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Absolute Quantification of Monoclonal Antibodies in Serum

Charlotte Hagman, 19 April 2010

Outline of presentation

- **Advantages of peptide and protein therapeutics with respect to small molecules**
- **Absolute quantification in comparison to relative quantification**
- **Strategies for reducing sample complexity in serum prior to quantification of peptides or protein therapeutics.**
- **Five albumin depletion kits were evaluated with regard to albumin depletion efficiency, specificity and reproducibility.**
- **Results after limited validation of the method for the quantification of a Hmab in two matrices.**

Different types of therapeutics: Small molecules, peptide and protein therapeutics

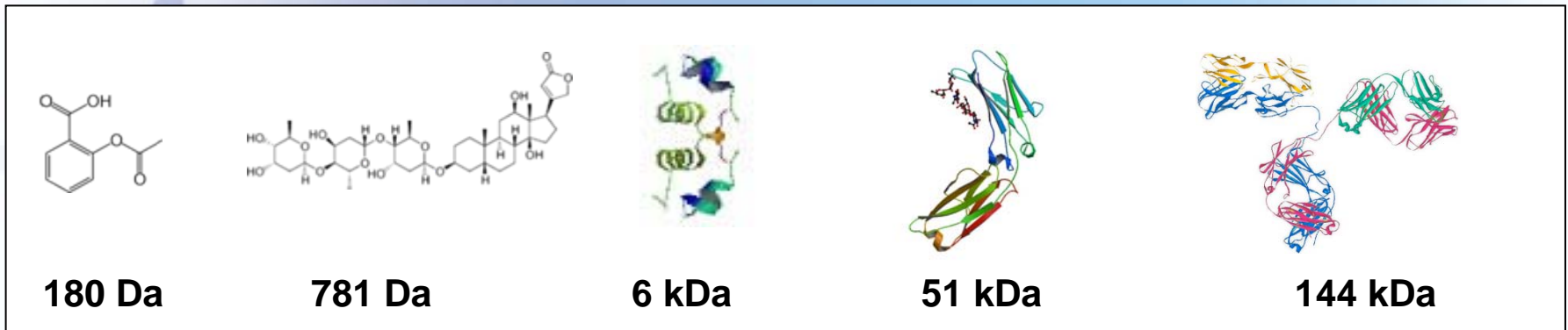
Acetylsalicylic acid

Digoxin

Insulin

Alefacept

Basiliximab



Acetylsalicylic acid - 1899, Aspirin - Headache and pain

Digoxin - 1785, Lanoxin - Atrial fibrillation, atrial flutter

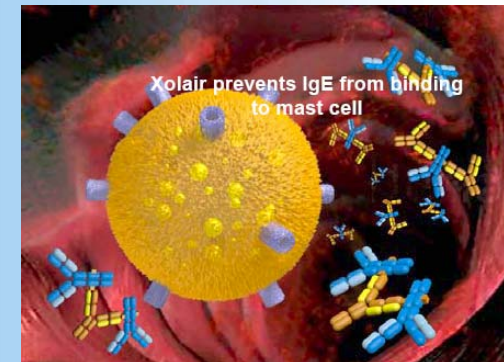
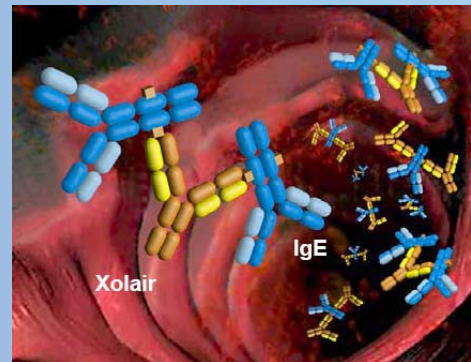
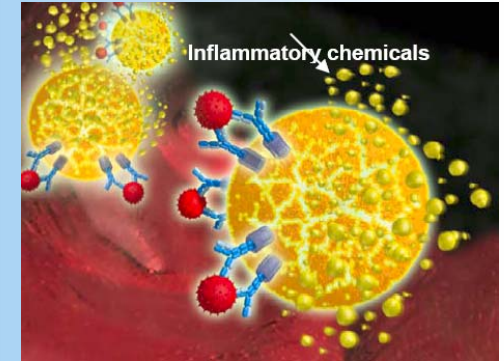
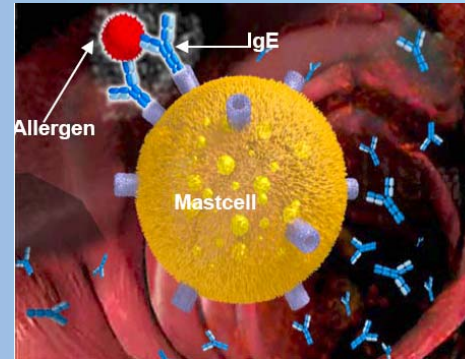
Insulin -1922 - Treating diabetes

Alefacept - 2005, Amevive - Immunosuppressive function, psoriasis

Basiliximab - 1998, Simulect - Immunosuppressive function, rejection in organ transplantation

Advantages of mAbs

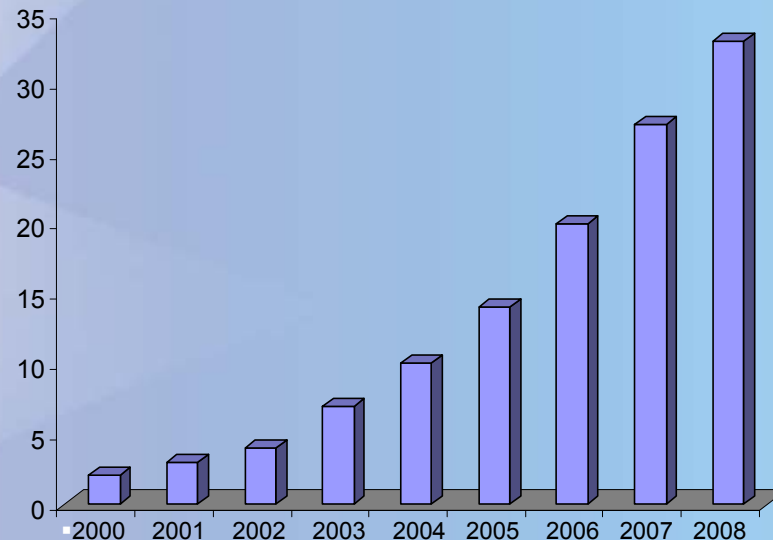
- The mAb's target specificity is important for example in cancer treatment.
- Possibility to engineer features such as: affinity, avidity, bio-distribution, and half-life.
- HmAb's can neutralize pathogens, toxins, and endogenous proteins



The market for protein therapeutics

Worldwide sales of antibody-based products

Billion \$



The table includes:

Rituxan®
Herceptin®
Avastin®
Erbix®
Mylotarg®
Campath®
Humira®
Remicade®
Synagis®

Currently approximately 150 mAb's are in development.

