Biochemical tests in diabetes

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Glycated Haemoglobin Analysis

The non- enzymatic addition of a sugar residue to amino groups of protein.

Haemoglobin HbA 97%, HbA_2 2.5 % and HbF 0.5%

Several minor haemoglobins migrate more rapidly than HbA in an electric field, called **HbA1, made up of HbA1a + HbA1b + HbA1c**.

Condensation of glucose and the N-terminal valine of each beta chain of haemoglobin is **HbA1c.**

HbA1a1 is fructose-1, 6 diphosphate and
HbA1a2 is glucose-6-phosphate attached to the amino terminal of the beta chain.
HbA1b is pyruvic acid linked to the amino terminal valine of the beta chain
HbA1c makes up 80% of HbA1.
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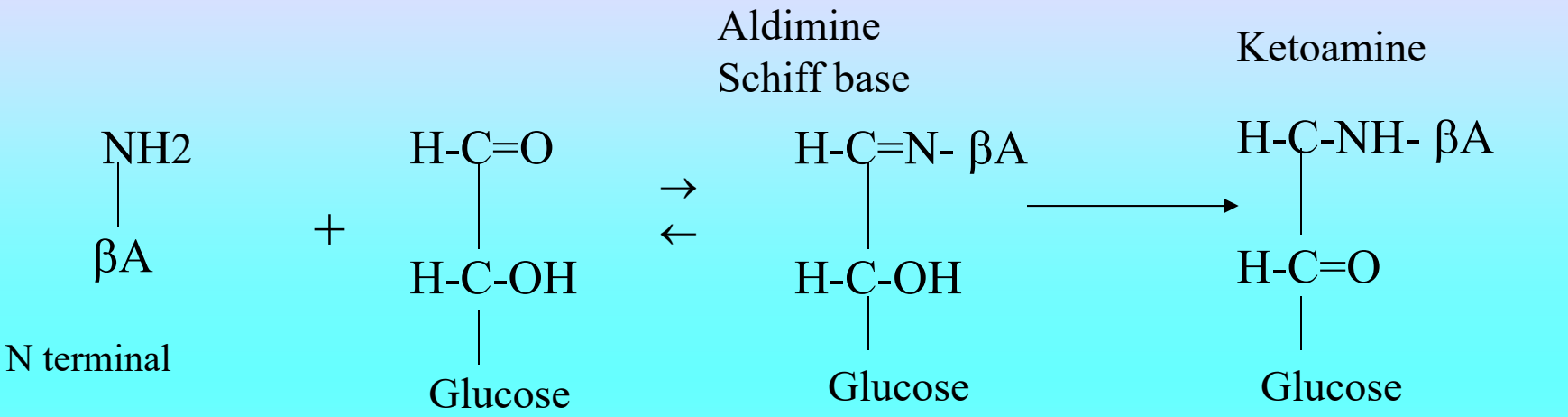

HbA + glucose ⇄ Pre-HbA1c ⟶ HbA1c

rapid slow

Methods for determining glycated haemoglobins

those based on **charge differences:**

ion-exchange chromatography, HPLC, electrophoresis, and isoelectric focusing

and those based on **structural differences**

affinity chromatography and immunoassay.

Chemical methods a third option rarely used.

Ion-exchange chromatography
Measures HBA1 – total glycated haemoglobins (A1a + 1b + 1c)
HPLC Both HbA1c and HbA1 can be reported,
Electrophoresis can measure HbA1c but less specific .
Isoelectrophoresis HbA1c adequately resolved from HbA1a1, HbA1b and S and F.

Immunoassay antibodies raised against the Amadori product of glucose (ketoamine linkage) plus the first 4-8 amino acids at the N-terminal of the beta chain by inhibition of latex agglutination. **Specific for HbA1c**

Affinity chromatography uses

m-aminophenylboronic acid bound to agarose or glass fibre matrix to react with cis-diol groups of glucose bound to haemoaglobin.

Measures HbA1

Diabetes Control and Complications Trial (DCCT) 1993 multicenter randomized trial

HbA1c measurement systems have been standardized through a process of alignment with the original DCCT method. This has been undertaken by the **US National Glycohemoglobin Standardisation Program (NGSP) .**

UK Consensus Statement

Glycemic control is best measured by HbA1c

The method should be a DCCT –aligned HBA1c method

The assay should have acceptable within assay precision <3% and between assay imprecision <5%

CMC METHOD BIORAD VARIANT HbA1c PROGRAM

Utilizes the principles of ion-exchange HPLC , without interference from labile A1c, lipaemia or temperature fluctuations.

