Challenges for International Standardization of Microalbumin in Urine

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Measurement Issues for Albumin in Urine

- Sample collection and pre-analytical considerations — J. Eckfeldt
- Quantitative urine albumin measurement procedures — M. McQueen
- IDMS candidate reference measurement procedure — J. Lieske
- Urine albumin as a measurand — G. Hortin
- ND industry practices for albumin calibration — J. Zakowski

Proteinuria is Important in Screening, Diagnosing and Treating CKD

- Early marker of kidney damage (ACR >30 mg/g) due to diabetes, glomerular disease, hypertension
- Hypothesized marker of generalized endothelial dysfunction Risk factor for progression
- Risk factor for CVD
- Modifier for efficacy of ACE inhibitor therapy in non-diabetic kidney disease
- Hypothesized surrogate outcome for kidney disease progression and CVD risk reduction

Clinical Issues

- Providers don’t understand meaning of albuminuria and are unable to use quantitative albumin effectively
  - Continuous risk factor
  - Changes in quantity
- Confusing reporting methods make provider education difficult

IMMUNOCHEMICAL METHODS TO DETECT MICROALBUMINURIA

(MOST CLINICAL LABORATORIES)

Turbidimetric (rate of formation of immune complexes)

Nephelometric (rate of formation of immune complexes)

Labelled Immunoassays

- RIA (concerns about safe handling and disposal of radioactive agents)
- Enzyme Immunoassay
  - Enzyme-linked immunosorbent (ELISA)
  - Enzyme-multiplied immunoassay (EMIT)
  - Cloned enzyme donor immunoassay (CEDIA)
- Fluoroimmunoassay
- Chemiluminescence immunoassay
- Electrochemiluminescence immunoassay

LABELS FOR NON-ISOTOPIC IMMUNOASSAY

Chemiluminescent:	Acridium Ester, Sulfonyl Acridium Ester, Isoluminol
Cofactor:	ATP, Flavin Adenine Dinucleotide
Enzyme:	Alk. Phos., Marine Bacterial Luciferase, B-Galactosidase, Firefly Luciferase, Glucose Oxidase, Glucose-6-PD, Horseradish Peroxidase, Lysozyme, Malate Dehydrogenase, Microperoxidase, Urease, Xanthine Oxidase
Fluorophore:	Europium Chelate, Fluorescein, Phycoerythrin, Terbium Chelate
Free Radical:	Nitroxide
Inhibitor:	Methotrexate
Metal:	Gold Sol, Selenium Sol, Silver Sol
Particle:	Bacteriocophage, Erythrocyte, Latex Bead, Liposome, Quantum Dot
Phosphor:	Up-converting Lanthanide-containing nanoparticle
Polynucleotide:	DNA
Substrate:	Galactosyl-Umbelliferone

(Tietz Ch9, 231)

COMPETITIVE IMMUNOASSAYS (Limited Reagent)

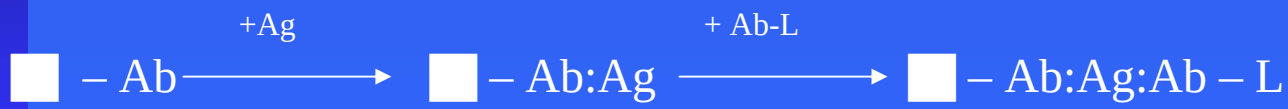
Simultaneous: $Ab + Ag + Ag - L \leftrightharpoons Ab{:}Ag + Ab{:}Ag - L$

(free) (bound)

Sequential: Step 1 $Ab + Ag \underset{k2}{\overset{k1}{\leftrightharpoons}} Ab{:}Ag + Ab$

Step 2 $Ab{:}Ag + Ab + Ag - L \leftrightharpoons Ab{:}Ag + Ab{:}Ag - L + Ag - L$

NON-COMPETITIVE IMMUNOASSAYS (Excess Reagent, Two-Site, Sandwich)



HETEROGENEOUS ASSAYS:

Require separation of free-label antigen from the bound-labelled antigen with physical separation techniques (precipitation, liquid phase and solid phase adsorption). Homogeneous do not require separation.