Total global monoclonal antibody sales are forecasted to reach \$ 49 billion by 2013

Quantification with ligand-binding assay

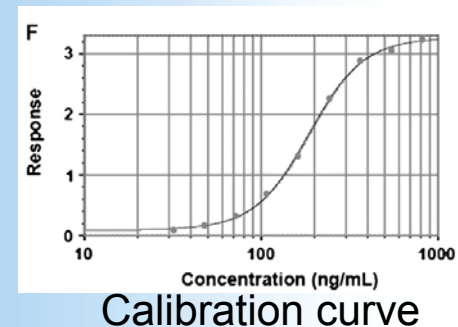
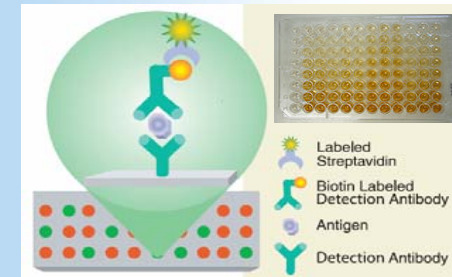
The ligand binding assay (LBA) –measurement depends on a high affinity biological interaction between the macromolecular reference standard and a capture/detection antibody.

Advantages: Fast and sensitive analysis.

Drawbacks: Endogenous anti-antibody response, unspecific binding, time for development, accuracy and precision.

Relative quantification, difficult to compare results

LBA-reader



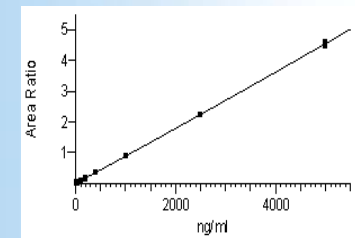
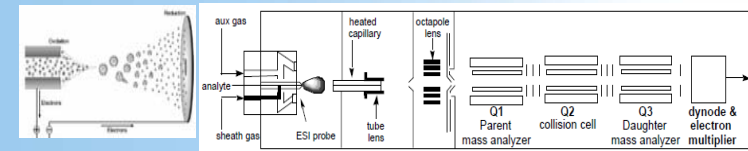
Quantification with tandem mass spectrometry

The basis for the LC-MS/MS measurement is the chemical properties of the reference standard.
Non-affinity based approach.



Advantages: Fast to develop, potential to overcome interference from anti-antibody response, improved assay precision, isotope labeled internal standard - absolute quantification is possible

Drawbacks: Currently lower sensitivity compared to LBA

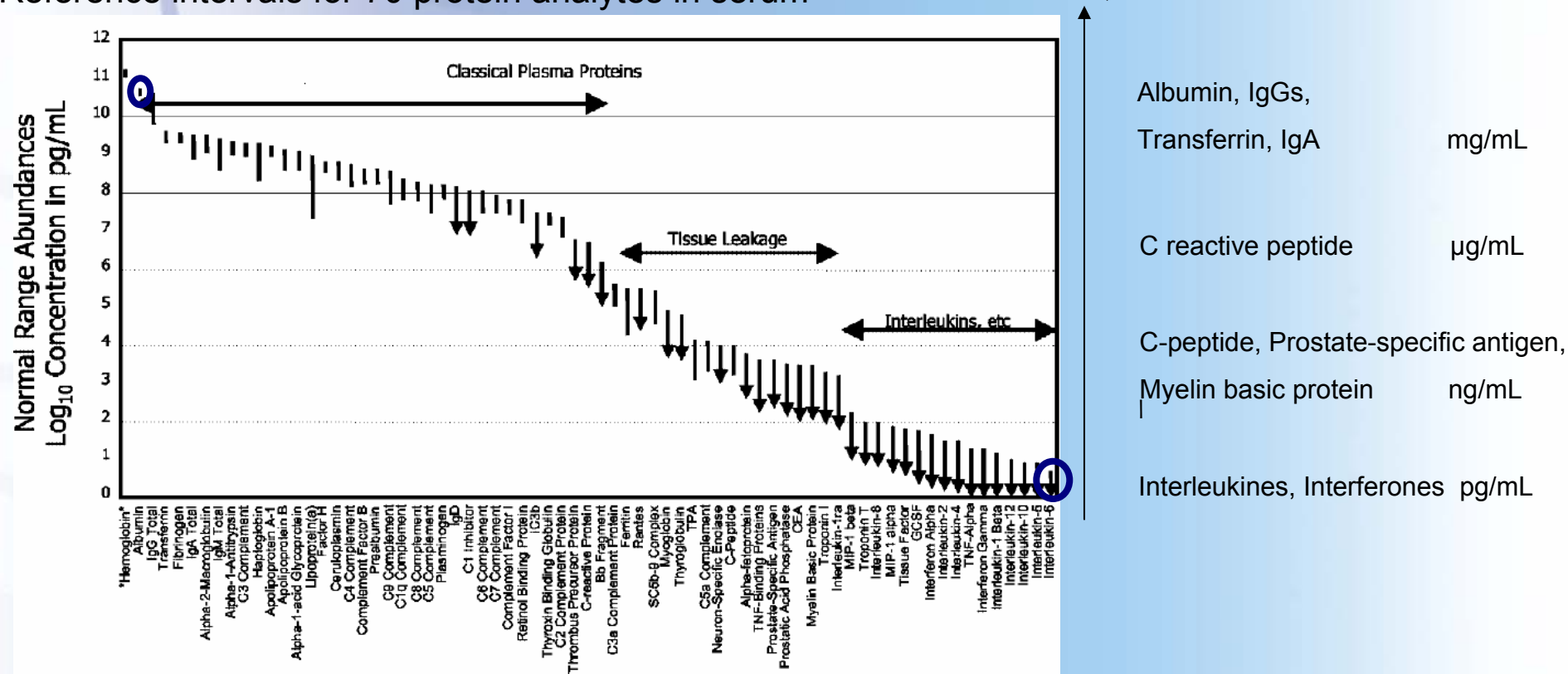


Calibration curve

Serum proteins and dynamic range

Reference intervals for 70 protein analytes in serum

L Andersson *et al.* 1:845-867, 2002 *Mol Cell Prot*



The dynamic range for albumin and interleukin 6 concentration is 10 orders of magnitude.

