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High-throughput determination of urinary hexosamines for diagnosis of mucopolysaccharidoses by capillary electrophoresis and high-performance liquid chromatography

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ABSTRACT

Mucopolysaccharidoses (MPS) diagnosis is often delayed and irreversible organ damage can occur, making possible therapies less effective. This highlights the importance of early and accurate diagnosis. A high-throughput procedure for the simultaneous determination of glucosamine and galactosamine produced from urinary galactosaminoglycans and glucosaminoglycans by capillary electrophoresis (CE) and HPLC has been performed and validated in subjects affected by various MPS including their mild and severe forms, Hurler and Hurler-Scheie, Hunter, Sanfilippo, Morquio, and Maroteaux-Lamy. Contrary to other analytical approaches, the present single analytical procedure, which is able to measure total abnormal amounts of urinary GAGs, high molecular mass, and related fragments, as well as specific hexosamines belonging to a group of GAGs, would be useful for possible application in their early diagnosis. After a rapid urine pretreatment, free hexosamines are generated by acidic hydrolysis, derivatized with 2-aminobenzoic acid and separated by CE/UV in ~10 min and reverse-phase (RP)-HPLC in fluorescence in ~21 min. The total content of hexosamines was found to be indicative of abnormal urinary excretion of GAGs in patients compared to the controls, and the galactosamine/glucosamine ratio was observed to be related to specific MPS syndromes in regard to both their mild and severe forms. As a consequence, important correlations between analytical response and clinical diagnosis and the severity of the disorders were observed. Furthermore, we can assume that the severity of the syndrome may be ascribed to the quantity of total GAGs, as high-molecular-mass polymers and fragments, accumulated in cells and directly excreted in the urine. Finally, due to the high-throughput nature of this approach and to the equipment commonly available in laboratories, this method is suitable for newborn screening in preventive public health programs for early detection of MPS disorders, diagnosis, and their treatment.

Mucopolysaccharidoses (MPS)[1] are a group of inherited lysosomal storage disorders characterized by a deficiency in one of the lysosomal enzymes required to degrade glycosaminoglycans (GAGs) [1–3]. In all MPS subtypes, partially degraded GAG(s) accumulate in the lysosomes of affected cells and/or are eliminated in the blood and excreted in the urine. MPS types I through IX involve deficiencies of one of the key 11 enzymes needed for the stepwise degradation of various GAGs, i.e., dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), chondroitin sulfate (CS), very rarely hyaluronan (HA), or in combination.

MPS incidence is estimated as one out of 25,000 live births [1], and, in general, MPS newborns are asymptomatic, although the accumulation starts in the fetal period forming a pathological base [4]. Development of clinical signs for MPS varies with some patients showing severe disease in the first few years of life. Therapeutic approaches toward MPS patients have offered various treatment options represented by exogenously supplied enzymes that are deficient (enzyme replacement therapy, ERT, approved for MPS I, II, and VI, with clinical trials for other types of MPS in progress) [5–8], by hematopoietic stem cell transplantation (HSCT) for severe MPS forms, suggesting that enzymes from the donor

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[1] *Abbreviations used*: AA, anthranilic acid or 2-aminobenzoic acid; AUC, area under the curve; CETAB, cetyltrimethylammonium bromide; CE, capillary electrophoresis; CR, creatinine; CS, chondroitin sulfate; DMB, 1,9-dimethylmethylene blue; DS, dermatan sulfate; ESI, electrospray ionization; ERT, enzyme-replacement therapy; Fd, fluorescence detection; GAG, glycosaminoglycan; Gal, D-galactosamine; GalNAc, *N*-acetyl-D-galactosamine; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; KS, keratan sulfate; HA, hyaluronic acid or hyaluronan; HexNAc, *N*-acetylhexosamine; HPCE, high-performance CE; HS, heparan sulfate; HSCT, hematopoietic stem cell transplantation; MPS, mucopolysaccharidoses; MS, mass spectrometry.

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bone marrow can reduce GAG storage [9]. Finally, experimental gene therapies have also been performed [10]. Generally, clinical benefits of ERT for MPS include improved growth and quality of life [8,9]. However, for treatment to be successful, patients need to be treated earlier in the course of their disease and early identification of the clinically asymptomatic subjects requires screening by means of specific and sensitive tests.

GAGs are structurally complex, sulfated, linear polymers [11] constituted by repeating disaccharides with an *N*-acetylhexosamine (HexNAc), *N*-acetyl-D-galactosamine (GalNAc), or *N*-acetyl-D-glucosamine (GlcNAc) (or lower percentages of *N*-sulfo-D-glucosamine in HS), as one of the sugars and hexuronic acid, glucuronic acid, or iduronic acid, with the exception of KS which contains galactose instead, as the alternating sugar. As a consequence, according to the nature of the hexosamine residue, they are classified into two groups: glucosaminoglycans with HA, KS, HS, and heparin, and galactosaminoglycans composed of CS and DS. Hexs are common monosaccharides forming more complex carbohydrates such as oligo- and polysaccharides, and they are determined to obtain structural information and quantitative evaluations. Generally, for analytical purposes, GalN and GlcN are produced by controlled chemical degradation of the (oligo)polysaccharides, labeled with a suitable tag [12], and separated by means of HPLC in fluorescence [13,14] or capillary electrophoresis (CE) with laser-induced fluorescence [15] or UV detector [16,17].

Depending on MPS types and regardless of the severity of the clinical findings, different high-molecular-mass GAGs [18,19] as well as fragments [2,20] are accumulated in tissues and excreted in biological fluids, in particular blood and urine. As a consequence, a single analytical approach able to quantitatively measure total abnormal amounts of GAGs in urine and qualitative differences between the various classes of these macromolecules as well as modifications related to the different types of MPS would be desirable. Furthermore, a similar laboratory approach would be useful to perform newborn screening in preventive public health programs for early detection of disorders, diagnosis, and treatment of these metabolic congenital disorders, thus leading to a significant reduction in terms of death, disease, and associated disabilities. Finally, a more accurate follow-up of treatments and new possible therapeutic interventions may be monitored by this new analytical application.

Enzyme-activity assays based on cultured fibroblasts, leukocytes, plasma, or serum are definitive for a specific MPS disorder and are considered the gold standard for diagnosis. However, none of the several different approaches developed to this aim, such as the direct multiplex assay of lysosomal enzymes in dried blood spots by tandem mass (MS/MS) spectrometry (MS) [21] or a multiplexed immune-quantification assay of lysosomal proteins from dried blood spots on filter paper [22], are useful for a early diagnosis of MPS in newborn screening due to complex procedures and laboratory equipment. Furthermore, to date, no genotype–phenotype correlations are evident due to the large number of mutations and limited by the rarity of the disorders [23].

