obtained, under no circumstances, with none of the matrices. Logically a quantitative relationship was absolutely out of the question.

Based on the different experiments using various operational conditions as well as different small molecules, in an effort to correlate ionisation success with physico-chemical characteristics, we conclude that the ionisation of small molecules using MALDI is largely based on coincidence and unsuitable for a screening analysis.

CAPILLARY-LC-MS/MS FOR THE QUANTIFICATION OF ENKEPHALINS.

B. S. Sinnaeve and J. F. Van Bocxlaer, Laboratory of Medical Biochemistry and Clinical Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, Ghent, Belgium.

The aim of our work is to absolutely quantify peptides, in our case the neuropeptides leucin-enkephalin and methionin-enkephalin. Sensitive determination of neuropeptides is necessary, certainly in view of the often small sample volumes and the low concentrations present in biological fluids. As sensitivity was the most important requisite, a miniaturised LC-MS/MS system was used. We have evaluated the possibility of a capillary LC-MS/MS system (300 µm) with on column focusing for the quantification of enkephalins.

Optimal separation of a standard mix of eight neuropeptides was achieved with reproducible retention times. LC solvents A and B consisted respectively of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in a 80/20 (v/v) acetonitrile/water mixture. The gradient consisted of initial 5 minutes at 100% A, in which the neuropeptides are trapped on the trapping column, followed by a linear gradient to 40% B.

The observed validation results show that it is possible to determine peptides as low as 5 fmol on column in MS/MS (MRM) using a triple quadrupole system. Linearity is good in a dynamic range of two to three orders of magnitude. Accuracy of quality control samples (QC1 and QC2, respectively 5 and 100 fmol on column) was below 30%.

The system indicates the possibility of capillary LC-MS/MS to quantify neuropeptides in the low femtomole range. However, at this stage, i.e. without isotopically labeled internal standardisation, validation results offer room for improvement.

REAL TIME ANALYSIS OF THE PHOTOOXIDATION OF BEER BITTERING PRINCIPLES BY QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY COMBINED WITH HEADSPACE GAS CHROMATOGRAPHY MASS SPECTROMETRY

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Significant taste and flavour changes are observed, when beer is exposed to light. A particular off-flavour, known as lightstruck flavour (LSF), results from formation of 3-methylbut-2-ene-1-thiol (MBT). The flavour threshold of MBT is very low and few ng per liter are sufficient to make beers unpalatable. Isohumulones, the main bittering principles in beer, are essential in the pathway to LSF formation. On visible light irradiation, these five-membered-ring hop derivatives release an electron on interaction with triplet excited flavins. In our model system, photooxidation of isohumulones, as well as their reduced forms, was achieved by excited beer flavins. However, the fate of the remaining one-electron oxidised species was not clear. Therefore, a detailed product investigation in order to reveal possible light-induced degradation mechanisms was aimed at.

Loading the reaction mixtures into transparent syringes and exposing them to visible light at the same time as they were injected into the ionisation source of a quadrupole time-of-flight hybrid mass spectrometer furnished us a real-time analysis technique. Thus, irradiation simultaneous with continuous flow injection in the electrospray ionisation source resulted in the elucidation of non-volatile reaction products. Further evidence was obtained from analysis of volatile reaction products by headspace gas chromatography mass spectrometry.

Visible-light irradiations of isohumulones and their reduced forms in the presence of flavin mononucleotide (FMN), readily induced decomposition. From identification of reaction products thus formed, feasible degradation mechanisms could be proposed. Degradation of these five-membered-ring hop derivatives resulted in a 3-methylbut-2-enyl radical, which is the key precursor on the route to the development of MBT. Remarkably, reduced isohumulones e.g. dihydroisohumulones, used in the brewing of light-stable beers, also showed radicaloid decomposition when exposed to visible light in the presence of FMN. This result contradicts the commonly held belief that these compounds withstand photoinduced degradation. The application of reduced derivatives of isohumulones as a substitute for isohumulones in so-called light-stable beer is, therefore, questionable.

CHARACTERISATION AND CLASSIFICATION OF REVERSED-PHASE LIQUID-CHROMATOGRAPHIC COLUMNS.