However, the dynamic range of LC-MS/MS analysis is only 3-4 orders of magnitude

Comparison of the Characteristics of Small Molecule and Macromolecule Compounds

Characteristic	Small molecule	Macromolecule
Size	Small < 1000 Da	Large > 5000 Da
Structure	Organic molecules	Amino acid biopolymers
Purity	Homogeneous	Heterogeneous, due to post translational modifications
Solubility	Often Hydrophobic	Often Hydrophilic
Stability	Chemical	Chemical, physical and biological
Presence in Matrix	Xenobiotic	Endogenous

Adsorption to surfaces

Chemical stability and stability in biological fluids

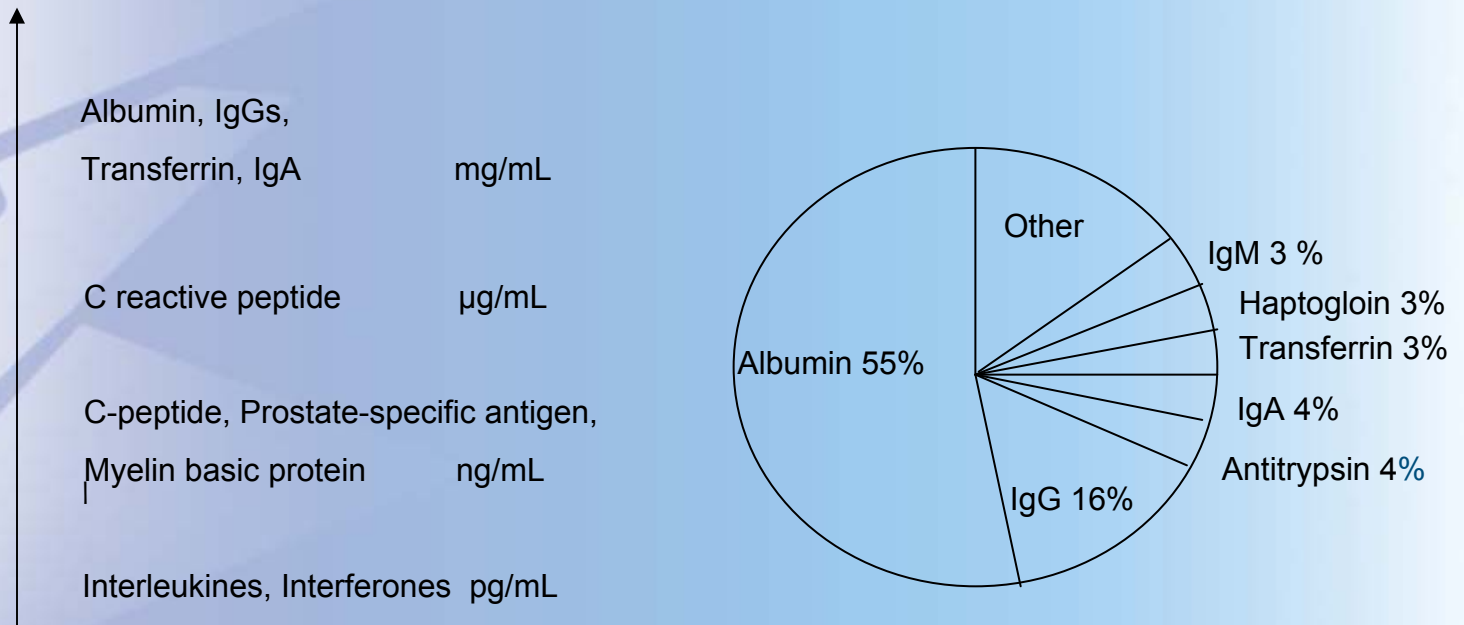
Endogenous versus exogenous peptide

Presence of endogenous peptide in control plasma

Peptide and protein therapeutics - sample preparation

- For peptide therapeutics
- Protein precipitation
- Solid phase extraction, mixed mode cartridges
- Quantification of intact target therapeutic.
- For protein therapeutics
- Targeted depletion of abundant proteins. Digestion and quantification of selected signature peptide.
- SISCAPA approach, antibodies against signature peptide
- Targeted enrichment of target protein with affinity purification. Digestion and quantification of selected signature peptide.

Dynamic range and composition of serum



- The most abundant proteins that constitute 95% of the bulk mass of proteins represent less than 0.1% of the total number of proteins.



Depletion with commercially available depletion kits

Different formats:
Single use columns
Permanent columns

Electrophoresis 2010, 31, 471–482 V. Polaskova et al.

	ProteoPrep	Seppro	MARC	Qproteome	Vivapure	Aurum
Albumin	•	•	•	•	•	•
IgG	•	•	•	•	•	
Transferrin	•	•	•			
Fibrinogen	•	•				
IgA	•	•	•			
α-2-Macroglobulin	•	•				
IgM	•	•				
α-1-Antitrypsin	•	•	•			
Complement C3	•					
Haptoglobin	•	•	•			
Apolipoprotein A-I	•	•				
Apolipoprotein A-II	•	•				
Apolipoprotein B	•					
α-1-Acid Glycoprotein	•	•				
Ceruloplasmin	•					
Complement C4	•					
Complement C1q	•					
IgD	•					
Prealbumin	•					
Plasminogen	•					
Number of depleted proteins	20	12	6	2	2	1