Certification/traceability of reference material

Certified by the NGSP as having documented traceability to the DCCT reference method. The haemoglobin A1c calibrators provided in the kit are **traceable to the Kyoto 2002 Calibrator set prepared by the IFCC working group on standardization of HbA1c. The specimens were prepared in the Netherlands at a hospital with ISO 9001:2000 certificate.**

NGSP = 0.906(IFCC) + 2.21.
This method reports performance data and reference ranges as NGSP values. The calibrator/diluent set includes both NGSP and IFCC values.

IFCC values are 1.5-2.0% lower than NGSP

Clinical Chemistry 2008; 54:240

Update 6 year progress report

IFCC recommends mmol/mol HbA1c as units

Sample EDTA whole blood stable 1 week at 4°C

HbA1c half life 35 days

A 1% increase in %HbA1c is equivalent to a rise in average blood glucose of 35 mg/dL.

Clin Chem 2009; 55: 1612-14

International Expert Committee says HbA1c should be the diagnostic test for diabetes.

The value of $\geq$ 6.5% decision point

6.0-6.4% indicate individuals at high risk of developing diabetes

DCCT –HbA1c (%)	IFCC-HbA1c (mmol/mol)
6.0	42
6.5	48
7.0	53
7.5	59
8.0	64
9.0	75

The HbA1c –derived average glucose (ADAG) calculated from the HbA1c result will also be reported.

Consensus by ADA,EASD, IFCC and IDF for worldwide standardization

Reference Ranges

< 6.5 % normal

6.5-7.0 % target in diabetic patients

7.0 -9.0% suboptimal diabetic control

> 9.0 % poor diabetic control

Interference

Icterus :

Lipemia

Hemoglobin variants S and C have no effect on the assay when they exist in the heterozygous forms HbAS and HbAC.

In homozygous **Hb SS or Hb CC** patients do not have HbA present or HbA1c thus criteria other than monitoring of HbA1c must be used to assess long term diabetic control in these patients.

HbF levels upto 30 % do not interfere

Interpretation of HbA1c relies on RBC having a normal lifespan

Conditions with shortened RBC survival or higher fraction of young RBC have reduced HbA1c

Higher HbA1c where older population of RBC exists

Haemoglobinopathies may increase or decrease HbA1c

Carbamylated Hb from attachment of urea may also interfere

Conditions which preclude HbA1c testing

Altered red blood cell turnover eg haemolytic anaemia, major blood loss or blood transfusion

Some Haemoglobin traits HbAS, Hb AC, Hb AE, Hb AD interfere with some methods but alternative methods are available. Values from 6.0% - 6.4 % are at high risk of developing diabetes. Methods should have CVs of =/< 5% between HbA1c values of 6% and 7%

HbA1c advantages for diagnosis of DM:

Low preanalytical and biological variation

Correlates with risk of developing microvascular complications

Values reflect overall glycaemic exposure

No requirement for fasting sample

Diagnois confirmed by HbA1c =/> 6.5% confirmed on a different day unless clinical symptoms and glucose > 200 mg//dL are present. Analysis to be performed on central laboratory instruments not point of care devices

Fructosamine

Generic name for plasma protein ketoamines

Glucose and ε lysine residues of albumin

Half life of circulating albumin is 20 days

Glycated albumin reflects control over a period of 2-3 weeks

Do not perfom when Albumin < 3 g/dL

GLUCOSE ANALYSIS

Specimen type, collection and storage

Plasma collected with EDTA/Fluoride Sodium EDTA 6mg, NaF 3mg/2ml blood) anticoagulant
and should be separated from the red cells within one hour of collecting the specimen.
CSF for glucose estimation is collected in a plain bottle.
Serum is not suitable due to continuing glycolysis by red cells in the absence of fluoride.

WBG 12-15% less than plasma glucose. Loss of glucose **approx 5-7% per hour (5-10 mg/dL)**
Fasting blood glucose (FBG) should be 10 hour fast not 16 hrs
EDTA/Fluoride specimen is stable for 7 days is a closed tube at 4^0C or 24 hours at 15-25^0C.
CSF should be analysed within 2 hours. Hexokinase and GOD/POD methods are not suitable for urine.

Clin Chem 2005; 51:1573-1576
Harmonisation of POCT devices with laboratory use a factor of 1.11 to convert POCT values in whole blood to plasma values

Principle of the method

Reaction sequence

$$\text{Glucose} \xrightarrow[\text{pH 7.0}]{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{POD}} H_2O + [O]$$

$$[O] + \text{4 – amino phenazone} + \text{Phenol} \longrightarrow \text{Pink Chromogen}$$

Measure absorbance at 505nm

Refs:
Trinder P Ann Clin Biochem 1969, 6: 24-27
Barham D, Trinder P. Analyst 1972; 97: 142-145.

Higher concentrations of bilirubin interfere in the peroxidase part of the assay causing a decrease in values

So do uric acid, ascorbate, haemoglobin, tetracycline, glutathione.

