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Screening for mucopolysaccharidoses in the Turkish population: Analytical and clinical performance of an age-range specific, dye-based, urinary glycosaminoglycan assay

Khaled El MoustafaMD [a], Serap SivriMDProfessor [b], Sevilay KarahanPhD [c], Turgay CoşkunMDProfessor [b], Filiz AkbıyıkMD, PhDProfessor [a], İncilay LayMD, PhDAssociate Prof. [a,d,*]

[a] Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, Turkey
[b] Department of Child Health and Diseases, Metabolism and Nutrition Units, Faculty of Medicine, Hacettepe University, Ankara, Turkey
[c] Department of Biostatistics, Faculty of Medicine, Hacettepe University, Ankara, Turkey
[d] Clinical Pathology Laboratory, Hacettepe University Hospitals, Ankara, Turkey

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ABSTRACT

Comprehensive analytical and diagnostic performance of urinary quantitative GAG analysis with dimethylmethylene blue (DMB) and the age-specific reference ranges were determined in Turkish population, which has a high incidence of MPSs. Precision, linearity, recovery and accuracy/trueness, limits, stability, and effect of interferents were tested according to CLSI guideline. Clinical performance was evaluated with ROC analyses including 45 MPS patients. Intra-day and inter-day precisions were <5% and <11% (CV), respectively. LoD was 9.12 mg/L and LoQ was 23.3 mg/L. The highest reference values for urinary GAG excretion were determined in an age-specific manner. In the 2–13 years age cohort, a cut-off of 89.86 mg/g creatinine resulted in 98.07% sensitivity and 93.33% specificity. Proteinuria and hematuria interfered with analysis in some instances. Neither leukocyturia nor pH changes affected the assay. Stability analysis indicated that freezing urine samples for transfer is unnecessary. Of the 45 MPS patient samples evaluated, only three tested negative including MPS II, IVA and VI. Despite limitations due to low levels of urinary GAG excretion in some cases, urinary GAG analysis with DMB with its technical simplicity, low cost, and precise quantitative results, is a valuable screening method, particularly in populations with a high rate of MPSs.

1. Introduction

Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders caused by deficiency of any one of 11 different lysosomal hydrolases required for glycosaminoglycan (GAG) catabolism. Impaired degradation of GAG leads to GAG accumulation in various tissues and biological fluids causing severe clinical manifestations [1]. Currently, enzyme replacement therapies (ERT) and hematopoietic stem cell transplantations are available for some types of MPSs [2–4]. Substrate reduction and gene therapies are also in development [5,6]. Starting these treatments at early stages of the disease has a positive impact on life in most patients. To provide a better efficacy, early diagnosis is crucial and definitive diagnosis is based on laboratory tests. Final diagnoses of MPS types are carried out by specific enzyme activity analyses and genetic investigations [7–10]. In classically presenting MPS disorder, individuals exhibit increased urinary GAG excretion, with the possible exception of some MPS IVA, adult and/or non-classically presenting individuals affected by MPS, and quantitative urinary GAG analysis is often the first line-screening test [9,11]. Numerous methods including dye-binding spectrophotometric methods, HPLC, ELISA, and LC-MS/MS are in use to detect urinary GAG excretion [12–21]. According to ERNDIM (European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism, Sheffield, UK) data, 82% of the metabolism laboratories are using 1,9-dimethyleneblue (DMB) analysis for the measurement of urinary GAG [22]. Dispersed and little data exist on analytic and diagnostic test characteristics of this quantitative urinary GAG measurement in the literature. Moreover, the incidence of MPSs is estimated more in populations with a high rate of consanguineous marriages, as seen 21% in Turkish population and determination of the age-specific highest reference values is essential to establish the use of urinary GAG measurement for high risk population MPS screening [23,24]. We aimed to determine analytical and diagnostic efficiency and age-specific reference intervals for urinary GAG analysis as performed by DMB-based primary screening for MPSs, the most common inherited metabolic disorder in the Turkish population.

* Corresponding author at: Hacettepe University Faculty of Medicine, Department of Medical Biochemistry, Hacettepe University Hospitals, Clinical Pathology Laboratory, 06100 Ankara, Turkey.
E-mail addresses: lincilay@gmail.com, isinici@hacettepe.edu.tr (İ. Lay).

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2. Materials and methods

2.1. Subjects

A total of 836 urine samples from healthy individuals who were referred to Hacettepe University Hospitals for check-up were sourced for the reference interval study. A detailed interview addressing personal, family history, and demographic information was performed and the individuals with chronic inflammatory and metabolic diseases, diabetes mellitus, and renal disorders were excluded. All subjects were categorized into 6 groups by age (0–3, 4–6, and 7–12 months, and 1–2, 2–13, and >13 years). Urine samples from 45 diagnosed MPS patients (13 Sanfilippo, 13 Morquio, 13 Maroteaux–Lamy, 4 Hunter, 2 Hurler–Scheie), admitted to the Pediatric Metabolism Department were also collected for the validation study and ROC analyses. The study protocol adhered to the Declaration of Helsinki Guidelines and was approved by the Ethics Committee of Hacettepe University (GO 2014/351). Informed consents were obtained for each individual.

2.2. GAG analysis with DMB

Random urine samples were collected in sterile bottles without any preservatives. Urine samples were analyzed promptly and also stored at different temperatures for further analyses. GAG analyses were performed in triplicate according to the method described by Whitley et al. [22]. Spot urine creatinine was also measured simultaneously by the kinetic Jaffe method (Beckman Coulter AU systems, USA). Results are expressed in mg/g creatinine.