Targeted depletion kits for serum proteins

Albumin depletion - removes approximately 55% of the total protein content

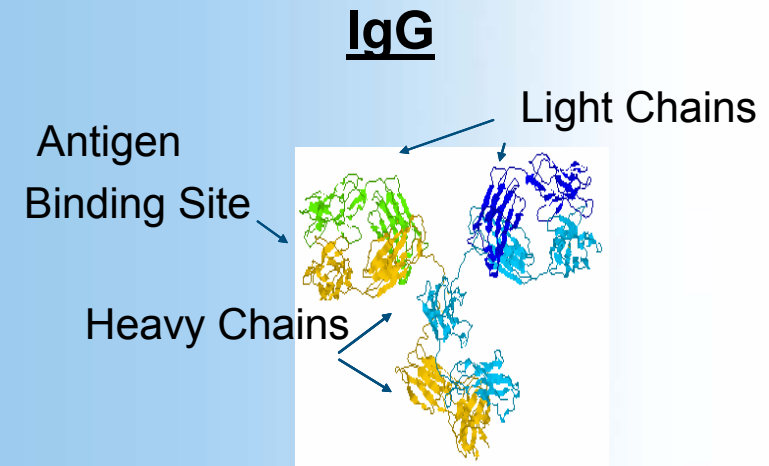
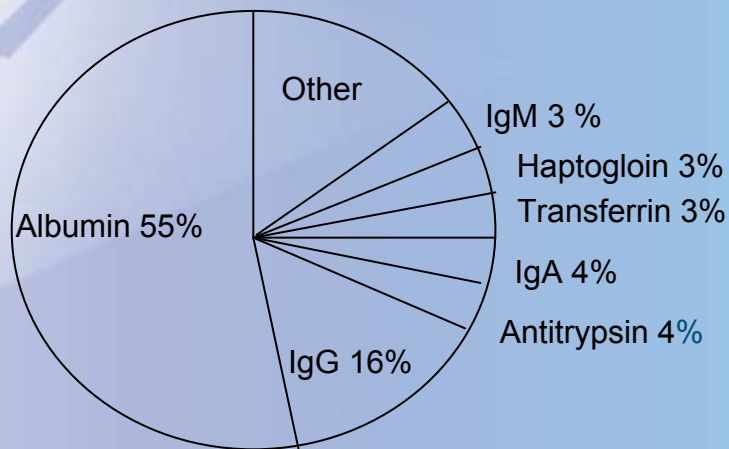
Albumin + IgG depletion - removes approximately 70% of the total protein content

Targeted depletion of the six most abundant proteins (albumin, IgG, IgA, transferrin, α -transferrin, α 1-antitrypsin and haptoglobin)

Targeted depletion of the twelve most abundant proteins (the above mentioned proteins plus: apo A-I and A-II, IgM, orosomucoid, fibrinogen, α 2-macroglobulin)

How to choose the appropriate sample preparation method?

- High sequence homology between the HmAb and serum IgGs
- Can not use depletion kits which removes IgG
- We focus on albumin depletion to reduce sample complexity





Cibachrome blue dye based albumin depletion kits

MILLIPORE

Based on a derivate of Cibachrome blue dye
 The kit should be able to process 100 μ L serum
 For 20 μ L serum, albumin should be depleted to 85%

Typical Human Albumin Depletion Measured by Radial Immunodiffusion Assay

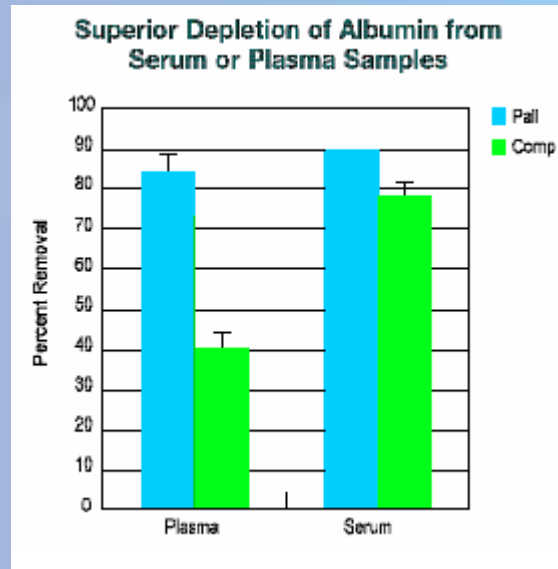
Human Plasma Volume (μ L)	Albumin Depletion (%)
20	85
50	75
75	65
100	58

PALL

- The Enhanced Albumin Depletion Kit uses a dehydrated Cibachrome blue dye based support
- Each disk removes >2 mg albumin
- Recommended sample load: \leq 30 μ L serum.

BIO-RAD

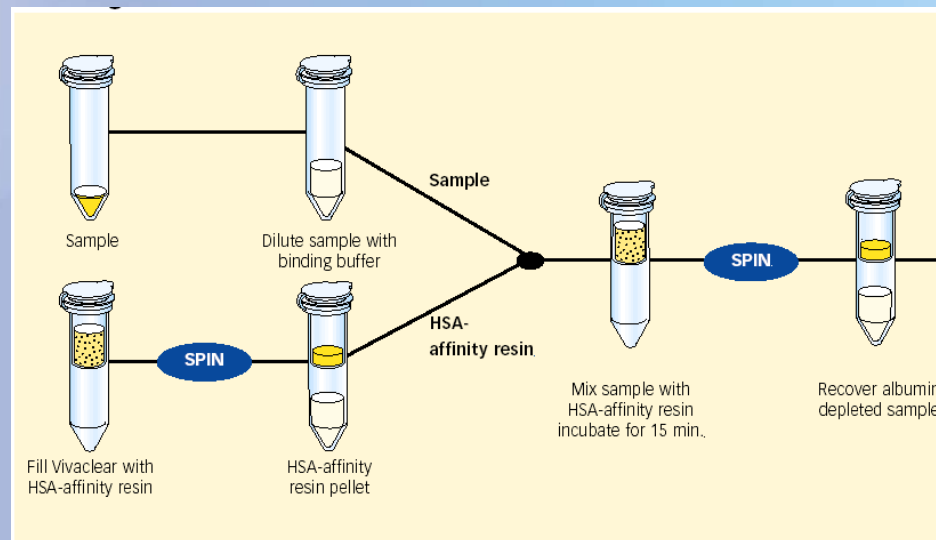
- The support is a cross linked agarose gel, with covalently attached Cibachrome blue F3GA-dye
- The drawback is the lack of specificity
- Recommended sample load: 125 μ L serum