PART I: COMPARING COLUMN CLASSIFICATION AND COLUMN PERFORMANCE FOR SEVEN DIFFERENT SEPARATIONS

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Keywords: RP-LC column, Column characterisation; Chromatographic test; PCA

The selection of RP-LC columns, which gives suitable selectivity for a particular separation is difficult if the brand name of the support is not known. Official compendia like the European Pharmacopoeia give only a general description of the stationary phase in the operating procedure of a liquid chromatographic method for drug analysis.

From the literature, 36 parameters were chosen and combined to a test procedure of 8 methods. Using principal component analysis (PCA) on 24 reproducible parameters, a final procedure of 4 parameters was obtained [1,2]. The correlation between the general test parameters and seven real separations was examined by comparing the classification obtained with the test results and the performance of separation of drugs and their impurities: acetylsalicylic acid [3], nimesulide, phenoxymethylpenicillin, buflomedil hydrochloride, clindamycin hydrochloride, chloramphenicol sodium succinate and dihydrostreptomycin sulfate [4]. Further investigation concerning the variability of batches has been done in part II: Study of the inter- and intrabatch variability of C18 columns using 4 selected chromatographic test parameters.

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CHARACTERISATION AND CLASSIFICATION OF REVERSED-PHASE LIQUID-CHROMATOGRAPHIC COLUMNS.
PART II: STUDY OF THE INTER- AND INTRA-BATCH VARIABILITY OF C18 COLUMNS USING 4 SELECTED CHROMATOGRAPHIC TEST PARAMETERS

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Keywords: Column classification, Chromatographic test, PCA

The European Pharmacopoeia and other official compendia give only general descriptions of the stationary phase to be used in a liquid chromatographic (LC) analysis of a drug. A test procedure to characterise reversed-phase LC columns was previously developed and introduced [1-2]. The final procedure is based on the determination of 4 different LC parameters, which were selected from 36 measured parameters on 69 columns [3] using principal component analysis (PCA). The classification, obtained from the 4 parameters, was correlated with seven real separations of drugs and impurities in the first poster: Part I. Comparing column classification and column performance for seven different separations.

In this study the final test procedure was expanded by involving newly commercialized stationary phases. On the other hand, the types of columns were limited to the most widely used columns for pharmaceutical purposes: C18 columns with a particle size of 5 µm and a length of 250 mm. About 50 different types of stationary phases (silica type A and B, end-capped/non-end-capped, base deactivated/not base deactivated and polar embedded) from several manufacturers were tested. In each case, two columns from the same batch were examined to be able to verify intra-batch deviations. Inter-batch deviations of the chromatographic parameters were also studied by including former results using the website [4]. The classification of the columns was performed using Statistica software as in the previous study and the reproducibility was calculated.

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CHIRAL CAPILLARY ELECTROPHORETIC METHOD FOR QUANTIFICATION OF APOMORPHINE ENANTIOMERS IN MEDIUM FOR IN VITRO PHARMACOKINETIC STUDY

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Keywords: Apomorphine; Capillary electrophoresis; Chiral

A new method for chiral determination of apomorphine enantiomers was developed and validated. Seven different neutral and charged cyclodextrins were tested for enantioselectivity on R,S-apomorphine. Sulfobutylether- α -cyclodextrin was found to offer the best resolution, but with this cyclodextrin system, 4 peaks were detected from a solution of the 2 enantiomers, which was proposed to be the result of different forms of the complex between the selector and apomorphine. A complexation constant was calculated for a complex of 1:1 ratio for the second and the fourth peak, whereas the other two peaks were fitted to a model ratio of 1:2 (analyte-selector). To avoid this phenomenon, hydroxypropyl- α -cyclodextrin was then chosen as the chiral selector. An optimisation study was performed to obtain optimum conditions with 14 mM of hydroxypropyl- α -cyclodextrin dissolved in 100 mM Tris-phosphate buffer pH 3.0, with 16 kV applied voltage. UV detection was at 200 nm. The method was validated at the chosen conditions, with an LOD of 0.2 μ M and LOQ of 0.5 μ M. The method was applied for the determination of R,S-apomorphine in a transport study with an in vitro cell culture model of the intestinal mucosa (Caco-2).