ANALYTICAL DETECTION LIMITS of competitive and non-competitive immunoassays are determined principally by the affinity of the antibody and the detection limit of the label used. Combination of amplification and an ultrasensitive detection reaction make noncompetitive chemiluminescent EIAs among the most sensitive immunoassays.

SIZE-EXCLUSION HPLC WITH UV DETECTION (ACCUMIN)

- FDA cleared. A Zorbax Bio Series GF-250 column, assay performed on an Agilent 1100/HP (Agilent Technologies, Los Angeles, Ca).
- Promoted to detect forms of albumin that are not reactive immunochemically.
- Detection in urine of diabetics “nicked” but intact albumin i.e. albumin containing 1 or more cleavages of the peptide chain but that remains intact through its numerous S-S bonds.
- Implies that there is more albumin in early diabetic urine than immunochemical methods.
- This assay consistently shows higher albumin values for urine specimens than found by immunoassay.

Chip Electrophoresis
(Chan and Herold, Clin Chem 2006; 52: 2141-2146)

Experion™ Automated Electrophoresis System (BIO-RAD), utilizes lab chip microfluidic separation technology and fluorescent sample detection.

- Sodium Dodecyl Sulphate in buffer binds to the protein
- Fluorescent dye binds to the sodium dodecyl sulphate micelles
- Dye fluorescence is quantified by laser

Sensitive (5mg/L); Imprecision 3%-13% for urines with MA concs. up to ~ 200mg/L; α-acid glycoprotein, α1-antitrypsin, and transferrin did not interfere by co-elution.

Application of LC-MS Technology

Babic et al, Clin Chem 2006; 52: 2155-2157

Reference Materials and Assay Methodologies

- Most assays are immunoassay (except dipstick)
- No Reference Method
- No Urine Reference Material
- Common, but not universal, use of CRM470
  - (by many names)
- CRM470 is a plasma preparation being diluted and used as a urine reference material
  - (an “off-label use”?)
- Probable variations between Manufacturers in use of CRM 470 or other standards (dilution, diluent, protocol, etc.)

Reference Materials and Assay Methodologies

Manufacturer	Primary Ref Material	Methodology
Abbott	CRM 470	Immuno-Turbidimetry
Bayer/Siemens		
Advia	Internal Standard	Immuno-Turbidimetry
Clinitek Dipstick	CRM 470	Colorimetric Dye Binding
Beckman Coulter		
Synchron	BCR 470	Immuno-Turbidimetry
Immage	BCR 470	Immuno-Nephelometry
Dade Behring		
Dimension	ERM 470	Immuno-Turbidimetry
BN Systems	CRM 470	Immuno-Nephelometry
J&J/Ortho Vitros	CRM 470	Immuno-Turbidimetry
Olympus	Albumin Standard	Immuno-Turbidimetry
Roche		
Tinaquant	BCR470/CRM470	Immuno-Turbidimetry
Cobas Integra	CRM 470	Immuno-Turbidimetry

Microalbumin Traceability Schema

- All Manufacturers use essentially the same Traceability Scheme
- Per ISO 17511
- Probable Variations in Traceability schemes in details of dilutions, diluents, plasma vs urine matrix, value transfer protocols.

American Diabetes Association Recommendations for Microalbumin

- ADA Recommendations for Microalbumin Reference Intervals:

CATEGORY	24-HOUR COLLECTION	TIMED COLLECTION	SPOT COLLECTION
Normal	<30 mg/24 hrs	<20 µg/min	<30 µg/mg creatinine
Microalbuminuria	30 – 300 mg/24 hrs	20 – 200 µg/min	30 – 300 µg/mg creatinine
Clinical albuminuria	>300 mg/24 hrs	>200 µg/min	>300 µg/mg creatinine

Reference Intervals and Analytical Ranges (all units in mg/L)

