METHODS

Chromatographic Determination of Methyl Glyoxal in Blood Plasma as the Test for Glycotoxicity and Accumulation of Glycation End-Products

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We developed a method of measuring methyl glyoxal concentration in blood serum using HPLC with UV detection. Methyl glyoxal concentration was determined in healthy subjects. The method was developed for indirect but reliable measurement of the levels of glycation end-products in patients with diabetes, hyperlipidemia, and cardiovascular pathologies.

Key Words: *methyl glyoxal; high performance liquid chromatography; o-phenylenediamine, glycation end-products*

Methyl glyoxal (CH_3-C-CHO, MG), aldehyde of pyruvic acid (2-oxopropanal), is the elemental representative of α-keto aldehydes [1]. It is formed from monosugars during fermentation and under the effect of microorganisms on glycerin. MG is the glycolysis metabolite and its level in intercellular fluid markedly increases in hyperglycemia. Although carbohydrates, phosphotrioses (glyceraldehyde-3-phosphate) are the main MG precursors, its other precursors are fatty acids, ketone bodies, and acetone. Under physiological conditions, MG undergoes detoxication in the glyoxylate pathway with D-lactate formation. Glyoxal, MG, and 3-deoxyglucosone are regarded as glycotoxins. Interest in MG is associated with its ability to interact with amine groups of Lys and Arg amino acid residues, thiol groups of the proteins [11], and nucleic acids [12] in irreversible reactions of glycation, so-called Maillard reaction. They start from condensation of aldehyde groups of monosugars with α-amine groups of Lys and Arg with Schiff base (aldimine) formation followed by their transformation in ketoamines through intramolecular rearrangement (Amadori products). Subsequent oxidation and condensation of Amadori products lead to the formation of glycation end-products, endogenous phlogogens, biological "garbage", which initiate *in vivo* development of inflammatory reaction and systemic inflammation response syndrome. These processes underlie specific diabetes mellitus complications, diabetic microangiopathy [10]. Clinical experimental evidences were obtained for GEP role in the development of other types of cardiovascular pathologies [5]. Simultaneously, MG may also exhibit antitumor activity [8].

The use of MG as a diagnostic test in pathological processes requires the development of specific methods for its measurement. Until now, no standardized methods for measuring MG concentration were developed and no international standard was produced. Therefore, the data on physiological and pathological MG concentrations considerably vary. The following physi-

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ological MG concentrations in normoglycemic blood plasma were reported 123±37 nM [4], 194±11 nM [10], 338±62 nM, 520±42 nM [13], and 650±160 nM [14]. Such variations are apparently caused by different analytic methods used. Derivatization is the essential stage of MG assay and its choice is a principal point. We compared methods of MG assay by their sensitivity and analytical specificity and chose reaction with 1,2-diamonobenzole (o-phenylenediamine) with measuring the reaction products using HPLC with UV detection [6]. Clinical biochemists usually measure N-carboxymethyl lysine (CML) and MG.

The objective of the study was to develop the method of measuring MG concentration in blood plasma using HPLC with registration in UV absorption.

MATERIALS AND METHODS

The previously described method [7] was taken as the basis and was modified with consideration for peculiarities of the equipment we use. The method is based on the reaction between MG and o-phenylenediamine yielding 2-methyl quinoxaline, which has specific absorption bands in UV spectrum band. The amount of 2-methyl quinoxaline in equivalent to MG was estimated using internal standard and calibration curves. The following reagents were used for standard solution, mobile phase preparation and for blood plasma probe preparations: MG (Sigma), 2-methyl quinoxaline (Sigma), 6-methyl quinoxaline (Lancaster), o-phenylenediamine dihydrochloride (Sigma), potassium dihydrophosphate (Merck), perchloric acid (Fluka), orthophosphoric acid (Fluka), acetonitrile (Merck). Distilled water with specific electric conductivity 5 μSim/cm was obtained using DVS -M/1H device (NPK Mediana-Filter). Venous blood was obtained from healthy subjects (males and females at the age of 18-65 years) selected in accordance with results of hematological analysis and infection analysis. Blood serum was stored in a freezer at -40°C.