COMPARISON AND IMPROVEMENT OF LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF RELATED SUBSTANCES IN INDINAVIR SULPHATE

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Keywords: Protease inhibitors, Indinavir sulphate, LC, Drug analysis

Indinavir sulphate (IND) is a synthetic protease inhibitor, which is used in combination therapy with other antiviral drugs for the treatment of human deficiency virus infected patients. So far, no method has been discussed in the scientific literature for assay and purity control of IND, but monographs have been published in the Indian Pharmacopoeia (IP) [1] and the United States Pharmacopoeial Forum (USPF) [2].

The Ind. Ph. method prescribes a C8 column kept at a temperature of 40 °C. The mobile phase consists of acetonitrile, citrate buffer pH 7.5 and isocratic elution is performed at a flow rate of 1.0 ml/min. UV detection is performed at 260 nm. High base line noise is observed at 220 nm due to the high absorbance of citrate buffer in the mobile phase. The USPF method prescribes a C18 column kept at room temperature. Two mobile phases consisting of acetonitrile and phosphate buffer pH 7.6 are used for gradient elution at a flow rate of 1.0 ml/min. UV detection is performed at 220 nm. With this method system peak problems were observed.

A gradient method is developed to improve the detectability of the impurities, which are eluted after the main peak. The method uses a base deactivated C18 column (Hypersil BDS), 5 μ m (25 cm x 4.6 mm I. D.) kept at a temperature of 40 °C. The mobile phases consist of acetonitrile, phosphate buffer pH 7.5 and water. The flow rate is 1.0 ml/min. UV detection is performed at 220 nm. Five commercial samples were examined using the method developed.

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ACCURATE MASS ANALYSIS OF GLYCOPROTEIN ISOFORMS BY ELECTROSPRAY IONISATION, ORTHOGONAL ACCELERATION TIME-OF-FLIGHT MASS SPECTROMETRY AND MAXIMUM ENTROPY

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The mass measurement of low molecular weight molecules (<1000 Da) to better than 5 ppm accuracy is currently a routine procedure, provided an internal calibrant or lock mass is introduced with the sample. However, it is only recently that this level of accuracy was extended to the measurement of intact proteins. The mass of the human haemoglobin β -chain (15867.2 Da) was determined with a standard deviation of ± 0.05 Da (± 3.2 ppm) using the α -chain (15126.4 Da) for internal calibration of the mass scale (1).

Within an electrospray spectrum of a pure protein, there is a series of multiply charged species whose mass-to-charge (m/z) ratio is given by $(M_r + nH)/n$, where M_r is the molecular weight of the protein, H is the mass of the proton and n is a series of integers representing the number of charges associated with each species. In order to simplify interpretation, particularly from mixtures of proteins, algorithms have been developed to deconvolute

these multiply charged spectra into a single peak on a true molecular weight scale. Of these algorithms, the one that uses a maximum entropy (MaxEnt) based approach is the most powerful because it is automatic, enhances the resolution, improves the signal-to-noise ratio and can deconvolute complex mixtures of proteins (2-3). Here we demonstrate accurate mass measurement of the isoforms of a glycoprotein (34 kDa) using an internal calibrant and Maximum Entropy deconvolution of the data obtained on an orthogonal acceleration time-of-flight (oaTof) instrument.

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3. Waters Corporation, MS Technologies Application note, number 212 (<http://www.waters.com>)

CHARACTERIZATION OF IGG GLYCOSYLATION USING INTACT PROTEIN ANALYSIS AND PEPTIDE MAPPING.

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Glycosylation is a cotranslational and a posttranslational modification found in proteins. N-linked glycosylation is found in the Fc region of immunoglobulins. The covalently bound oligosaccharides vary in composition and branching within different classes of immunoglobulins. Glycosylation is vital for bioactivity and pharmacokinetics of biotherapeutic proteins. We characterized glycosylation in monoclonal IgG by "top down" and "bottom up" approaches using intact protein mass analysis and peptide mapping. The intact protein analysis was carried out using SEC-MS and RP-MS. Global mass analysis of glycosylated, partially glycosylated and deglycosylated IgG revealed that the sugar moiety has mass of approximately 1468 Daltons corresponding to the asialo-biantennary N-linked oligosaccharide with core fucose. Signature ion scan for mass 204 in the peptide map of IgG revealed 3 distinct glycol-peptide peaks. Based on the mass of these resolved peaks we were able to confirm that they were composed of different glycoforms, confirming the heterogeneity observed at the intact protein analysis. The MS/MS analysis of the G0 glycopeptide was also obtained and the composition of the oligosaccharides confirmed by daughter ion assignments.