To date, there are several established procedures to diagnose MPS by the evaluation of the accumulated GAGs, such as total urinary GAGs measurement using dye-binding assay generally with 1,9-dimethylmethylene blue (DMB) [24,25] or electrophoresis on cellulose acetate [8]. However, dye-binding assays are generally used to obtain a quantitative evaluation but they are incapable of identifying individual GAGs and electrophoresis is unable to evaluate related polysaccharide structures and characteristics useful for the characterization of specific modifications. A sandwich ELISA method has also been developed for HS and KS quantitative evaluation on blood and urine [26]. However, DS measurement has not been developed along with the incapacity to evaluate its (and HS and KS) composition. Electrospray ionization (ESI) MS and the tandem mass techniques have also been used to analyze GAG oligosaccharides and mono- and disaccharides in biological samples [2,20,27]. However, this approach generally requires time-consuming and complex preparative and labeling measures before sample analysis, in addition to very expensive equipment. An HPLC-ESI-MS/MS method able to detect nanomolar amounts of HS-, DS-, and KS-derived disaccharides in MPS patient serum and plasma has been reported [26,28]. This approach requires the digestion of sample GAGs to disaccharides with various lyases prior to analysis, which is potentially costly and time-consuming. Moreover, a new reversed-phase HPLC-ESI-MS/MS assay for the determination of the urinary intact HS- and DS-derived di- to pentasaccharides has been recently described for MPS diagnosis [29]. A main drawback of this analysis is the incapacity to determine high-molecular-mass urinary GAGs demonstrated to be present in high amounts in MPS urines [18,19], as well as the time-consuming and complex preparation of samples and derivatization with 1-phenyl-3-methyl-5-pyrazolone. Finally, all these methods are not appropriate for mass screening since they are excessively costly for sample analysis due to the high cost of the equipment and they have never been found able to correlate analytical data with the clinical diagnosis and severity of MPS signs and symptoms.

In our preliminary data, we present a high-throughput procedure for the determination of urinary Hexs performed by means of CE and HPLC for possible application in the early diagnosis of MPS. Important correlations between analytical response and clinical diagnosis and the severity of the disorders were also observed. Finally, results from this study may be useful for newborn screening in preventive public health programs, as well as for a more accurate follow-up of treatment and new possible therapeutic interventions.

Materials and methods

Materials

D-(+)-GlcN hydrochloride, D-(+)-GalN hydrochloride, D-ribose, anthranilic acid (2-aminobenzoic acid, AA), and sodium cyanoborohydride were from Sigma-Aldrich. CS A from bovine trachea, DS from porcine intestinal mucosa, and HS from beef spleen were from Sigma-Aldrich. Standard HA/CS/DS unsaturated disaccharides were purchased from Sigma-Aldrich.

All the other reagents were analytical grade generally supplied by Sigma-Aldrich.

Control subjects and MPS patients

Normal subjects and patients affected by MPS type I (Hurler, Hurler-Scheie, Scheie), MPS II (severe and mild forms), MPS III, MPS IV, and MPS VI (Table 1) were registered in the Pediatric Division, Department of Clinical Sciences, Polytechnic University of Marche, Presidio Salesi, Ancona, Italy.

The diagnosis of MPS was performed on the basis of the pathological pattern of urinary GAGs and the enzymatic deficiency. The different forms of the MPS I (Hurler, Hurler-Scheie, Scheie) and MPS II (severe and mild forms) were established on the basis of the peculiar clinical signs and of the presence or not of mental retardation.

In all subjects the parents gave informed consent for the collection of urinary samples.

Urine sample collection

Urine samples were collected from healthy volunteers and subjects affected by various forms of MPS at the Department of Clinical

Table 1
Characteristics of the healthy subjects and various MPS-affected patients.

	Subjects (number)	Female/male	Mean age (years)
Healthy group	83	27/56	3.96 ± 0.38 (0.01–13)
MPS I Scheie	6	3/3	9.17 ± 2.41 (1–15)
MPS I Hurler-Scheie	1	0/1	8.33
MPS I Hurler	7	4/3	1.70 ± 0.27 (1–3)
MPS II mild	4	0/4	4.25 ± 0.48 (3–5)
MPS II severe	7	0/7	2.86 ± 0.72 (0.3–6)
MPS III	12	6/6	5.68 ± 1.17 (3–15)
MPS IV	7	1/6	4.98 ± 1.29 (1–12)
MPS VI	1	1/0	2.5

Data are reported as mean ± standard deviation. Minimum and maximum values are illustrated in parentheses.

Sciences, Polytechnic University of the Marche, Presidio Salesi, Ancona, Italy, and frozen at −20 °C for analytical investigation. According to Andrade et al. [30] the stability of GAGs allowed urine samples to be sent to the Department of Biology, University of Modena & Reggio Emilia, Modena, Italy, for analyses without the need to freeze samples, as this would not affect results.

Sample preparation

Stock solutions of GlcN or GalN standard were prepared by dissolving an accurately weighed amount of 50 mg in 5 mL (10 mg/mL) of doubly distilled water. A series of standard solutions were obtained by dilution of the stock solution in a standard volume of water (200 μL) and lyophilized [17].

The amount of 500 μL of urine samples was centrifuged at 10,000*g* for 10 min, and 1 mL ethanol was added to 200 μL of supernatant. After 2 h at −20 °C, samples were centrifuged at 10,000*g* for 10 min and pellets were dissolved in 500 μL of freshly accurately prepared 4 M HCl. After 120 min at 110 °C, the samples were lyophilized.

Optimal hydrolysis conditions were obtained by performing various time courses of chemical treatment under different conditions of HCl molarity, temperature, and times.

Derivatization of hexosamines with AA

Lyophilized GlcN or GalN standard solutions or treated samples, in the presence of internal standard ribose, were dissolved in 50 μL 1% fresh sodium acetate and 50 μL of AA (30 mg) and sodium cyanoborohydride (20 mg) dissolved in 1 mL of methanol–acetate–borate solution (120 mg sodium acetate and 100 mg boric acid in 5 mL methanol) [17]. Tubes were heated at 80 °C for 60 min. After cooling to room temperature, the samples were analyzed by CE and HPLC.

Capillary electrophoresis

CE was performed on a Beckman HPCE instrument (P/ACE System 5000) equipped with a UV detector set at 214 nm. Separation and analysis were performed on an uncoated fused-silica capillary tube (50 μm i.d., 85 cm total length, and 65 cm from the injection point to the detector) at 25 °C. The operating buffer was composed of 150 mM boric acid and 50 mM NaH_2PO_4 buffered at pH 7.0 with NaOH solution. Before each run, the capillary tube was washed with 0.1 M NaOH for 1 min and doubly distilled water for 2 min, and then conditioned with the operating buffer for 2 min. After each run, the capillary was rinsed with 0.1 M NaOH for 1 min and then conditioned with the operating buffer for 2 min. Samples were injected automatically, using the pressure injection mode, in which the sample is pressurized for 5 s. Electrophoresis was performed at 15 kV (about 60 μA) using normal polarity. Peak areas were recorded and calculated using the Beckman Gold V810 software system.