Non-dye based albumin depletion kits

VivaScience

- The high affinity for albumin is achieved by coupling unique antibody fragments to a low binding cross-linked agaros
- This kit should have no cross reactivity with other human proteins
- When loaded with 20 μ L of serum the kit should remove 95% of the albumin content. Allows preparation of several samples in parallel



Non-dye based albumin depletion kits

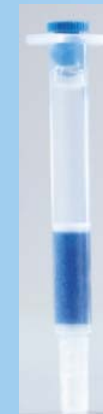
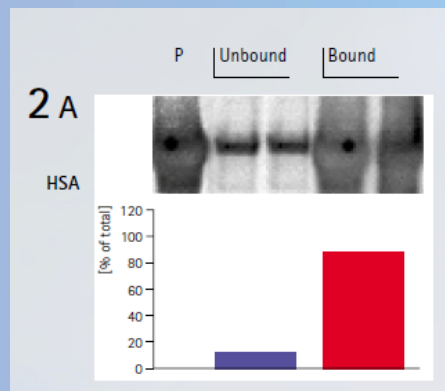
CalBioChem

The ProteoExtract Albumin Removal Kit is based on a new affinity resin, highly specific for albumin, which is not based on Cibachrome blue dye

The columns are pre-filled disposable gravity flow columns. Allows preparation of several samples in parallel

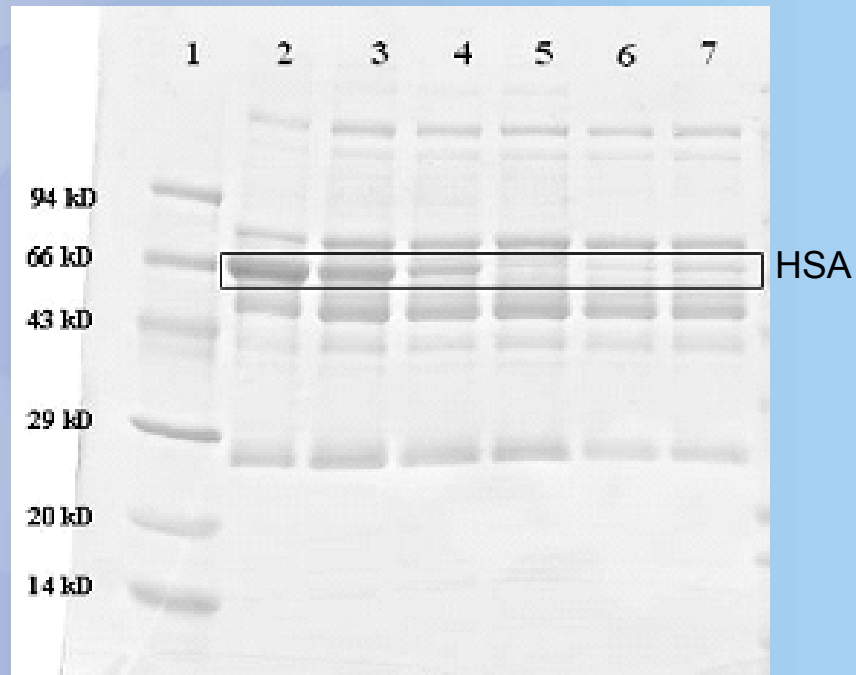
Samples volumes of 35 μ L results in > 80% removal of albumin

Loading the column with 35 μ L serum, 90 % of other protein markers such as transferrin, antithrombin III and factor VII are recovered in the albumin depleted sample.



SDS-PAGE of albumin depleted serum

In each lane 7 µg of sample



2 Crude serum

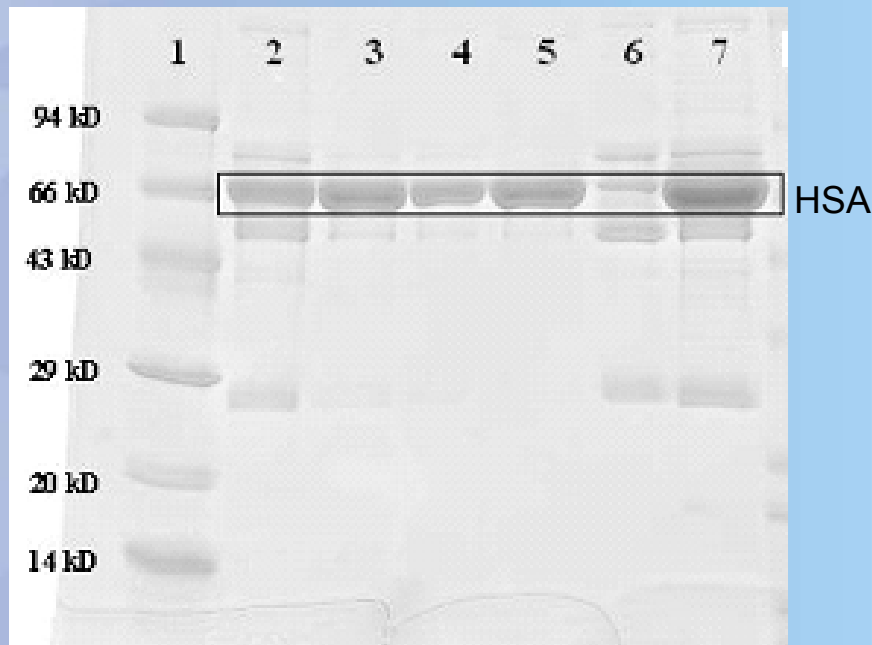
● 3	Millipore
4	VivaScience
5	CalBiochem
● 6	PALL
● 7	BioRad