Hexokinase assay

Uses hexokinase and G6PDH enzymes, ATP and NADP+ cofactors

Haemolysis 0.5 g/dL,

lipaemia > 500 mg/dL, positive effect

bilirubin > 5 mg/dL negative effect

Reference Values

ADA 2 fasting plasma values ≥ 126 mg/dL (7.0 mmol/L)

Impaired fasting glucose	101- 124 mg/dL (5.6-6.9 mmol/L)
Glucose AC fasting	70-110 mg/dL
Glucose PC (2 hours)	80-140 mg/dL
Glucose random	70-140mg/dL

Semi-quantitative measurement of urine glucose

Benedicts test based on reduction of copper ions by glucose to give green to brick red colour. Protein free urine

All other urine reducing substances interfere.
Analytical sensitivity 250 mg/dl

Dip-stix method GOD/POD

Analytical sensitivity 100 mg/dL

Ketones, ascorbic acid, salicylates false negative

Bleach false positive

ESTIMATION OF SERUM CREATININE

Specimen type, collection and storage

Serum or plasma can be analysed and can be stored at 4^0C, for 24 hrs.

Collect 24 hr urine in a plastic container with thymol as a preservative. Stable at 4^0C for 24 hr.

Centrifuge all urines before analysis.

Principle of the method

Creatinine + picric acid $\xrightarrow{\text{NaOH}}$ Creatinine picramate (red colour) at 505 nm

Source of the Method Protocol

Slot C. J Clin Invest. 1965: 17: 381 –87

Seation B, Ali A. Med Lab Sci 1984; 41: 327 -36

Haemolysis /Hemoglobin up to 0.68 g/dL bilirubin up to 7.8 mg/dl, lipaemia /triglyceride upto 2200 mg/dl, do not have any significant interference.

Interference from **β -OH butyrate and acetoacetate** minimized by using a rate reaction. Cephalosporin antibiotic and other drug reactions with picric acid overcome by using a rate reaction.
All specimens which are icteric, having a **bilirubin > 7.8 mg/dL** must be repeated using the alternative blank creatinine method, all specimens with a negative or unexpectedly low creatinine should be repeated by this method.

Refs: Recommendations for improving serum creatinine measurement: A report from the Laboratory Working Group of the National Kidney Disease Education program. Clin Chem 2006; 52: 5-18 GL Myers, WG Miller, Coresh J et al.

Summary:
We require better standardization and improved accuracy (trueness) of serum creatinine including the use of the estimating equation for GFR from the Modification of Diet in Renal Disease Study (MDRD). The current variability in SCr estimation renders all equations for GFR less accurate in the normal and slightly increased range < 1.5 mg/dL (<133 μmol/L) which is the relevant range for detecting chronic kidney disease (CKD). Defined as GFR < 60 $ml.min^{-1}$ $(1.73m^2)^{-1}$.

SCr should be reported in mg/dL to 2 decimal places ie 0.92 mg/dL not 0.90, μmol/L will still be reported to whole numbers.

Use of compensated creatinine methods:

After recalibration of assays to IDMS the goal for total error is maximum 10%

Estimation of serum cholesterol

Specimen type, collection and storage

Serum, heparinised plasma or EDTA plasma

Specimen stable for 6 days at 4^0C or 20-25^0C. Patient should be fasted over night if the specimen is also for triglycerides estimation as part of a lipid profile otherwise, it can be random.

Principle of the method

Cholesterol esters are hydrolyzed by cholesterol esterase to cholesterol and fatty acids.

$$\text{Cholesterol esters} \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{fatty acids}$$

Cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone with the simultaneous production of hydrogen peroxide:

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \Delta 4\text{-cholestenone} + 4H_2O_2$$

In the presence of peroxidase, hydrogen peroxide oxidizes phenol and 4-aminoantioyrine to give quinoneimine dye colored in red:

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O$$

The intensity of the color produced (at 505 nm) is proportional to the concentration of cholesterol in the sample.

Interference

There is no interference for haemoglobin up to to 0.68 g/dL, bilirubin to 16 mg/dl or triglyceride up to 2200 mg/dl.

Reference Range	*Cholesterol*
Desirable	< 200 mg/dL
Borderline	200 – 230 mg/dL
High	> 240 mg/dL

Reference Range *Creatinine*

0.5 –1.1 mg/dl (women)

0.7 – 1.3 mg/dL (men)

1.0 –2.0 g/24 hr (urine)

ESTIMATION OF DIRECT HDL

Summary and explanation of the test

The reaction proceeds in 2 steps. **Step 1**: Elimination of chylomicron, VLDL-cholesterol and LDL-cholesterol by cholesterol esterase, cholesterol oxidase, and subsequently catalase.