RP-HPLC analysis

All separations were performed on a Jasco HPLC 1500 Series equipped with an integrated degasser, a quaternary pump, Rheodyne injector with a 20-μL loop, a fluorescence detector Model FP-1520, and software Jasco-Borwin release 1.5. For fluorescence detection the excitation wavelength was set at 360 nm and emission at 425 nm. HPLC column was a 3-μm Gemini C18 110 Å (4.6 × 150 mm) from Phenomenex (Torrance, CA, USA) equipped with a precolumn. According to Racaityte et al. [14], eluent A contained 50 mM sodium acetate buffer, pH 4.1, in water, and eluent B contained 20% eluent A in methanol. The gradient program was 0–10 min 3% B isocratic, 10–35 min linear gradient 3–10% B, 35–45 min linear gradient 10–100% B, 35–50 min 100% B isocratic. In order to ensure the reproducibility from run to run, the column was reequilibrated with 3% B for 5 min. The flow rate was 1.0 mL/min.

Validation of the analytical methods

Quantitative CE/UV and RP-HPLC-fluorescence detection (Fd) method validations were established according to the guideline on the validation of bioanalytical methods by the European Medicines Agency (EMA) published in 2009 (guideline on the validation of bioanalytical methods, committee for medicinal products for human use. European medicines agency, London, 2009), including specificity, linearity, detection (LOD) and quantification (LOQ) limit, precision, accuracy, recovery, and robustness tests. The detection limits were estimated as the quantity of GlcN and GalN

Table 2
Effect of acid concentration, temperature, and incubation time on hydrolysis of standard GAGs, HS, CS, and DS, to produce hexosamines.

	CS GalN%	DS GalN%	HS GlcN%
HCl 1 M/60 min/110 °C	0	0	0
HCl 1 M/90 min/110 °C	5	3	3
HCl 1 M/120 min/110 °C	14	12	8
HCl 1 M/150 min/110 °C	14	15	13
HCl 2 M/60 min/110 °C	0	0	0
HCl 2 M/90 min/110 °C	11	14	64
HCl 2 M/120 min/110 °C	17	24	97
HCl 2 M/150 min/110 °C	35	47	98
HCl 4 M/60 min/110 °C	79	85	95
HCl 4 M/90 min/110 °C	89	93	101
HCl 4 M/120 min/110 °C	**103**	**98**	**102**
HCl 4 M/150 min/110 °C	97	101	73
HCl 6 M/60 min/110 °C	86	72	93
HCl 6 M/90 min/110 °C	96	103	75
HCl 6 M/120 min/110 °C	84	95	42
HCl 6 M/150 min/110 °C	53	46	12
HCl 4 M/120 min/110 °C	**103**	**98**	**102**
HCl 4 M/120 min/100 °C	98	93	96
HCl 4 M/120 min/90 °C	63	57	88
HCl 4 M/120 min/80 °C	42	33	48

The absolute amount of both produced hexosamines from standard GAGs was determined by performing hydrolysis and derivatization procedures and calculating the response on hexosamine calibration curves. Values are reported as % hexosamines generated in comparison with theoretical expected values. Values are mean of six independent analyses performed both by HPLC and by HPCE having an overall coefficient of variation generally lower than 15%. In bold are underlined the optimum obtained results.

producing a signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. The specificity of the two analytical techniques was determined with migration time (MT) and peak area (PA) of the two Hex peaks through the precision analysis assay. The calibration curves were constructed from PA versus concentrations of GlcN/GalN standard. Linear regression analysis was used to calculate the slope, intercept, and correlation coefficient (r^2) of the calibration curve (from 1 to 20 μg of Hexs for CE and from 5 to 100 μg for RP-HPLC-Fd). The precision of the method was assessed by determination of Hexs with five replicates ($n = 5$) of five different concentrations of standard solutions. Intra- and inter-day precision and accuracy of the methods were estimated by relative standard deviation percentage (CV%) from the analysis of freshly prepared solutions on 3 separate days.

For recovery of Hexs from urine, GlcN and GalN were added to samples and the solutions were spiked with standards at three concentration levels (5, 10, and 20 μg for CE and 10, 50, and 100 μg for RP-HPLC-Fd) and then analyzed in comparison with samples with no external standards. The solutions were replicated three times each, and the Hexs amounts determined were compared to the theoretical amounts. The recovery ratio percentage (REC%) and their CV% were calculated.

Robustness of analytical procedures was assessed by analysis of the standards under different analytical conditions, in particular temperatures, voltage, flow rate, and buffers composition no more and no less than 10% of the adopted values.

Statistics

Micrograms of Hexs were calculated by means of specific calibration curves (not shown) and reported as microgram per milligram creatinine (CR) determined according to Coppa et al. [24] and de Lima et al. [25]. Data are expressed as means ± SD or SE. Statistical analysis was performed by analysis of variance (ANOVA), Student–Newman–Keuls test, and Mann–Whitney *U* test as appropriate by means of SPSS Statistics software Version 17.0 for Windows. The statistical significance of differences was set at $P < 0.05$.

Results

Validation of the analytical procedures applied to urine samples

After a rapid (2-h) urine pretreatment procedure consisting in precipitating complex heteropolysaccharides along with related oligomers and fragments [11,17], free Hexs are generated by acidic hydrolysis. The hydrolysis process involves two acid-catalyzed steps, the hydrolysis of glycosidic linkage and the *N*-acetyl (and the *N*-sulfo in minor percentages for HS) group (resulting in de-*N*-acetylation or de-*N*-sulfonation), with the formation of the Hexs. The standard GAGs, HS, CS, and DS, hydrolysis procedure was performed using HCl after evaluating both the effect of the acid