Manufacturer	Claimed Reference Interval	Default → Extended Analytical Ranges
Abbott	ADA Only	5 - 500
Bayer/Siemens		
Advia	<30 mg/day	3 – 200
Clinitek Dipstick	<20	10 - 300
Beckman Coulter		
Synchron	<19 + ADA	2 – 300 → 970
Immage	<19	2 – 40 → 8640
Dade Behring		
Dimension	<30mg/day+ADA ref	1.3 - 100
BN Systems	<30 + ADA ref	2 – 340 → >10,000
J&J/Ortho	<16.7 + ADA	6 - 190
Olympus	ADA Only	5 - 300
Roche		
Tinaquant	<23	3 – 400
Cobas Integra	<29	0 – 193 → 3860

Summary and Conclusions

- No Reference Method
- No Urine Reference Material
- Many Manufacturers standardize to CRM470 (by any name)
- Use of a plasma preparation as a urine standard
- Variations in Traceability schemes in details of dilutions, diluents, plasma vs urine matrix, value transfer protocols.
- Many quote ADA Reference Intervals. Most list method-specific values.

Hope

MA as Marker of Future CV Events

	RR	95% CI
Major CV Events	1.83	1.64 – 2.05
All-Cause Death	2.09	1.84 – 2.38
Hospitalization for CHF	3.23	2.54 – 4.10

Similar With and Without Diabetes

- Every 0.4 mg/mmol increase in ACR increased adjusted hazard of major CV events by 5.9% (95% CI, 4.9% - 7.0%).
- Graded relationship between baseline ACR, CV disease, and mortality, extending to at least 0.5 mg/mmol. (Well below current screening thresholds for MA.)

CV Risk and ACR Cut-Points

- Optimal for Whole Population:
  - RIA 0.9 mg/mmol (Sens 0.48, Spec 0.72)
  - HPLC 3.4 mg/mmol (Sens 0.38, Spec 0.77)
- People with Diabetes:
  - RIA 1.4 mg/mmol (Sens 0.29, Spec 0.84)
  - HPLC 5.2 mg/mmol (Sens 0.32, Spec 0.81)
- People Without Diabetes:
  - RIA 0.7 mg/mmol (Sens 0.22, Spec 0.89)
  - HPLC 3.1 mg/mmol (Sens 0.23, Spec 0.89)

CAP Urine Survey
Overall Performance Since January 2006
Microalbumin mg/L

Specimen	Median	Low	High	Mean	CV(%)
2006 U-01	923	385.0	1300.0	925.19	9.9
2006 U-02	26.7	17.7	37.0	26.80	9.0
2006 U-05	14.0	0.0	48.0	13.05	35.9
2006 U-06	91.0	62.6	122.0	90.48	8.6
2006 U-09	26.0	9.0	38.0	26.06	10.1
2006 U-10	10.0	4.0	15.8	9.79	16.6
2007 U-01	86.0	66.0	104.0	86.19	6.2
2007 U-02	12.0	0.0	20.6	10.97	34.4
2007 U-03	11.5	3.9	16.7	11.40	15.7

Urine Albumin Lyophilized Sample Survey 2007 – U01

	N	Mean mg/L	CV (%)
Beckman Array	11	88.11	5.2
Beckman Immage	79	85.55	5.3
Dade Behring Nephelometer	69	92.79	4.5
Dade Behring Dimension	194	82.78	3.1
Abbott Arch/Aeroset	59	87.90	2.9
Siemens Advia 1200/1650/2400	59	88.07	4.3
Siemens DCA 2000	44	87.16	7.6
Beckman Sync CX3, CX9	39	90.57	4.2
Beckman Sync LX20	207	87.48	5.1
Beckman Unicell	112	86.65	4.9
Olympus 400-640/2700/5400	86	84.11	3.8
Roche Cobas Integra	123	87.99	4.5
Roche Modular	82	89.33	4.9
Roche Hitachi 917	18	87.15	8.1
Vitros	66	76.25	4.9
All (76.25 - 92.79)	1398	86.19	6.2

CVs on any given sample range from 6% to 36%

Problem not simply a lack of harmonization of values.

3 individual peer groups, representing

2 different manufacturers

with CVs 26.2%; 45.0%; 77.7%.

