16. PRE-ANALYTICAL, ANALYTICAL AND POST-ANALYTICAL FACTORS INFLUENCING SPECIFIC TESTS FOR DIAGNOSIS AND MONITORING OF DM-National network in quality assessment

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The clinical laboratory plays a key role in both the diagnosis and monitoring of diabetes mellitus. Appropriate use of the state-of-the-art technology and quality assurance of laboratory and technical procedures used for the diabetes management have been recognized as important parts needed to attain the goals proclaimed by the Saint Vincent Declaration, i.e. to decrease the morbidity and mortality due to diabetes and its complications (1).

Elevated fasting plasma glucose is considered to be a basic diagnostic indicator of diabetes mellitus (2). While the diagnosis of type 1 diabetes seems relatively simple because of the acute clinical onset accompanied with significant hyperglycaemia and metabolic disturbances, type 2 diabetes often requires a more sophisticated diagnostic approach, due to the absence of classical clinical symptoms. Thus, the oral glucose tolerance test (oGTT) still remains a standard diagnostic tool for discriminating between the impaired glucose tolerance and diabetes mellitus (3).

Once diagnosed and treated, diabetes mellitus as a chronic illness requires regular control and assessment of the patient condition. The importance of maintaining good metabolic control in reducing the risk of the development and progression of late diabetic complications was amply evidenced by the results of Diabetes Control and Complication Trial (DCCT) and UK Prospective Diabetes Study (4,5). At the same time, the measurement of glycohaemoglobin (HbA1c) has been definitely identified as the most reliable tool in the assessment of intermediate (2-3 months) metabolic control and prediction of the risk for the development of late complications in patients with diabetes mellitus. Analogous to glycated haemoglobin, measurement of fructosamine may be used as an index of the average concentration of blood glucose over an extended period of time (2-3 weeks).

This presentation aims to provide an overview of pre-analytical, analytical and post-analytical factors influencing specific tests for diagnosis and monitoring of diabetes mellitus, with special emphasis on quality assessment of fasting plasma glucose, oGTT, haemoglobin A1c and fructosamine.

1.1. Plasma glucose determination

Specific and sensitive enzymatic assays, routinely used for the plasma glucose measurement, have considerably improved the quality parameters, in both accuracy and reproducibility terms.

The glucose assays most widely used in Croatia may be determined by inspecting quality control surveys conducted by the Croatian Society of Medical Biochemists. Results from 168 medical biochemistry laboratories reported in the surveys reveal that 81% of the laboratories used a glucose assay based on the glucose-oxidase/peroxidase principle (6). The second step of this reaction, i.e. transfer of hydrogen peroxide to a chromogenic oxygen acceptor, resulting into colour formation, is not specific. The presence of any reducing compound, like urate, ascorbate, glutathione etc., in the sample, negatively interferes with the glucose measurement. However, only extremely high, non-physiological concentrations of these compounds could result into a clinically significant interference (e.g. plasma glucose measurement immediately after intravenous administration of ascorbate).

More relevant for the plasma glucose measurement are pre-analytical variations, due to improper sampling, processing and storage of analytical samples. Approximately 5-7% decrease in glucose concentration per hour occurs due to the glycolytic processes in vitro, which could be further accelerated by concomitant leukocytosis and/or bacteraemia. The influence of glycolysis could be prevented by either immediate separation of plasma (within 60 minutes from sampling) followed by determination of glucose within next 60 minutes, or by collecting blood specimens in special tubes, containing glycolytic inhibitor (sodium fluoride or iodoacetate) with an anticoagulant (e.g. potassium oxalate). It should be stressed, however, that glucose preservatives do not totally prevent glycolysis (3). Whole blood samples preserved with fluoride show an initial rapid fall in glucose up to 10% at room temperature, but subsequent decline is slow. However, the initial fall is easily prevented by immediate centrifugation.

Most laboratories prefer serum to plasma for the glucose measurement, because serum is the most prevalent sample for other biochemical analyses. However, these laboratories should bear in mind that the results of serum glucose will be reliable only if serum is separated within 1 hour from blood sampling. No significant differences between plasma and serum glucose, obtained under these circumstances, should be expected.

However, there is a difference in glucose concentration between venous and capillary plasma, which becomes especially pronounced in post-load samples during oGTT. Both haemodynamic and metabolic differences between venous and arterial blood contribute to the usual finding of capillary plasma glucose being 7-10% higher than corresponding venous values. Thus, the type of sample should always be clearly identified to provide relevant clinical information.

1.2. Oral Glucose Tolerance Test

The oral glucose tolerance test (oGTT) is a standard diagnostic tool, which involves a two-point measurement of plasma glucose, before, and two hours after oral administration of the standard glucose amount. The oGTT should be performed in the morning after at least 3 days of unrestricted diet (>150 g of carbohydrates) and normal physical activity. An overnight fast (8-14h), during which only administration of water is allowed, should precede the test. The presence of factors that may influence test results (medication, infection etc.) should be avoided or recorded.