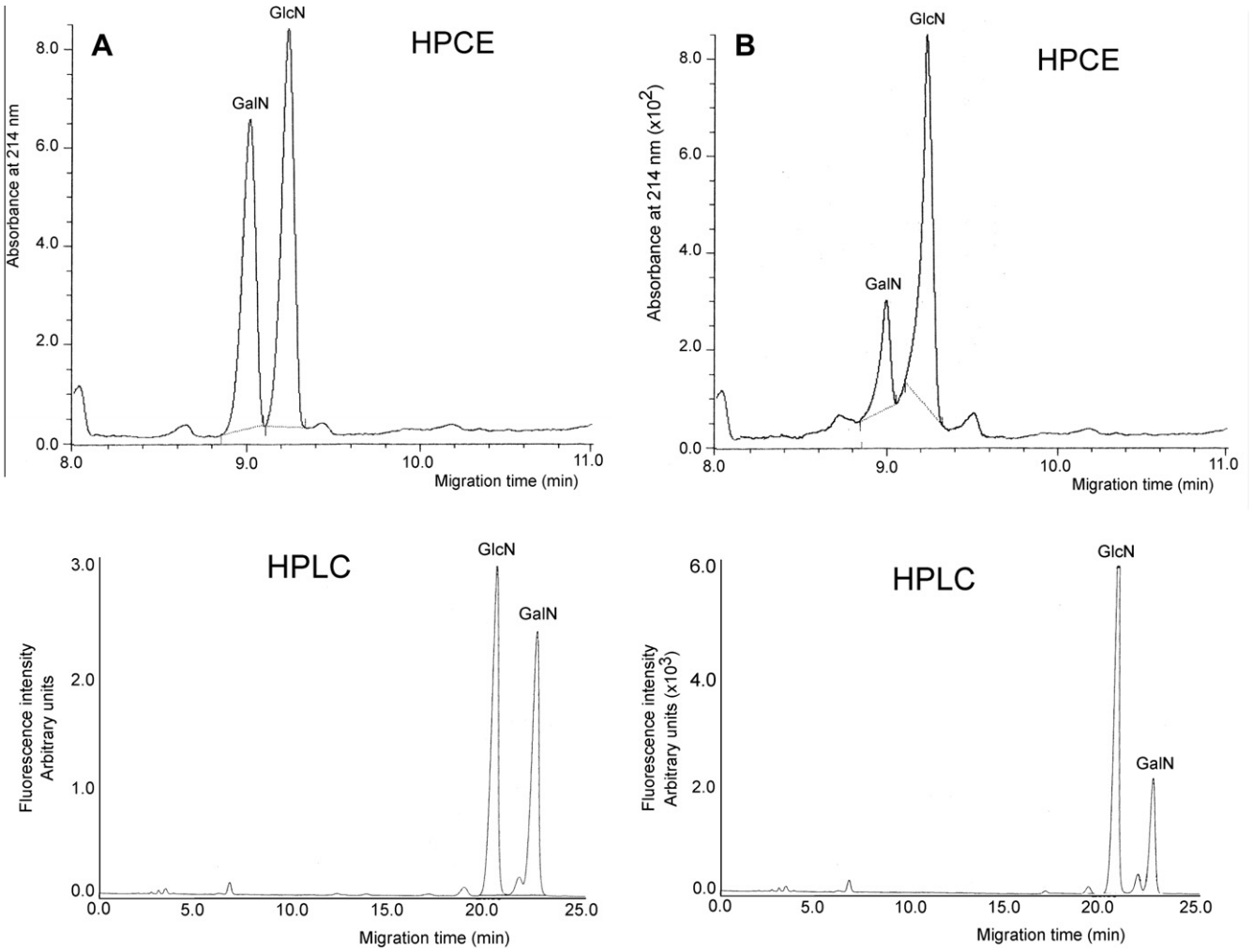

Fig.1. (A) Examples of HPCE/UV electrophoregram and RP-HPLC-Fd chromatogram of GlcN and GalN derivatized with AA and obtained after the hydrolysis procedure of urinary GAGs from (A) controls, (B) MPS III subjects, and (C) MPS VI patients. Ribose used as internal standard is out of the two panels having greater migration times (see also Refs. [14–17]).

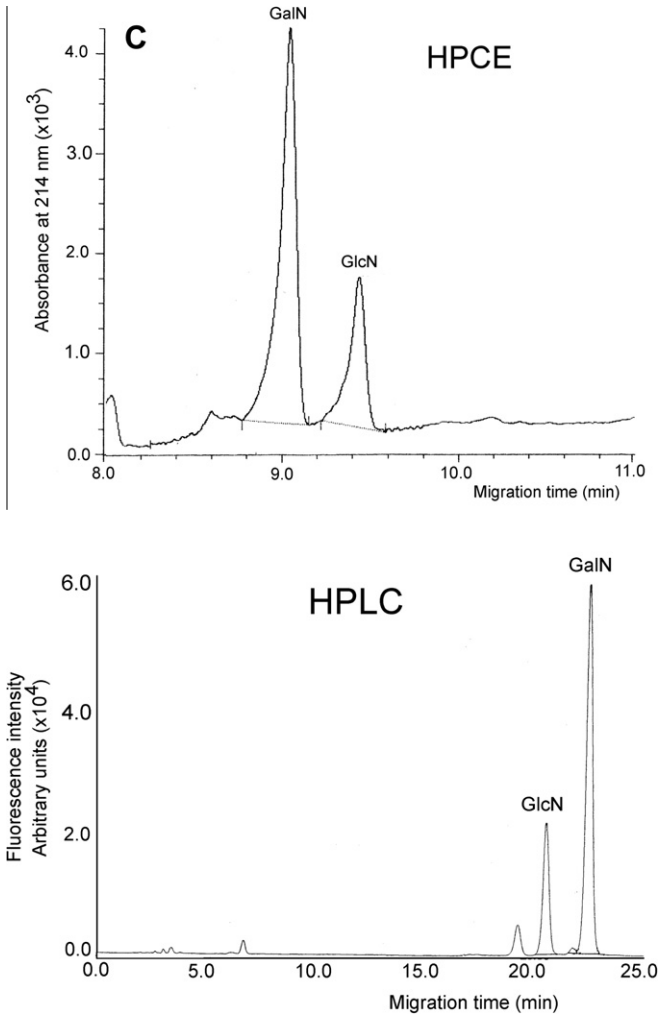

Fig. 1 (*continued*)

concentration and the influence of the temperature and the incubation time (see Table 2). The absolute amount of both Hexs in standard GAGs was determined by performing hydrolysis and derivatization procedures on standard and calculating response on Hexs calibration curves (see Supplemental Data). Results are illustrated in Table 2. Optimal conditions were found at 110 °C using 4 M HCl for 120 min, affording a maximum percentage of hydrolysis. Lower temperatures or times were found to produce lower Hexs percentages and stronger conditions were shown to degrade GlcN and GalN. Furthermore, in order to ensure that both Hexs prepared are not degraded or altered after preparation prior to analysis, their stability was investigated analyzing representative test samples directly after preparation and after 1, 3, 5, and 30 days. According to previous publications [13,14,17], it was found that both Hexs in analysis and reference samples are stable at least 30 days when stored at 4 °C.

The percentage recoveries of both Hexs in urine were determined by performing the above illustrated procedure with pure CS, DS, and HS and standard related disaccharides. Under these conditions, the GlcN and GalN recovery from urine was observed to be about 100% for polysaccharides and close to 95% for disaccharides. As a consequence, the total recovery for high-molecular-mass polymers and disaccharides (and obviously for fragments having intermediate mass values as demonstrated to be present in MPS subjects urine [18,20]) was found to be 95–100% assuring a complete quantitation of urine saccharidic species.

According to previous studies [12,13,17], GlcN and GalN (and other monosaccharides) are derivatized under optimum conditions with 2-aminobenzoic acid in methanol-acetate-borate reaction medium to produce derivatives capable of strong absorption at UV 214 nm and fluorescence. After a very short time, 1 h, required for the derivatization process, samples were separated by CE/UV in ~9–10 min and RP-HPLC-Fd in ~20–22 min (Fig. 1A). The presence of internal standard ribose enabled the control of the entire quantitative procedure [17] (see Supplemental Data for validation of the entire procedure).