● Cibachrome blue dye kit

Tris-Gly 4-20 % gel, 200 V for 1 hour

SDS-PAGE of proteins eluted from depletion columns

In each lane 7 μ g of sample



2 Crude serum

● 3	Millipore
4	VivaScience
5	CalBiochem
● 6	PALL
● 7	BioRad

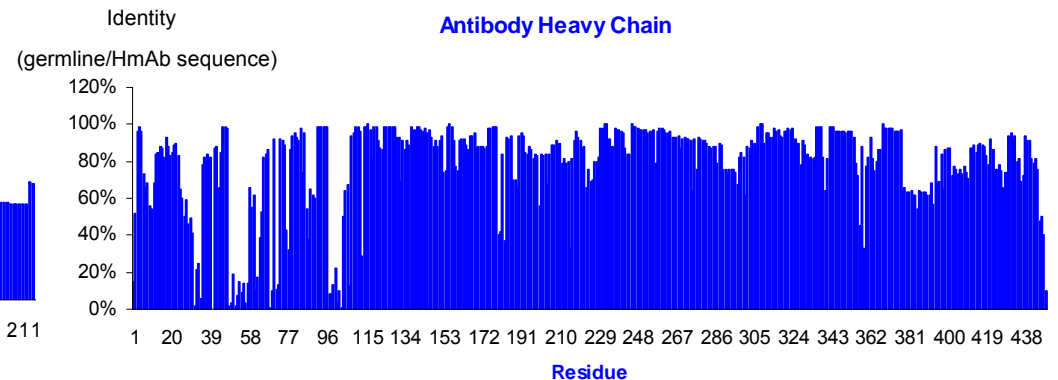
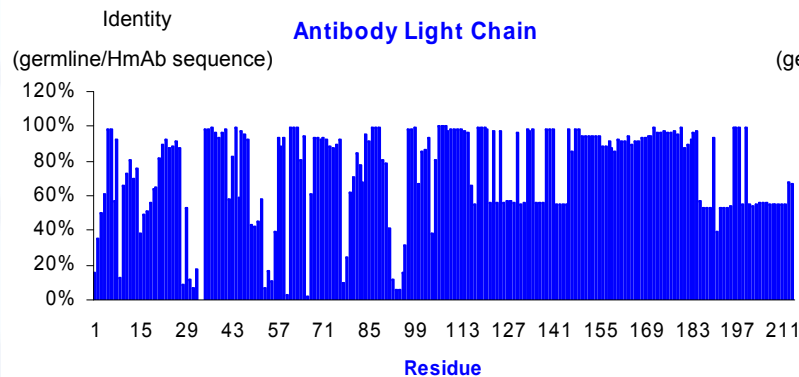
● Cibachrome blue dye kit

Tris-Gly 4-20 % gel, 200 V for 1 hour

By spectrophotometric analysis it was established that after depleting 50 μ L of serum using the Cal BioChem kit, 49% of the total protein content was removed.

Sequence homology between the HmAb and IgGs

- The total protein content in serum consists to $\approx 16\%$ of IgGs
- Identify sequences that are specific for the HmAb
- A bioinformatics tool was constructed based on alignment with germline and HmAb sequence.
- Residues with a low identity score are regarded as unique



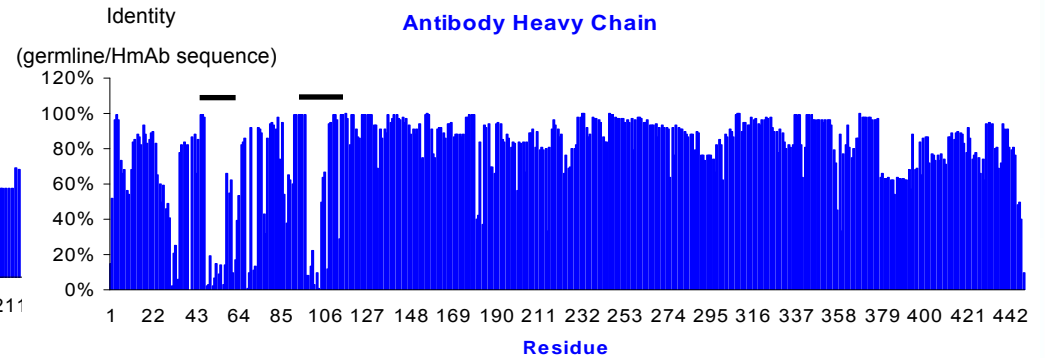
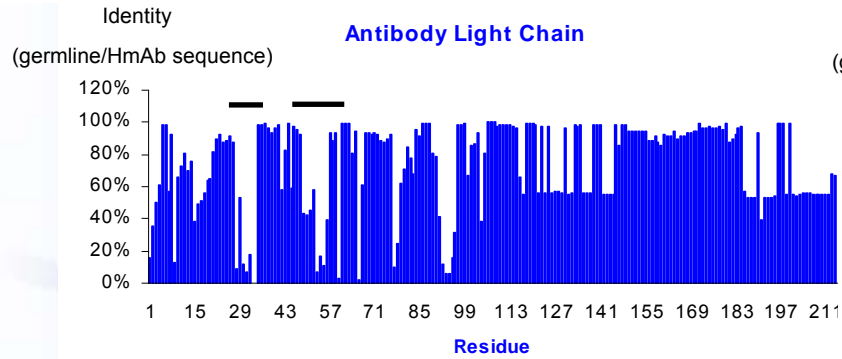
The NIH genetic sequence database: GenBank contains a collection of all publicly available DNA sequences, ≈ 80 billions base pairs

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences.

Dennis A. Benson, Ilene Karsch-Mizrachi, David J. Lipman, James Ostell, and David L. Wheeler

Nucleic Acids Research 2006 Jan 1;34:D16-20

Finding signature peptides for the quantification of the HmAb in serum



25 ---peptide1---39

50---peptide2---61

39---peptide3---59

99---peptide4---119

To think of when selecting signature peptides:

- Avoid KK and RR-sequences
- Avoid residues that are prone to chemical modifications eg. Met, Trp and Cys
- The most relevant selection parameter is the peptides ionization properties and MS/MS fragmentation properties