After collection of the fasting blood sample, the patient should drink 75 g of glucose (anhydrous) dissolved in 250-300 mL of water. For children, the test load is 1.75 g per kg body weight, up to a total of 75 g of glucose. The timing of the test starts with the beginning of the drink, and the glucose should be consumed within 5 minutes. Smoking, drinking and eating are not permitted during the next two hours. Another blood sample must be collected exactly 2 hours after the test load.

Collection, processing and storage of samples, as well as analytical aspects of glucose measurement, are described in details in the previous section. The interpretation of results is presented in Table 1.

Table 1. Diagnostic criteria for diabetes mellitus and impaired glucose tolerance

	Glucose (mmol/L)	
	Venous plasma	Capillary plasma
Diabetes mellitus		
Fasting or 2-h post-glucose load	>7.0 >11.1	>7.0 >12.2
Impaired Glucose Tolerance (IGT)		
Fasting and 2-h post-glucose load	<7.0 >/=7.8	<7.0 >/=8.9

Modified according to Ref 3

1.3. Glycated hemoglobin/HbA1c determination

Glycohaemoglobin (GHb) is a common term for post-translational modified molecules of haemoglobin A, resulting from a non-enzymatic binding of glucose (glycation) to the amino acid residues in a- and/or b-globin chains (7). Given the normal life-span of erythrocytes, the amount of glycohaemoglobin is directly proportional to the average blood glucose concentration over the preceding 6-8 weeks. Haemoglobin A1c (HbA1c), the specific product defined as haemoglobin irreversibly glycated at one or both N-terminal valine of the b-chains, comprising about 80% of total GHb, was used as a central determinant of metabolic regulation in the DCCT, and subsequently implemented in the recommended goals of metabolic control for diabetic patients (4). Based on these data, HbA1c testing is recommended quarterly and semi-annually, for patients with type 1 and type 2 diabetes mellitus, respectively.

The results of the DCCT not only significantly influenced the clinical care for patients with diabetes mellitus, but also clearly emphasized the need for a reliable and reproducible measurement of HbA1c, particularly regarding a narrow range of HbA1c values discriminating the patients at low and high risk for the development of late diabetic complications. Should these results be applied to routine clinical practice the specific measurement of HbA1c, defined as haemoglobin irreversibly glycated at one or both N-terminal valine of b-chains, should be provided (7,8).

The major difficulty associated with the glycohaemoglobin determination is a variable and unstandardized methodology, often measuring different chemical moieties of glycated haemoglobin(s) and thereby giving irreproducible and incomparable results. Despite the technological advances, widely used methods based on the charge differences (ion-exchange chromatography, electrophoresis) are still lacking specificity, due to the influence of various interfering factors. Procedures employing the boronate-affinity principle measure total glycohaemoglobin (i.e. haemoglobin moiety glycated on multiple sites in both the a- and b-chains). Among pre-analytical interferences, the presence of HbF and or other haemoglobinopathies, as well as different types of anaemia are the commonest cause of inconsistent results, although the extent of a particular interference is again method-dependent.

Results from a recent survey revealed that GHb/HbA1c testing in Croatia is far from being standardized and readily available (9). The analytical methodology is very variable, included both manual and automated procedures for GHb/HbA1c measurement, employing various physical and chemical principles and measuring different chemical moieties. Thus, almost half of the laboratories (48%) reported to use the boronate-affinity method, measuring total GHb (i.e. haemoglobin moiety glycated on multiple sites in both the a- and b-chains), a value which is not interconvertible with the haemoglobin A1c and therefore questionable in terms of clinical comparison with the DCCT data and actual clinical recommendations. Apart from this, the methods based on the charge differences (ion-exchange chromatography, electrophoresis) measuring either HbA1c or HbA1, and recently developed light-scattering immunoassays, specifically measuring HbA1c, routinely used by the other laboratories, further complicate the picture of GHb/HbA1c testing in Croatia, leading to highly variable and incomparable test results in both analytical and clinical terms.

Thus, a very clear communication between laboratory professionals and clinicians should be of highest priority when evaluating metabolic control by using GHb/HbA1c results on a regular basis, especially considering that even within-laboratory comparability of the test-results is not attained in 17% of diabetic centres in Croatia (covering almost 20 000 patients), which reported on the alternating use of two different methods.

This communication presumes a mutual responsibility of both laboratory professionals and diabetologists, in providing and seeking information on the methodology, analyte, interferences, precision, quality control and reference values, before interpreting glycohemoglobin/HbA1c results.

1.4. Fructosamine

Fructosamine is the generic name for plasma protein ketoamines. The name refers to the structure of the ketoamine rearrangement product formed by the interaction of glucose with the e-amino group on lysine residues of albumin. Because serum proteins turn over more rapidly than haemoglobin (the circulating half-life for albumin is about 20 days), the concentration of glycated albumin reflects glucose control over a period of 2 to 3 weeks. Although the fructosamine assay can be automated, gives better precision, and is cheaper than glycated haemoglobin, there is a lack of consensus on its clinical utility. Over the succeeding decade, the assay underwent numerous modifications as several artefacts were

identified. These include an apparent lack of specificity for glycated proteins, lack of standardization among laboratories, difficulty in calibrating the assay and interference by urates and hyperlipidaemia. It is generally accepted that the test should not be performed when serum albumin is less than 30 g/L (10).

Recommended literature:

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