Samples were defrosted, 50 μl 5 M $HClO_4$ solution was added to 500 μl plasma for protein precipitation, and the mixture was stirred and placed on ice for 10 min. After centrifugation (Abbott TDx LN9527-15, 10,000 rpm, 2 min), 350 μl supernatant was taken and 70 μl 5 M $HClO_4$ solution was added together with 10 μl 100 μM 6-methyl quinoxaline solution (internal standard) and 70 μl 9.2 mM o-phenylenediamine. Incubation was carried out at room temperature (25°C) for 30 min with stirring. Thereafter, the samples were filtered by centrifugation in tubes (Corning SpinX, pore diameter 0.45 μ). Chromatographic separation of the mixture was performed on a liquid chromatograph consisting of a Beckman-114M high pressure pump, a Rheodyne 7125 injector with loop volume of 50 μl, a column with Gold-turbo silica gel (4.6×33 mm, sorbent granulation 1.5 μ), and a Kontron 430 UV-detector at λ=315 nm. The data were processed using MultiChrome 1.5 software. Solution samples were taken using a Hamilton microsyringe with a volume of 100 μl. The mobile phase contained 80% 10 mM KH_2PO_4 solution (pH 2.8) and 20% CH_3CN; flow rate 1.5 ml/min. For construction of the calibration curve, standard MG and 6-methyl quinoxaline solutions were prepared; concentration range was 62.5-1000 nM. Values were selected with respect to the data on MG concentrations under normal and pathological conditions [13]. Calibration curve was plotted using five points; the graph was linear in this concentration range (Fig. 1). The data were processed using Statistica 6.0 software.

RESULTS

To assess within-run precision in the series, 10 MG measurements were carried out successively for one blood plasma; $V_{\%}$ was 7.9%. Reproducibility of the results was determined on the basis of measurements for the pool of frozen and aliquoted serum for 8 days; $V_{\%}$ – 14.6%. If the peak magnitude was insufficient for reliable concentration estimation, the sample was evaporated (ECTS 10 evaporator, Aquilon) to two-fold volume reduction at 50°C. Figure 2 demonstrates chromatogram of blood serum. MG concentration in donor's serum samples (n=27) was 251.3±53.6 nM, confidence interval 21.2 (p=0.95, f=26). The obtained concentrations fit unimodal normal distribution (Fig. 3).

The method was developed for indirect, but reliable estimation of GEP level in patients with diabetes, hyperlipidemia, and cardiovascular disorders. In the

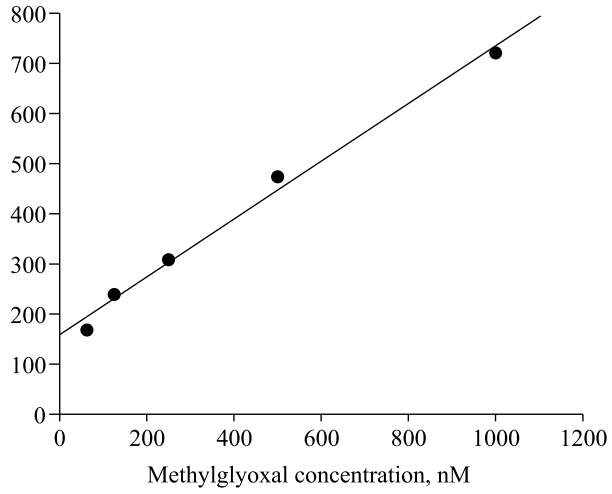

Fig. 1. UV absorption (peak area) as a function of 2-methyl quinoxalinea (MG) concentration (calibration plot).

absence of reference methods for GEP determination, unitary standard samples, difficulties of determination of one glycated protein among many others, plasma CML and MG were regarded as quantitative GEP markers *in vivo*. Mass-spectrometer is needed to estimate CML levels, however, highly significant positive correlation between CML and MG allows quantitative assessment of pooled GEPs *in vivo* with HPLC assessment of plasma MG. In diagnostics of diabetic microangiopathies, of particular importance is direct interaction of MG as glycotoxin with NH_2-groups of Lys and Arg residues with the formation of GEP, rather that its successive interaction with these residues similar to that of glucose in all Maillard reactions. This results in abnormal cross-linking between collagen fibers of the loose connective tissue with further development of microcirculation pathology of the cardiovascular system. Under these conditions MG is a unspecific, but reliable test of pathological changes [3]. Strict positive correlation between MG, 8-isoprostanes, and MDA levels can be explained by the necessity of physiological denaturation of early glycation products and GEP via oxidation with active O_2 species before utilization by resident macrophages, monocytes, or hepatocytes [2]. High MG level in blood plasma reflects blood GEP levels and is a risk factor for ischemic heart disease morbidity and mortality also in non-diabetic patients [9]. Increased plasma MG level is also a risk factor for renal function impairment with predominance of glomerular sclerosis and renal failure, and a marker of endoecology impairment and activation of biological reaction of innate immunity.