Absolute quantification using isotope labeled peptides as internal standard

The unique peptide was the template for the internal standard

Signature peptide

39----peptide3----59

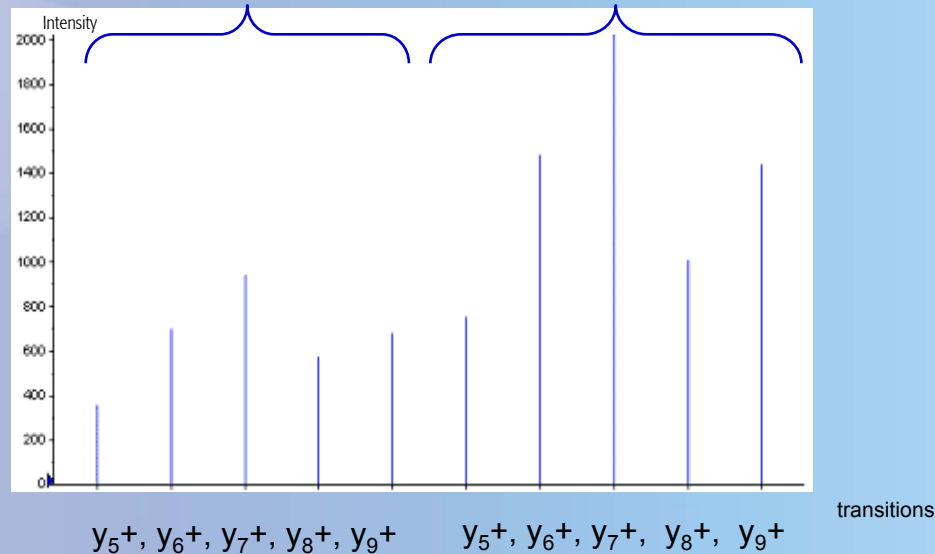
Internal Standard

39----peptide3----59

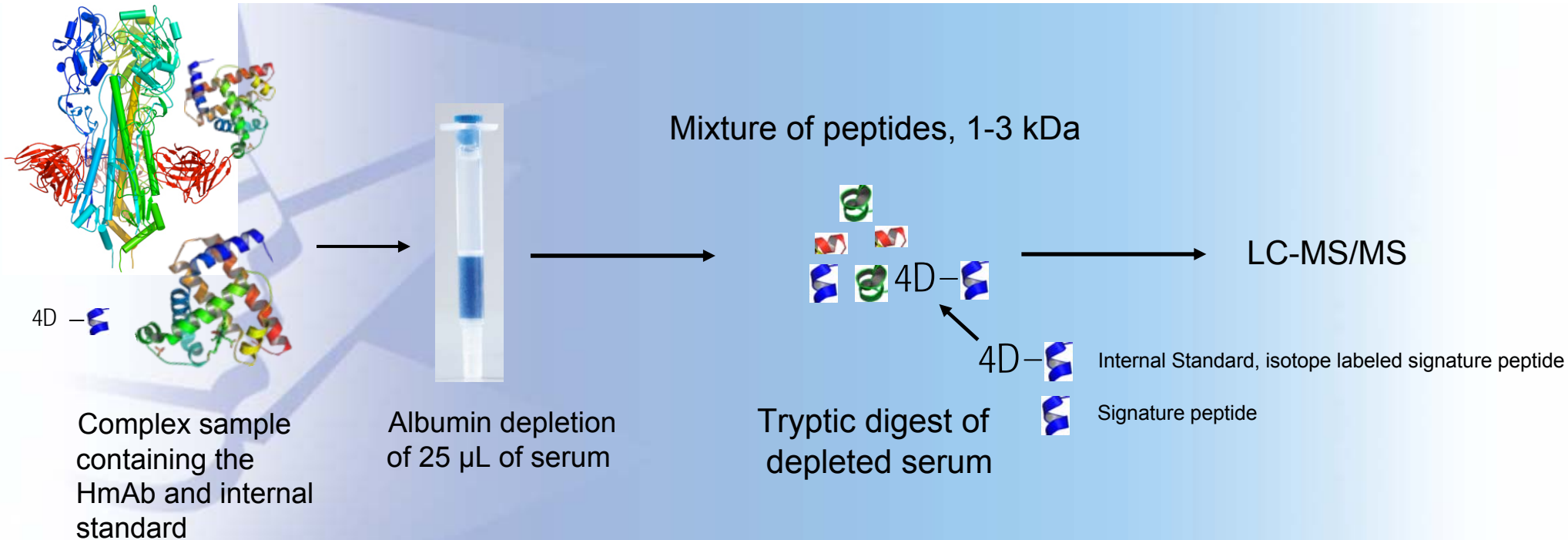
4D

Unique peptide

Internal standard

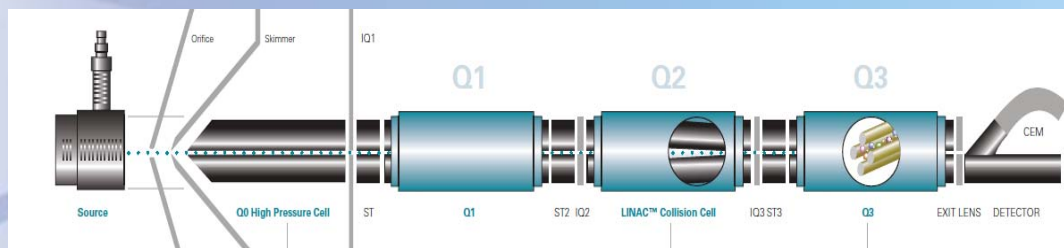


Workflow for sample preparation



Digest of therapeutic protein – analyze signature peptide that represent the entire protein.