Hexs profile in urine of control subjects

Hexs were determined in the urine of 83 healthy subjects, 27 females and 56 males, ranging from less than 1 month old to 13 years old (Table 1). The ratio between the two Hexs was calculated as a ratio of μg/mg CR determined by using specific calibration curves or by a simple ratio between the area under the curve (AUC) determined by the two analytical procedures. Even if a slightly lower value was calculated for the ratio determined by calibration curves (Table 3), no significant differences were found between the two data using both methods. In fact, after derivatization with AA, GlcN and GalN showed fairly similar calibration curves both in UV and fluorescence (this work and [17]), making this method useful for direct and simple quantification of the two Hexs using AUC data directly output by detectors. As a consequence, fairly similar amounts of the two Hexs were found in the controls with a small increase in GlcN. Finally, no significant differences were found between values observed for the two analytical procedures, as clearly emerges also from Figs. 2 and 3.

No significant differences were obtained for the total content of Hexs depending on the age of subjects up to 13 years old, while a trend toward a decrease in the GalN/GlcN ratio was observed depending on age (Fig. 2).

MPS I patients

The total content of urinary Hexs in six patients with the mild form of MPS I, Scheie type (Table 1), significantly increased in both analytical methods by ~303% (CE/UV) and ~211% (HPLC) (Table 3 and Figs. 2 and 3). This greater value of the total Hexs is due to a significant increase in both Hexs with GalN greater than GlcN. The GalN/GlcN ratios determined by calibration curves and directly by AUC increased by ~78 and ~50% (CE/UV), and ~24 and ~32% (HPLC) (Table 3). However, these values were found to be borderline in regard to statistical significance with HPLC data calculated as nonsignificant.

Seven patients affected by the severe form of MPS I, Hurler type (Table 1), were analyzed using both procedures. The results illustrated in Table 3 and Figs. 2 and 3 showed a significant strong increase in the total amount of Hexs (~500%) and the GalN/GlcN ratio due to a strong increase in the GalN content (more than ~1000%) along with GlcN. Finally, significant differences were also calculated between the total content of Hexs between the mild and severe forms of MPS I (see Figs. 2 and 3).

The only subject affected by an intermediate form of MPS I, Hurler-Scheie type, showed intermediate values of total Hexs content and a value of GalN/GlcN ratio fairly similar to the MPS I patients Hurler form (Table 3 and Figs. 2 and 3).

MPS II patients

Four subjects affected by the mild form of MPS II (Table 1) showed slightly increased values of total Hexs (~20–30%) found to be nonsignificant due to their high coefficient of variation (Table 3). On the contrary, a significant high Hexs ratio was

Table 3
Single and total hexosamines values along with their ratio determined by HPCE and HPLC in control group and various MPS I and II syndromes.

	Healthy group	MPS I Scheie	MPS I Hurler-Scheie	MPS I Hurler	MPS II Mild	MPS II severe
HPCE/UV						
GalN (μg/mg CR)	20.1 ± 12.6 (0.1–55.0) (62.7%)	110.6 ± 22.5 (92.4–152.7) (20.3%)	197.9	320.0 ± 91.3 (229.5–476.7) (28.5%)	32.5 ± 14.5 (18.6–45.4) (44.6%)	124.9 ± 30.7 (93.2–175.0) (24.6%)
Difference vs control		+450.2%	+884.6%	+1492.0%	+61.7%	+521.4%
ANOVA/student		$P < 0.05/P < 0.001$		$P < 0.02/P < 0.000$	$P = NS/P = NS$	$P < 0.05/P < 0.01$
GlcN (μg/mg CR)	30.9 ± 18.1 (4.8–79.0) (58.7%)	95.3 ± 26.3 (73.3–138.8) (27.6%)	110.2	175.7 ± 62.9 (117.9–285.8) (35.8%)	29.3 ± 10.6 (18.1–42.3) (36.3%)	82.0 ± 19.1 (40.5–95.5) (23.3%)
Difference vs control		+208.4%	+256.6%	+468.6%	−5.2%	+165.4%
ANOVA/student		$P < 0.05/P < 0.002$		$P < 0.02/P < 0.001$	$P = NS/P = NS$	$P < 0.05/P < 0.01$
Total Hexs (μg/mg CR)	51.1 ± 30.1 (4.9–120.8) (58.9%)	206.0 ± 45.7 (166.1–291.5) (22.2%)	308.1	495.7 ± 151.7 (364.8–762.5) (30.6%)	61.8 ± 24.7 (77.7–87.7) (40.0%)	206.9 ± 36.2 (157.0–265.2) (17.5%)
Difference vs control		+303.1%	+502.9%	+870.0%	+20.9%	+304.9%
ANOVA/student		$P < 0.05/P < 0.001$		$P < 0.005/P < 0.001$	$P = NS/P = NS$	$P < 0.05/P < 0.01$
GalN/GlcN (μg/mg CR)	0.65 ± 0.2 (0.6–1.2) (17.5%)	1.16 ± 0.2 (0.9–1.4) (16.8%)	1.80	1.82 ± 0.3 (1.6–2.4) (16.5%)	1.11 ± 0.2 (0.9–1.4) (17.6%)	1.52 ± 0.7 (1.0–2.9) (41.2%)
Difference vs control		+78.5%	+176.9%	+180.0%	+70.8%	+133.8%
ANOVA/student		$P < 0.05/P < 0.05$		$P < 0.02/P < 0.000$	$P < 0.05/P < 0.02$	$P < 0.05/P < 0.01$
GalN/GlcN (AUC)	0.93 ± 0.2 (0.7–1.3) (17.7%)	1.40 ± 0.2 (1.0–1.7) (16.8%)	2.09	2.15 ± 0.4 (1.9–2.7) (16.9%)	1.62 ± 0.3 (1.4–1.9) (15.9%)	1.76 ± 0.5 (1.2–2.7) (29.8%)
Difference vs control		+50.5%	+124.7%	+131.2%	+93.3%	+89.2%
ANOVA/student		$P < 0.05/P < 0.05$		$P < 0.02/P < 0.000$	$P < 0.05/P < 0.02$	$P < 0.05/P < 0.01$
HPLC/Fd						
GalN (μg/mg CR)	28.3 ± 14.7 (4.2–58.9) (51.9%)	98.2 ± 13.0 (81.9–114.5) (13.2%)	198.9	290.9 ± 93.6 (167.1–436.7) (32.2%)	43.5 ± 31.6 (13.6–72.0) (72.6%)	101.7 ± 15.9 (82.0–126.2) (15.6%)
Difference vs control		+247.0%	+602.8%	+927.9%	+53.7%	+259.4%
ANOVA/student		$P < 0.05/P < 0.001$		$P < 0.02/P < 0.000$	$P = NS/P = NS$	$P < 0.01/P < 0.005$
GlcN (μg/mg CR)	35.6 ± 18.0 (5.7–73.9) (50.4%)	100.5 ± 35.5 (62.9–162.9) (35.3%)	138.7	219.1 ± 83.2 (111.9–338.0) (37.9%)	40.7 ± 23.3 (19.4–64.3) (57.4%)	82.5 ± 10.7 (70.6–100.0) (13.0%)
Difference vs control		+182.3%	+289.6%	+515.4%	+14.3%	+131.7%
ANOVA/student		$P < 0.05/P < 0.01$		$P < 0.02/P < 0.001$	$P = NS/P = NS$	$P < 0.01/P < 0.005$
Total Hexs (μg/mg CR)	63.9 ± 32.3 (10.3–124.4) (50.5%)	198.7 ± 46.6 (144.8–277.4) (23.5%)	337.6	510.0 ± 172.9 (279.0–774.7) (33.9%)	84.1 ± 54.8 (33.0–136.3) (65.1%)	184.2 ± 19.0 (157.1–218.6) (10.3%)
Difference vs control		+211.0%	+429.3%	+698.0%	+31.6%	+188.3%
ANOVA/student		$P < 0.05/P < 0.05$		$P < 0.02/P < 0.000$	$P = NS/P = NS$	$P < 0.01/P < 0.000$
GalN/GlcN (μg/mg CR)	0.79 ± 0.1 (0.5–1.0) (14.4%)	0.98 ± 0.2 (0.7–1.3) (16.8%)	1.40	1.33 ± 0.2 (1.0–1.6) (15.5%)	1.07 ± 0.2 (0.7–1.2) (24.2%)	1.23 ± 0.1 (0.9–1.5) (19.2%)
Difference vs control		+24.0%	+77.2%	+68.3%	+35.4%	+55.7%
ANOVA/student		$P = NS/P = NS$		$P < 0.02/P < 0.005$	$P < 0.05/P < 0.02$	$P < 0.05/P < 0.01$
GalN/GlcN (AUC)	0.96 ± 0.1 (0.6–1.3) (14.5%)	1.27 ± 0.3 (0.8–1.6) (21.7%)	1.69	1.62 ± 0.3 (1.2–1.9) (16.4%)	1.52 ± 0.1 (1.4–1.7) (8.6%)	1.55 ± 0.3 (1.0–2.0) (22.6%)
Difference vs control		+32.3%	+76.0%	+68.8%	+58.3%	+61.5%
ANOVA/student		$P = NS/P = NS$		$P < 0.02/P < 0.005$	$P < 0.05/P < 0.02$	$P < 0.05/P < 0.01$