2.3. Validation and reference interval study

Validation of GAG analysis was performed according to Clinical and Laboratory Standards Institute (CLSI) guideline. Appropriate wavelength, stability, precision, linearity, recovery and accuracy/trueness, effect of interferents (proteinuria, hematuria, leukocyturia, pH changes), limit of blank (LoB), limit of detection (LoD), limit of quantitation (LoQ), receiver operating characteristic (ROC curve), and age-related reference intervals were determined according to CLSI protocols.

Different levels of chondroitin sulphate A (20, 40, 60, 80, 120, 160 mg/L) and one urine sample from a healthy individual (low GAG level) and one urine sample from an MPS patient (high GAG level) were used to determine the appropriate wavelength. Absorbance readings were carried out between 500 and 600 nm at intervals of 5 nm.

Urine samples with 4 different GAG levels (<100, 100–200, 200–300, and >300 mg/g creatinine) were aliquoted into portions and kept at 4 different storage temperatures (room temperature, +4 °C, −20 °C, and −80 °C) for 33 days. GAG analyses were performed with 3 days intervals for the first 21 days and 6 days apart for the rest in the stability analysis.

A 20 day precision study (morning and evening) was performed at 5 different levels of GAG (13.64–390.67 mg/g creatinine). Intra-day and inter-day CV% values were <5% and <11%, respectively for five different levels of urinary GAG. Intra-day precisions for 13.64–158.01–246.04–372.42–390.67 mg/g creatinine were 4.64–0.88–2.48–2.61–3.87% (CV) and inter-day precisions were 8.30–2.08–10.85–5.33–9.66% (CV), respectively.

A sample set that was predicted larger than the linear range of 20–30% was prepared for linearity analysis. Ten different levels of chondroitin sulphate A (0–280 mg/L) were used. Calibration solutions with 7 different levels of chondroitin sulphate A (0–160 mg/L) were prepared and repeated 10 times for verification of assay linearity.

Recovery was evaluated by spiking 7 different pooled urines of known GAG levels (8.7–166.96 mg/L) with two distinct chondroitin sulphate concentrations (15 and 30 mg/L). GAG analyses were carried out in 5 cycles before and after adding the standards. Accuracy and trueness were evaluated with ERNDIM external quality urine mucopolysaccharides scheme and recovery analyses, respectively.

The effects of proteinuria, hematuria, leukocyturia, and pH changes on GAG analysis were examined. Interference of proteinuria was evaluated by adding bovine serum albumin (BSA) to give a final protein concentration of 0, 15, 30, 45, and 60 mg/dL into each urine sample with low, moderate, and high GAG levels (15, 85, and 190 mg/L). For hematuria interference, a urine sample with 400 red blood cell count in every field was added into each urine sample with low, moderate, and high GAG levels (15, 85, and 210 mg/L) to observe 0, 5, 10, 15, and 20 erythrocytes in all fields. For leukocyturia interference, a urine sample containing 760 leukocytes in every field was added into each urine sample with low, moderate, and high GAG levels (15, 100, and 210 mg/L) to give 0, 5, 10, 15, and 20 leukocytes in all fields. pH of urine samples with low, moderate, and high GAG levels (25, 80, and 200 mg/L) were changed by HCl or NaOH to obtain pH values of 4, 6, 8, 10, and 12.

GAG analyses were performed in 20 blanks, 20 samples containing the lowest amount of analyte, and 5 samples containing 20, 30, 60, 80, and 120 mg/L GAG for 20 days to determine LoB, LoD, and LoQ, respectively.

Evaluation of clinical test performance was assessed via ROC analyses by use of urinary GAG measurements from 836 healthy individuals and 45 MPS patients of all ages, and by use of 196 healthy individuals and 30 MPS patients in the 2–13 years specific age range. Diagnoses of MPS patients were confirmed through GAG patterns in cellulose acetate electrophoresis (qualitative GAG assay), leukocyte specific enzyme activity assays, and mutation analyses.

From a total of 836 healthy individuals, age-related reference intervals were determined for 0–3, 4–6, and 7–12 months and 1–2, 2–13, and >13 years, in both genders. Outliers and extreme values were shown by box-plot analyses. Urinary GAG values from patients and healthy individuals were plotted with respect to age and the logarithmic equation with R^2 was calculated.

2.4. Statistics

Statistical analyses were performed with SPSS software v.21.0. Distribution normality and extreme outliners were tested by D'Agostino Pearson's and Tukey method, respectively. The relationship between age and GAG values were given by non-linear regression model. The differences between the age groups were tested with Kruskal-Wallis test.

3. Results

The maximum absorbance from urine samples having low levels of GAG was obtained at 595 nm. The highest absorbance of urine samples with high levels of GAG was observed at 530 nm (Fig. 1A).

Stability analysis was carried out for the evaluation of transfer conditions to the laboratory at room temperature, +4 °C, −20 °C and −80 °C in the urine samples containing 4 different concentrations of GAG. Urine samples with GAG concentrations of <100, 100–200, and 200–300 mg/g creatinine remained stable for at least 1 month at all temperature conditions tested The stability was maintained for urine samples with GAG concentration of >300 mg/g creatinine at room temperature for 21 days (Fig. 1B).

Deterioration in the linearity was observed after reaching the level of 240 mg/L chondroitin sulphate A and up to this value $y = 0.022 + 0.010x$ ($R^2 = 0.975$). Therefore, calibration solutions between 0 and 160 mg/L of chondroitin sulphate A were used for linear calibration interval ($y = 0.0117x$ and $R^2 = 0.9972$). The lowest and the highest expected values in healthy and patient urine samples were found measurable within the limits of this range. Correlation between measured and target levels of analyte was assessed for verification of linearity. Slope, standard deviation (SD), intercept, and correlation were given in the associated table in Fig. 2. Linearity was acceptable with R^2 of 0.99.