Whether problems caused by calibration issues, antigen excess effects, or low concentration.

Imprecision problems is not yet clear.

[CAP MA (2)]

Health Metrx Canada Albumin/Creatinine Ratio mg/mmol)

	N	Mean	SD	CV (%)	Range
March 2007					
Sample A	35	13.92	1.07	7.7	11.7 – 16.1
Sample B	35	11.17	1.09	9.8	8.9 – 13.4
June 2007					
Sample A	34	1.84	0.19	10.4	1.4 – 2.3
Sample B	34	7.35	0.65	8.8	6.0 – 8.7

Health Metrx Canada Microalbumin (mg/L)

	N	Mean	SD	CV (%)	Range
March 2007					
Sample A	37	95.11	7.81	8.2	66.5 – 123.7
Sample B	37	73.68	6.19	8.4	51.5 – 95.8
June 2007					
Sample A	38	35.9	4.04	11.3	25.1 – 46.7
Sample B	38	143.37	9.58	6.7	100.3 – 186.4

QMP-LS (Ontario) Urine Albumin (mg/L)

	N	Mean	Median	SD	Range	SE
May 2007 (0705 AE)						
All Methods (Vial 1)	29	4.6	4.50	0.73	3.2 – 20.3	0.19
EIA	2	--	--		4.9 – 5.9	
Nephelometry	9	4.3	4.20	0.42	3.7 – 5.0	0.19
Turbidimetry	18	4.8	4.75	1.08	3.2 – 20.3	0.39
All Methods (Vial 2)	29	9.7	9.10	1.47	7.3 – 13.8	0.34
EIA	2	--	--	1-		
Nephelometry	9	9.6	9.00	1.25	8.3 – 11.58	0.52
Turbidimetry	18	9.2	9.05	1.03	7.3 – 13.8	0.30

- Unacceptable range of results
- Upward trend in results grouped by method
- EIA > Turbidimetry > Nephelometry

Albumin : Creatinine (mg alb/mmol creatinine)

	N	Mean	SD	Range
May 2007				
Vial 1 0705 AE	22	1.3	0.20	0.8 – 6.1
Vial 2 0705 AE	28	2.2	0.37	1.7 – 3.3
Vial 3 0705 AE	28	8.4	1.07	6.7 – 11.3
Jan 2007				
Vial 1 0701 AE	23	0.4	0.12	0.2 – 0.7
Vial 2 0701 AE	27	0.8	0.18	0.4 – 1.1
Vial 3 0701 AE	28	11.3	1.09	9.1 – 13.8
Oct 2006				
Vial 1 0610 AE	22	0.9	0.22	0.3 – 1.4
Vial 2 0610 AE	27	2.2	0.38	1.3 – 3.2
Vial 3 0610 AE	27	4.0	0.41	2.8 – 5.5

Albumin Structure and Structural Variation

- 585 amino acids, 66,473 mass, SwissProt P02768
- No biosynthetic glycosylation
- 17 disulfides (highly crosslinked)
- 1 Unpaired Cys (residue 34) variable modification:
  Cys, CysGly, GSH, oxidation
- 4 Globular domains
- Relatively high thermal stability to 65 C
- High solubility under variable salt and pH
- Multiple noncovalent ligands
  Fatty acids, Trp, bilirubin, Ca
- Nonenzymatic glycation – a minor fraction
- Sequence variations recognized but uncommon

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Potential Difference Between Plasma and Urinary Albumin

- **Increased fragmentation in urine**
  - Selective glomerular filtration of plasma fragments
  - Proteolysis by tubular proteases
  - Lysosomal uptake and release into urine
  - Microbial or WBC proteases
- **Aggregation** (potential masking of epitopes, change of valency)
  - Homo- or heterodimers via disulfides (Cys-34)
  - Noncovalent complexes
  - Entrapment in sediment
- **Denaturation**
  - Exposure to variable pH (5-8), ionic strength, urea to 1 M
- **Chemical modification**
  - Oxidation or other chemical modifications
- **Different ligand concentrations**
  - Free fatty acids, calcium, hippuric acids, drugs

Do any of these actually differ for urine specimens?