The results are the mean of three different analyses. Data are reported as μg/mg creatinine (CR) ± standard deviation. Minimum and maximum values are illustrated in parentheses along with the coefficient of variation%. Differences vs control and significance determined by analysis of variance (ANOVA) and by the Student–Newman–Keuls test are also indicated. NS, nonsignificant.

calculated (Table 3 and Figs. 2 and 3) mainly due to a strong increase in GalN (~50–60%) as opposed to GlcN (~0–14%) (Table 3). It is worth noting that the mild form of MPS II clinically observed is characterized by a slight increase of total Hexs in the urine (and as a consequence complex polysaccharides, i.e., DS/CS and HS, along with fragments). On the contrary, the urine samples of seven patients affected by the severe form of MPS II presented very high levels of total Hexs, over ~300% in CE and over ~180% in HPLC mainly due to a strong significant increase in GalN (Table 3 and Figs. 2 and 3). In fact, a high Hexs ratio was significantly observed versus the controls both in HPCE/UV (~90–130%) and HPLC/Fd (~60%). Finally, both forms showed a relative increase in GalN rather than GlcN, producing significantly high values of Hexs ratios.

MPS III subjects

The urine of six patients affected by MPS III type A, five by type B and 1 by type C (Table 1), were subjected to Hexs analyses. As appears evident, in comparison to the controls, a strong significant increase in the total Hexs content was determined both by CE (~440%) and HPLC (~300%) (Table 4 and Figs. 2 and 3). Interestingly, this high amount of total Hexs was mainly due to a strong increase in the content of GlcN (~450–550%) as opposed to GalN (Fig. 1B) with a significant decrease in the value of the GalN/GlcN ratio, ~0.3 compared to ~0.9 of healthy subjects (Table 4). Finally, no significant differences were observed for the patients affected by the three types of MPS III, A, B, and C.