HT QUANTITATIVE ANALYSIS FOR A DRUG MIXTURE IN MOUSE URINE AND PLASMA - A COMPARISON OF HPLC/MS/MS AND UPLCTM/MS/MS

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One major application of LC/MS/MS is the quantification analysis. An optimum quantification protocol is a fast separation with sufficient resolution to obtain low limit of detection. Major challenges in achieving an ultra-sensitive quantification protocol include matrix interference, limitations of LC/MS system such as column efficiency, system volume, system back pressure etc., as well as the data collection speed of the MS. Improvement in LC/MS/MS quantification relies on either to improve LC separation (column efficiency, peak capacity etc) via increasing flow rate, reducing column dimension/particle size, and reducing system volume, or to improve the data collection speed in the mass spectrometer. This work demonstrates the advantages in quantification analysis by systematically improving both LC and MS.

The mixture has 5 compounds: Alprazolam, Diphenhydramine, Ibuprofen, Naproxen, and Prednisolone.

Alprazolam was calibrated with the deuterated internal standard, and the other compounds were each analyzed by external calibration. Two tandem quadrupole mass spectrometers were used for this study: MS1 and MS2.

These two MS were coupled with a HPLC system (LC1) or a novel ultra high pressure LC system (LC2: i.e.

Waters Ultra Performance LCTM = UPLC system). A binary gradient was used for the separation at a flow rate of 0.3 ml/minute: mobile phase A is 10 mM ammonium acetate in water at pH 5.0, and mobile phase B is 10 mM ammonium acetate in acetonitrile. The data monitoring was by multiple reaction monitoring (MRM) with on-line polarity switching.

A new quantification protocol was established using a novel ultra high pressure LC technique and a fast acquisition mass spectrometer, providing a significant improvement in sensitivity over conventional LC/MS and allowing low fg on-column detection, with linear range at three and half orders of magnitude.

VENTURE A - HYPERFORMANCE AFFINITY COLUMNS FOR ANTI-BODY PURIFICATIONS

Noud Grimberg, Aurora Borealis Control B.V., P.O. Box 2, NL - 7760 AA Schoonebeek, The Netherlands

Grace Vydac introduces the VENTURE A column, the first in the VENTURE line of affinity chromatography columns utilizing the ICET (Inert Coating Enhancement) surface passivation technology to eliminate non-specific binding on the silica surface.

ICE technology enabled the VENTURE A columns to be the first affinity column to take advantage of silica gel's rigid porous structure, providing greater productivity and capacity to users. Ventures A columns use a recombinant protein-A ligand for binding antibodies. The rigidity of the silica particles enables the columns to run under HPLC and FPLC modes with linear velocities from 150 up to 5000 cm/h and its optimized structures in capacities of 40 mg/ml for human polyclonal globulins.

The performance characteristic of this new affinity HPLC Columns will be demonstrated as well as its performance against competitive materials

IMMOBILIZED ARTIFICIAL MEMBRANE (IAM) CHROMATOGRAPHY

Noud Grimberg, Aurora Borealis Control B.V., P.O. Box 2, NL - 7760 AA Schoonebeek, The Netherlands

Immobilized Artificial Membrane (IAM) chromatography phases mimic the lipid environment found in cell membranes. Since phosphatidylcholine (PC) is the major phospholipid found in cell membranes, IAM phases prepared from PC analogs are models of cell membranes. These materials model the hydrophobic and hydrophilic contribution of a drug's partitioning and can be used for elucidating drug-membrane interactions. Therefore, IAM HPLC Drug Discovery columns are useful tools for predicting drug membrane permeability.