Absolute Quantification and HPLC-MS/MS



y_9^+ , y_8^+ , y_7^+ , y_6^+ and y_5^+ -ions were selected

Mass spectrometry – 4000 QTRAP

Declustering potential, 60V

Entrance potential, 10 V

Ion source parameters: Optimized at 200 μ L/min

- HPLC- Agilent 1100

A 2.1x150 mm C-8, 3.5 μ m particle size, (SymmetryShield™) column

Eluent A: H₂O containing 0.1% formic acid

Eluent B: Acetonitrile containing 0.1% formic acid

C.Hagman *et al. Anal Chem.* 2008 Feb 15; 80(4):1290-6

Limited validation of the method in cynomolgus monkey serum

Standard Curve

Six calibration points

2, 4, 100, 300, 800, 1000 $\mu\text{g/mL}$ HmAb

Each concentration analyzed in triplicates

Quality Control Samples

The intra-assay variability

4, 200, 400 $\mu\text{g/mL}$ HmAb

- the variability of the method itself

The inter-assay variability

4 $\mu\text{g/mL}$ HmAb

- different matrices

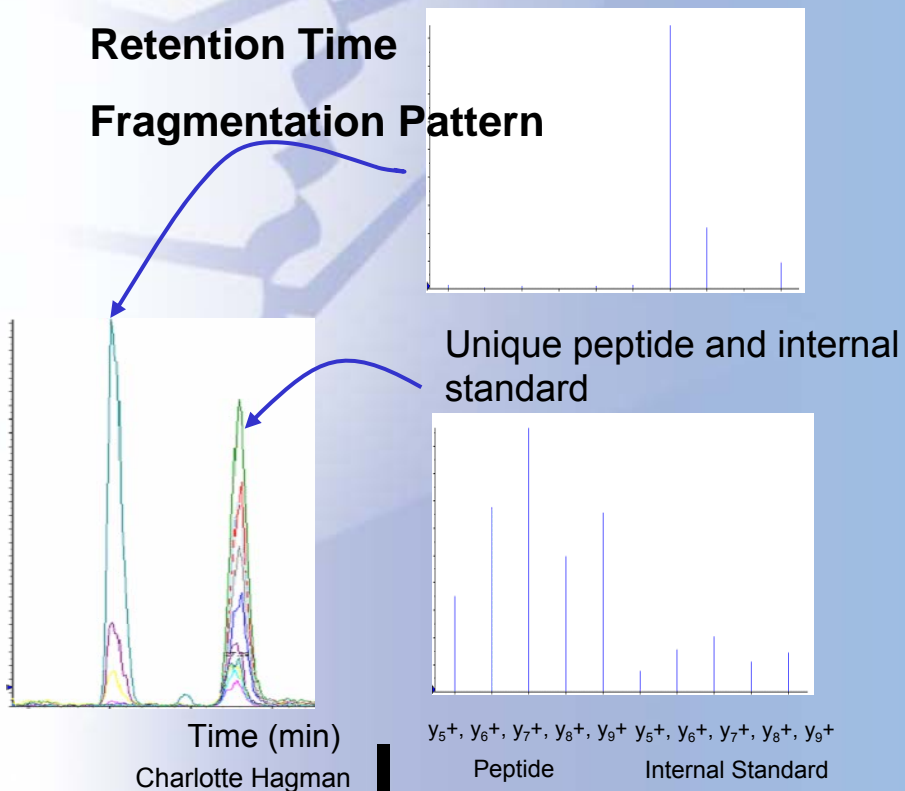
081-sample: Serum, Cynomolgus males pool

083-sample: Serum, Cynomolgus female pool

Each concentration analyzed in triplicates

Identification of signature peptide and internal standard and the specificity of the method

Identification of signature peptide and internal standard

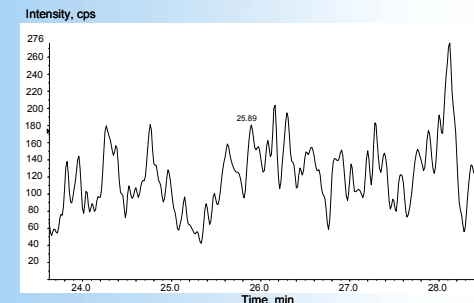
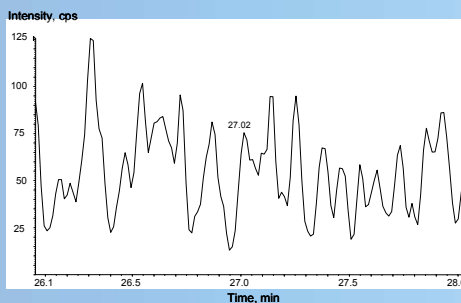


Specificity

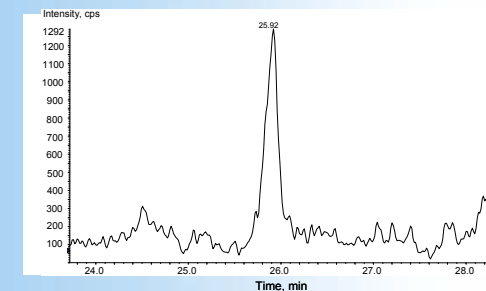
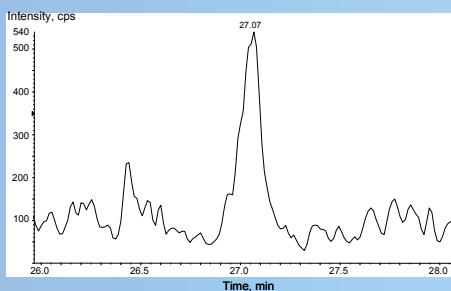
Human serum

Cynomolgus serum

Blank



2 μ g/mL HmAb



Results of the validation

$Y = ax + b$, weighting factor of $1/x$

The method was linear from 2-1000 $\mu\text{g/mL}$ with $R^2 \geq 0.998$

The accuracy ranged between 99 and 112%

The precision at 2 $\mu\text{g/mL}$ was $\pm 5.4 \%$ and the $S/N > 5$.

After albumin depletion of 25 μL of serum, a LLOQ of 2 $\mu\text{g/mL}$ HmAb was reached

Intra-assay variability

Concentration ($\mu\text{g/mL}$)	Mean	Standard deviation	R.S.D. %	Accuracy %
4	4.05	± 0.16	± 3.95	101
200	198.82	± 6.62	± 3.33	99
400	448.92	± 40.34	± 8.97	112

Inter-assay variability

QC pool	Concentration ($\mu\text{g/mL}$)	Mean	Standard deviation	R.S.D. %	Accuracy %
081	4	4.43	± 0.32	± 7.24	111
083	4	4.13	± 0.33	± 8.00	103

Conclusions

- A generic method for quantifying a HmAbs in serum has been developed and validated by liquid chromatography tandem mass spectrometry.
- Advantages offered includes improved assay accuracy, direct inter-assay comparison, absence of unspecific matrix binding effects and reduced assay development and validation time to approximately 6-8 weeks compared to 4-5 months required for a LBA.
- A LLOQ of 2 $\mu\text{g/mL}$ HmAb was reached in cynmolgus monkey serum after albumin depletion of 25 μL serum.
- The sensitivity is at this point is sufficient for early drug development phases, for which a LLOQ of 3-5 $\mu\text{g/mL}$ HmAb is typically required.



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