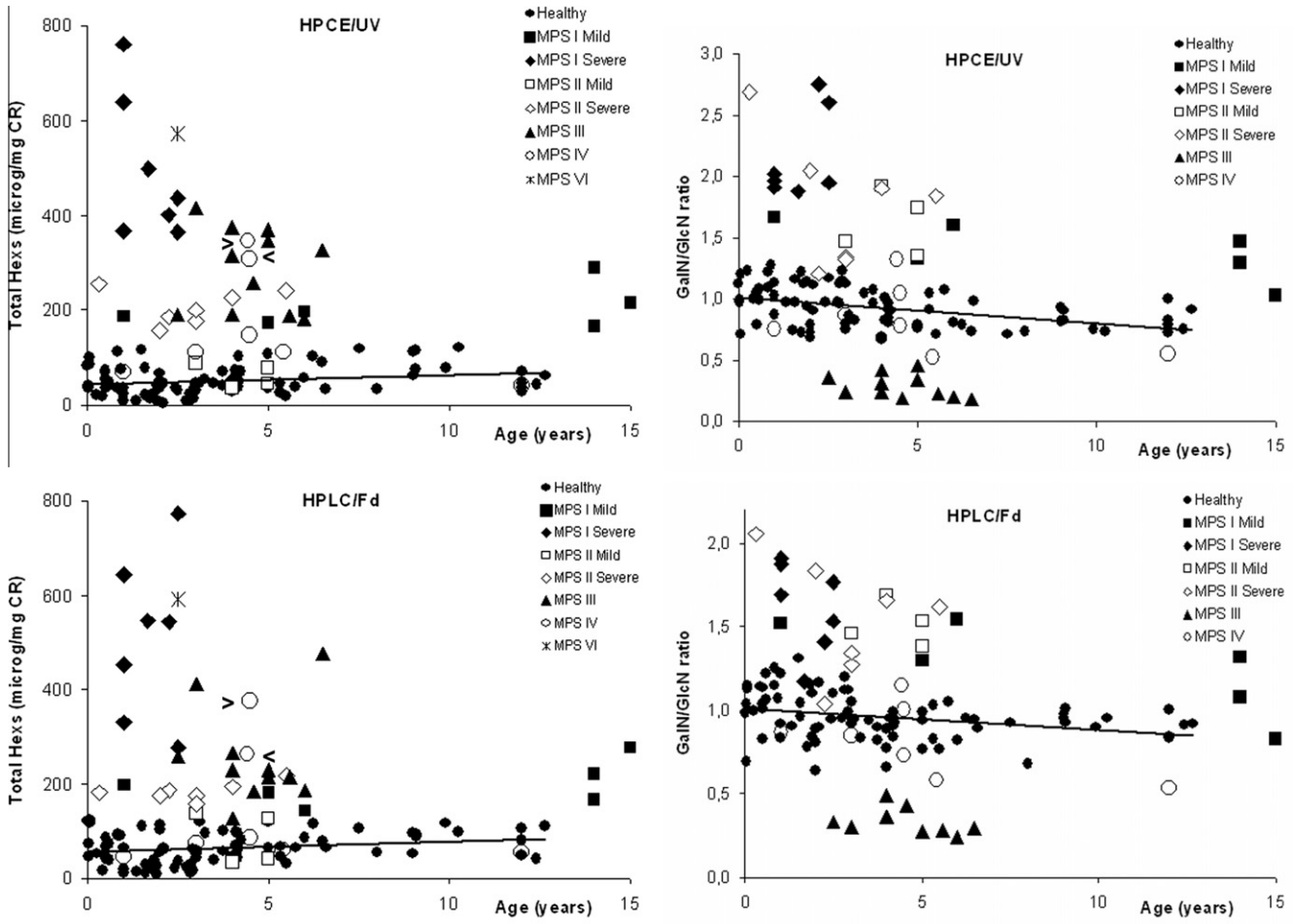

Fig.2. Single values of total hexosamines and their related ratios obtained by HPCE/UV and HPLC/Fd for each healthy subject and various MPS patients. Regression plots are also illustrated for the control group depending on age. Arrows indicate total hexosamines for two MPS IV subjects having higher values when compared to other MPS IV patients. Ratios of the unique MPS VI patients are not reported in the figure due to their very high values (see Table 4).

MPS IV subjects

We analyzed the urine of six subjects affected by MPS IVA and of one patient suffering from MPS IVB (Table 1). No differences were calculated between the two subtypes. Furthermore, as clearly emerges from Table 4 and Fig. 3, an increase in the total amount of Hexs was observed even if these differences were found to be nonsignificant compared to the controls. This was essentially due to low values fairly similar to the controls even if two patients affected by MPS IVA showed very high levels of urinary Hexs (indicated by arrows in Fig. 2) mainly due to a strong increase in GalN. In fact, these two subjects showed a GalN/GlcN ratio of ~1.0–1.3 (determined by both CE and HPLC) greater than the other MPS IV patients (Fig. 2). As can be seen, compared to the other Morquio patients, these two subjects showed anomalous values unrelated to clinical diagnosis and studies are in progress to clarify possible analytical–clinical links.

MPS VI subjects

The only subject affected by MPS VI showed very high values of total Hexs content (+870% by CE and +~700% by HPLC) mainly due to a strong increase in the GalN percentage (+~1500% by CE and +~930% by HPLC) (Fig. 1C), producing a very high value of GalN/GlcN ratio with respect to the controls (+~130% by CE and +~70% by HPLC) (Table 4 and Figs. 2 and 3).

Discussion

AA is a small fluorescent tag commonly used for labeling carbohydrates in the determination of the monosaccharide composition of glycoproteins and complex polysaccharides, i.e., GAGs, with high sensitivity [12–16]. AA-monosaccharide derivatives can be detected by a UV detector and/or in fluorescence with high sensitivity both in CE and in HPLC. In our Laboratory, this analytical approach has been applied for the determination of complex GAGs such as heparin, CS and oversulfated CS, and DS [17]. However, to date, this analytical approach has never been applied for the quali-quantitative detection and diagnosis of MPS. This is most important, considering that the various MPS disorders are characterized by the deficiency of different lysosomal enzymes [1] producing a wide range of high-molecular-mass heteropolysaccharides, CS/DS/HS/KS [18,19], as well as fragments [2,20] that are accumulated in the lysosomes of affected cells and excreted in biological fluids. As a consequence, the evaluation of MPS urinary Hexs by AA derivatives of GAGs could offer several advantages. In fact, all known heteropolysaccharides produced in MPS urine are formed by disaccharides in which one of the two monosaccharides is always GalN or GlcN (acetylated or sulfated in HS), depending on the GAG. Furthermore, this approach is able to measure high-molecular-mass polysaccharides and lower-molecular-mass fragments after a controlled chemical degradation step, as well as Hexs still present in samples like simple monosaccharides.