Glyoxale, MG, MDA, and glutaric dialdehyde are isoforms of dialdehydes and exhibit similar chemical properties. Being bifunctional cross-linking reagents, they all act as immobilization reagents and form cross-links of biopolymers with each other and with other macromolecules. In biotechnology, immobilization of proteins on a matrix or selective electrode was performed using glutaric aldehyde, which is an isoform of glyoxal, MG, and MDA. Hence, they can also form cross-links between collagen chains in the loose connective tissue. Cross-linking is a characteristic reaction for glyoxal, MG, and MDA. When glutaraldehyde interacts with hydroxyl or thiol (-SH) groups of amino acid residues, with lipids and nucleic acids, the same Schiff's bases (primary glycation products) are formed. They are unstable and intramollecular transformations result in GEP formation. When increased amounts of glyoxal and MG appear *in vivo* in diabetes mellitus and inactivation systems have no time no eliminate it, glycotoxins start to form cross-links between primarily collagen fibers in the loose connective tissue with alteration in its physicochemical properties. It is accompanied by glycation of structural proteins

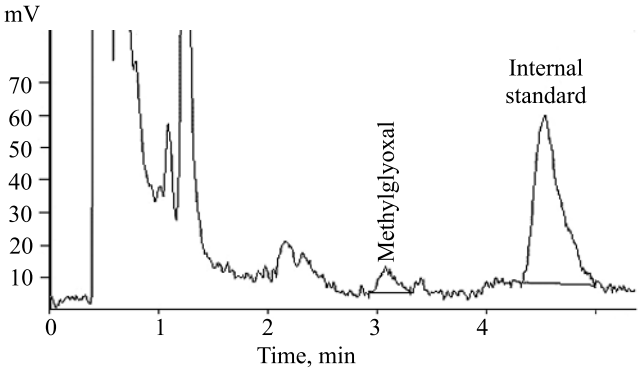

Fig. 2. Representative chromatogram of blood serum from a healthy donor. MG peak magnitude corresponds to concentration of 246 nM.

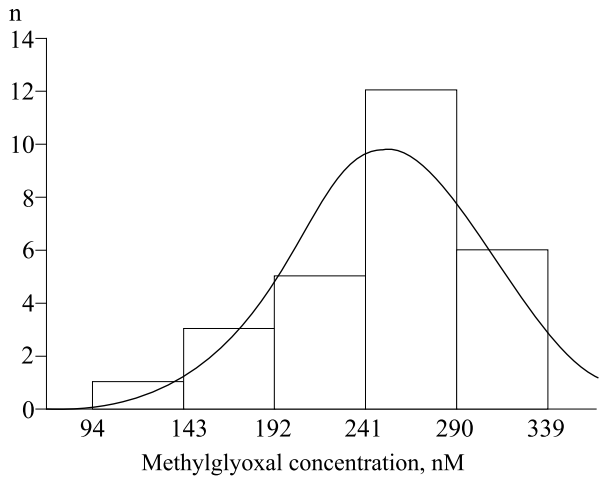

Fig. 3. Density histogram of MG concentrations in healthy donors. N: number of donors.

of the basement membrane in renal capillaries and glomeruli with alteration the microcirculation stage in blood circulatory system. Ample experimental and clinical data suggest that:

a) pathological effects of hyperglycemia on cell function is eventually realized through glycotoxin formation, *e.g.* MG;
b) plasma MG levels significantly and positively correlate with tissue content of all GEP, including CML [15];
c) quantitative measurement of MG plasma level using HPLC is a prognostic factor (diagnostic test) of slow arteriolar sclerosis in the form of microangiopathies of muscular type arterioles with clinical manifestations of diabetic cardiopathy, nephropathy, and skin pathologies. We assume that simultaneous MG and MDA measurements is a reliable diagnostic test of the risk of formation and accelerated development of microangiopathies in the form of both arteriolar sclerosis (microvascular complications) of the distal part of arterial bed and atherosclerosis

(marcovascular complications) of the proximal part of arterial bed.

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