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Conclusions from Studies of Fragmented Albumin

- A turbidimetric assay was found to react nearly equivalently with albumin fragments
- Antibody specificity was directed to epitopes distributed across all 3 of the CNBr peptides
- Other assays had variable reactivity with fragments. Competitive assays formats or use of monoclonal antibodies may affect reactivity.

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Conclusions

- Albumin fragments occur but need further quantitative analysis and studies of mechanism of formation.
- Reactivity of each assay with fragments should be defined as an operating characteristic – assays are nonequivalent.

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An HPLC-MS Method for the Quantification of Urinary Albumin Using In-Source Collision-Induced Fragmentation

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Mayo Clinic Renal Lab Approach

- Develop a Liquid Chromatography-Mass Spectroscopy (LC-MS) assay to definitively quantitate urinary albumin
- Previous studies demonstrated the feasibility of source-induced fragmentation of large molecules within an electrospray ionization source, including albumin (Loo et al: Annal Chem 1991; 63: 2488-99)
- We achieved fragmentation of the first 30 amino acids of albumin during ionization in the MS electrospray ion source by raising the declustering potential to 336-350V
- The multiple ion mode was used to detect the resulting *m/z* fragment ions:

	B_{24}^{4+}	B_{24}^{3+}
HSA	685.1	913.2
N^{15}-HSA	693.6	924.1
BSA	698.15	930.9

Current LC-MS Assay: Conclusions

- **Strengths**
  - Measures any albumin (whole or fragment) that contains N-terminal 24 amino acids
- **Weaknesses**
  - Measures any albumin (whole or fragment) that contains N-terminal 24 amino acids
  - BSA as an internal standard interferes with the HSA ion limiting the detection limit
  - Using MS to detect the molecular ion for the whole/intact albumin (as opposed to the terminal 24aa) shows poor sensitivity and low dynamic range is very poor
- Overall the LC-MS assay shows good potential as a reference assay for urinary albumin

Next Steps

- **Assay Specific:**
  - Improve low-end sensitivity to allow precise quantification in the low range
  - Quantitate specific fragments?

- **Clinical Studies:**
  - Complete comparative normal value study (LC-MS vs. immunoassay vs. HPLC)
  - Complete clinical study using banked diabetic samples
  - Protocols to assess albumin fragments in disease states

Mayo Clinic

Japanese Initiative for Standardization; Preparation of Working Reference Material for Urine Albumin and Total Protein Measurement" Prof. Y. Ito

- Working reference material for urine albumin and total protein measurement was prepared, which consists of monomeric albumin >97.5% in purity.
- The properties almost fulfill the demand for what will be required for a reference material.
- Value assignment is possible using CRM 470 by selected methods with good performance.
- Primary reference material should be prepared for the establishment of traceability chain in the future.

Japanese Initiative (2)

- Reference Material – 5000 vials have been prepared, value assignment will be finished in 2007 and completed in international format.
- The reference material is to be submitted to JCCLS.

  Question: CRM470 is a serum material so a large dilution using pure water is made to use it in a urine method; making a large dilution may have its own set of issues.

Need to Define a Full Reference System for Urine Albumin

- Measurand, reference materials, reference measurement procedures.

- Answer critical measurand questions
  - Intact vs. fragments of albumin molecule
  - Rule out presence of N-terminal 24 peptide epitope as normally occurring fragment in patient samples
  - Ensure constant ratio/relationship of N-terminal 24 peptide to “urine albumin” measurements determined with common field methods
  - Refer to IFCC “Nomenclature, Properties and Units Committee” for technical measurand name.

- Complete and publish data from Japanese standardization project to demonstrate that albumin (urine) = albumin (serum) to validate application of dilute CRM 470 as reference material

(Y. Ito)

Reference System (2)

- If CRM 470 serum albumin is not suitable, determine suitability of JCCLS candidate reference materials (purified human albumin); confirm commutability with native urine (Y. Itoh)
- Encourage parallel development of alternative candidate urine albumin reference materials
- Encourage/support further development of Mayo candidate reference method