Step 2 is specific measurement of HDL-cholesterol after its release by detergents in reagent 2. The intensity of the quinoneimine dye produced is directly proportional to the HDL concentration, and is monitored at 600nm

Specimen type, collection and storage

Serum, heparinised plasma or EDTA plasma

Specimen stable for 6 days at 4^0C. Patient should be fasted over night if the specimen is also for triglycerides estimation as part of a lipid profile otherwise, it can be random.

Reference

Izawa S, Okada M, Matsui H, and Horita Y. J Med and Pharm Sci 1997; 37: 1385-88

Reference Range	35- 70 mg/dL
Negative risk factor	> 60 mg/dL

Estimation of serum direct LDL

Principle of the method

The assay consists of two distinct steps.

1. Elimination of chylomicron, VLDL-cholesterol and HDL-cholesterol by cholesterol esterase (CHE), cholesterol oxidase (CO) and subsequently catalase

2. Specific measurement of LDL –cholesterol after release of LDL cholesterol by detergents in reagent 2 . Then action of CHE and CO to given hydrogen peroxide and subsequent reaction. The intensity of the quinoneimine dye produced is directly proportional to the LDL cholesterol concentration when measured at 600 nm.

References
Weiland H and Seidel D. J Lip Res 1983; 24: 904-909
Friedewald WF et al. Clin Chem 1972; 18: 499-502

Target Values

< 100 mg/dL (2.59 mmol/L)	therapy target in diabetic patients
<130 mg/dL	diabetics
<160 mg/dL	non diabetics
160 – 189 mg/dL	high
> 190	very high

NATIONAL CHOLESTEROL EDUCATION PROGRAM (NCEP SEPT 2002)

LDL-C the primary determinant in hypercholesterolaemia

Estimated by a direct LDL-C method. Friedewald formula cannot provide values with the recommended precision and accuracy limits

ie total error =/< 12%, accuracy ± 4%

CV =/< 4%

LDL-C value for calibration and QC material traceable to the reference method for LDL-C

Friedewald formula overestimates LDL in the presence of Type II hyperlioproteinaemia (increased β-VLDL)

Estimation of serum triglycerides

Principle of the method

Triglycerides $\longrightarrow$ glycerol *using the enzyme lipoprotein lipase*

Glycerol $\longrightarrow$ glycerol –3-phosphate *using glycerol kinase*

Glycerol-3-phosphate$\longrightarrow$ dihydroxyacetone phosphate + H_2O_2 *using glycerolphosphate oxidase*

H_2O_2 + 4-aminophenazone/N –ethyl-methylanilin-propan-sulphonate (ESPT) $\longrightarrow$ purple quinoneimine *using the enzyme peroxidase*

Specimen type, collection and storage

Serum, heparinised plasma or EDTA plasma

The separated specimen can be stored for 3 days at 4°C.

The specimen should be taken after an overnight fast.

Reporting of results

Reference Range 45—190 mg/dL

Source of the Method Protocol

Bucolo G, and David M. Clin Chem 1973; 19: 476

Werner M, Gabrieson DG and Eastman G. Clin Chem 1981; 27: 268

Estimation of urine microalbumin

Summary and explanation of the test
Immunoturbimetric assay.
In solution the precipitate formed by an antigen-antibody complex between albumin in the urine and albumin antibody scatters light. The intensity of transmitted light is compared to that of the incident light. The antigen antibody reaction is enhanced by polyethylene glycol Absorbance is measured at 234nm

Specimen type, collection and storage
Random urine sample. Stability one week at 4^0C.
Source of the Method Protocol
Based on the optimised standard method of Van Munster PJJ et al Clin Chim Acta 76,377-388, 1977.

Reporting of results

Lower limit reporting range values < 5 mg/L

Upper Limit reporting range values >150 mg/L

Reference Range < 25mg/g creatinine

Calculation of results

Microalbumin result in mg/L divided by urine creatinine result in g/L to give result as mg/g creatinine.

An albumin excretion rate of >25 mg/g creatinine is considered as microalbuminuria. Persistent urinary UAE albumin excretion of > 25 mg/g creatinine represents a 20 fold greater risk of development of renal disease in diabetic patients. In type 2 diabetes increased UAE is a predictor of progressive renal disease, atherosclerotic disease and cardiac vascular mortality.

μg/min	mg/24hr	mg/g
<20	<30	<30 normal
20-200	30 – 300	30 – 300 increased UAE
>200	>300	>300 overt diabetic nephropathy

POINT OF CARE DEVICES (POCT)

UK Medicines and health care Products regulatory Agency (MHRA)

Guildford Evaluation Unit

Surrey.ac.uk/GMEC/pages/MHRA/Home

Reports : methodology

Analytical performance

Ease of use, reliability and safety

Thank you