QUANTITATIVE ANALYSIS OF OLIGONUCLEOTIDES IN PLASMA USING HYBRIDIZATION ASSAY AND LC-MS

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In the discovery of new medicines the importance of the so called biopharmaceuticals is growing. Therapeutic oligonucleotides (like anti-sense oligonucleotides or silencing RNA) are biopharmaceuticals which can be used as therapeutic agents interfering with the expression of genes involved in disease processes. Quantitative bioanalysis of single and double stranded oligonucleotides is mandatory. For the quantitative bioanalysis of anti-sense oligonucleotides in plasma we successfully implemented an ultra sensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay. In this hybridization ELISA oligonucleotide concentrations down to 0.5 pmol/ml (~1 ng/ml) could be measured without any sample pretreatment. To demonstrate the suitability of the hybridization ELISA for pharmacokinetic studies a 20-mer phosphorothioate anti-sense oligonucleotide was administered to rats and the plasma concentrations of the oligonucleotide at different time points after intravenous bolus injection were determined using the hybridization ELISA. In parallel with the ELISA-based method we have developed a generic LC-MS method for the quantitative instrumental analysis of oligonucleotides in plasma. An extraction of single stranded oligonucleotides from plasma using strong anion-exchange and solid phase extraction was combined with an LC-MS method. The LC-MS method has been set up using triethylamine (TEA) ion pairing and hexafluoroisopropanyl alcohol (HFIP) to improve ionization. By using a high resolution triple quadrupole mass spectrometer (TSQ Quantum, ThermoElectron, API4000, Sciex) oligonucleotides differing only one nucleotide can easily be chromatographically separated and uniquely identified.

AN IMPROVED COATING FOR THE ISOLATION AND QUANTITATION OF INTERFERON- γ IN SPIKED PLASMA USING SURFACE PLASMON RESONANCE (SPR)

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A study was initiated to investigate the use of surface plasmon resonance (SPR) for the detection in plasma of a high pI model protein, recombinant human interferon- γ (IFN- γ). Initially a number of self assembled monolayers (SAMs) and hydrogel-derivatised SAM-coatings were characterised for the adsorptive and desorptive properties of plasma components. Next a monoclonal anti-IFN- γ antibody, MD-2, was covalently attached to dextran-modified mercaptoundecanoic acid surfaces that performed best.

On coatings consisting of carboxyl-modified dextran (CMD) different interaction behaviour was observed when IFN- γ was injected in either buffer or diluted plasma. During the injection of IFN- γ in buffer, the binding rate accelerated and the signal continued to increase after the injection was finished. Upon injection of diluted plasma spiked with IFN- γ , the response increased without acceleration of the binding process. After the injection was finished, some of the bound material desorbed resulting in a signal decrease.

On non-charged dextrans, the interaction between the antibody modified surface and IFN- γ in either plasma or buffer was similar. During sample injection the response increased with a binding rate depending on the concentration of IFN- γ present in solution. When the injection was finished, some of the bound material was washed from the surface and only a minor contribution of non-specific adsorbed plasma components was noticeable.

From the coatings tested, the non-modified dextran-coated SPR sensor disks prove to be best suited for the detection of IFN- γ in complex matrices like plasma. The interaction of IFN- γ in both diluted plasma and buffer is comparable and concentrations of IFN- γ of 250 ng/ml and higher can be detected in both matrices. The non-specific adsorption of plasma components is low whereas the specific IFN- γ response is relatively high.

Locatie / reizen / overnachten

Lokatie



Het symposium wordt gehouden in dit prachtige gebouw "Het Pand" in de fraaie historische stad Gent. Voor meer informatie verwijzen wij U graag naar de desbetreffende pagina's van de site van de Universiteit van Gent:



Het Pand is gelegen naast de Sint-Michielskerk, Onderbergen 1

(http://www.ugent.be/nl/personeel/dienstfacil/pand/Algemene_informatie_Het_Pand/Historiek_Pand)

Openbaar vervoer

Voor reizen naar Gent met de trein adviseren wij U om gebruik te maken van de sites van de Belgische spoorwegen: <http://www.b-rail.be/main/index.html> (nationaal) en/of <http://plannerint.b-rail.be/bin/query.exe/en?L=b-rail> (internationaal). U kunt hier zelf de route en uurtabel opmaken. Let wel, het eindstation hier in Gent is "Gent St. Pieters", het hoofdstation in Antwerpen is "Antwerpen centraal" maar voor internationale treinen kan dit ook dikwijls "Antwerpen Berchem" zijn (zeker nu er grote werken aan de gang zijn in Antwerpen centraal). Het station verbonden aan de luchthaven in Brussel is "Brussel nationaal luchthaven" of "Bruxelles national aeroport".

Overnachten in Gent

Voor een of meerdere overnachtingen in Gent adviseren wij U een hotel in het centrum van de stad te kiezen. Voor enkele suggesties zie: [Hotels](#)
(<http://www.bioanalyse.org/symposia/2004/hotels.htm>)