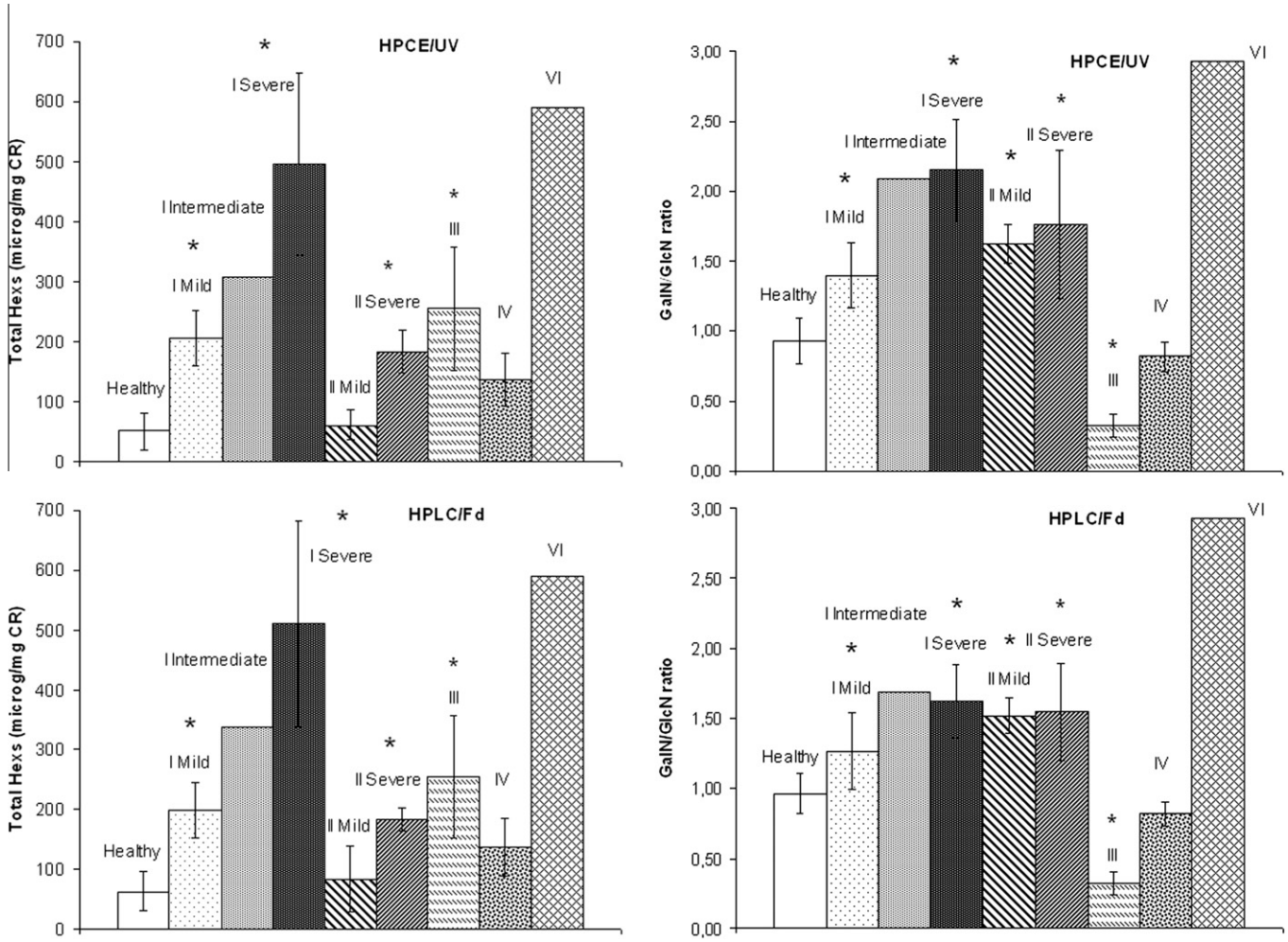

Fig.3. Total hexosamines values (expressed as μg/mg creatinine ± standard error) and their related ratios obtained by HPCE/UV and HPLC/Fd for control subjects and various MPS patients along with differing severity of the disorders. *indicates significant differences.

Finally, the simultaneous evaluation of both Hexs allows us to detect the two main families of GAGs, i.e., glucosaminoglycans composed of GlcN such as HS and KS (HA and heparin are not common in urine) and galactosaminoglycans formed of GalN (CS and DS) producing a further useful disease marker.

Early diagnosis and treatment improves outcomes in MPS [8,36,37], particularly in those disorders that can be treated with ERT or HSCT. Furthermore, because the MPS disorders produce a wide variety of clinical presentations, diagnosis is often delayed, particularly in those patients without cognitive impairment [37]. Even those with severe cognitive and somatic disease may not be diagnosed until 12 to 18 months after the onset of symptoms, during which time irreversible organ damage can occur. This is more important considering that the earlier ERT and HSCT are initiated, the better the potential outcome because of the irreversible nature of some of the abnormalities associated with the MPS disorders [37]. This highlights the importance of early and accurate diagnosis and given the clinical heterogeneity and rarity of MPS, newborn screening may be the key to identifying individuals before the onset of irreversible clinical disease. To date, several established procedures have been adopted in clinical laboratories to diagnose MPS. Apart from enzyme-activity assays that are the gold standard for diagnosis but not still useful for a early diagnosis, these analytical procedures are in general aspecific [24–26], or they require very expensive equipment and/or complex, costly, and time-consuming preparative measures [2,20,27,28] or they are merely capable of determining oligosaccharides [29] and, as a consequence, they are unable to detect the entire pattern of pathological GAGs. On the contrary, HPLC equipped with UV or fluorescence detectors is a common equipment in normal analytical and clinical Laboratories, and CE is a recent modern analytical tool due to its high resolving power, sensitivity, and high-throughput capacity useful in the analysis of complex and simple carbohydrates [31]. As a consequence, we decided to develop and validate both procedures to also obtain a comparison of data to strengthen this new analytical approach. After a rapid, 2-h urine pretreatment procedure, free Hexs are generated by hydrolysis over a further 2 h, and samples are lyophilized for a total period of 4 h, a step required to ensure the removal of all the possible water known to inhibit reductive amination of Hexs [12]. After 1 h for the derivatization process, samples are ready to be analyzed for a total preparation process of ~8–10 h. Furthermore, in a single day it would be possible to analyze several samples due to the relatively short CE/UV, ~9–10 min, and RP-HPLC-Fd, ~20–22 min, separation courses and the possibility to use common autosamples.

More than 80 control children of ~0.1–13 years old having no enzymatic deficiency comparable to MPS were analyzed using the proposed procedure. Fairly similar low levels of both Hexs were observed by CE and HPLC with no significant differences depending on age. Furthermore, fairly similar amounts of the two Hexs were found in the controls with a small increase in GlcN, and no signif-

Table 4
Single and total hexosamines values along with their ratio determined by HPCE and HPLC in control group and various MPS III, IV, and VI syndromes.

	Healthy group	MPS III	MPS IV	MPS VI
HPCE/UV				
GalN (μg/mg CR)	20.1 ± 12.6 (0.1–55.0) (62.7%)	65.9 ± 37.5 (11.5–132.2) (56.9%)	71.7 ± 66.9 (12.8–185.5) (93.3%)	451.2
Difference vs control		+227.9%	+256.7%	+2144.8%
ANOVA/student		$P < 0.01$/$P < 0.005$	P = NS/P = NS	
GlcN (μg/mg CR)	30.9 ± 18.1 (4.8–79.0) (58.7%)	204.0 ± 75.2 (65.8–337.5) (36.9%)	91.0 ± 52.8 (28.1–163.1) (58.0%)	122.4
Difference vs control		+560.2%	+194.5%	+296.1%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
Total Hexs (μg/mg CR)	51.1 ± 30.1 (4.9–120.8) (58.9%)	278.1 ± 89.4 (177.2–416.1) (32.1%)	162.7 ± 118.6 (41.0–347.4) (72.9%)	573.6
Difference vs control group		+444.2%	+218.4%	+1022.5%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
GalN/GlcN ratio (μg/mg CR)	0.65 ± 0.2 (0.6–1.2) (17.5%)	0.32 ± 0.1 (0.1–0.5) (36.7%)	0.79 ± 0.3 (0.4–1.1) (41.1%)	3.69
Difference vs control		−50.8%	+21.5%	+467.7%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
GalN/GlcN ratio (AUC)	0.93 ± 0.2 (0.7–1.3) (17.7%)	0.27 ± 0.1 (0.2–0.5) (35.4%)	0.83 ± 0.3 (0.5–1.3) (33.5%)	5.02
Difference vs control		−74.2%	−10.7%	+439.8%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
HPLC/Fd				
GalN (μg/mg CR)	28.3 ± 14.7 (4.2–58.9) (51.9%)	53.1 ± 18.9 (27.3–78.1) (35.5%)	59.6 ± 64.9 (13.9–173.9) (109.0%)	377.7
Difference vs control		+87.6%	+110.6%	+1234.6%
ANOVA/student		$P < 0.01$/$P < 0.005$	P = NS/P = NS	
GlcN (μg/mg CR)	35.6 ± 18.0 (5.7–73.9) (50.4%)	201.6 ± 88.4 (96.9–399.2) (43.9%)	77.6 ± 64.6 (32.7–201.6) (83.2%)	212.9
Difference vs control		+466.3%	+118.0%	+498.0%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
Total Hexs (μg/mg CR)	63.9 ± 32.3 (10.3–124.4) (50.5%)	254.7 ± 102.2 (126.4–476.4) (40.1%)	137.2 ± 129.3 (46.6–375.5) (94.2%)	590.6
Difference vs control		+298.6%	+114.7%	+824.3%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
GalN/GlcN ratio (μg/mg CR)	0.79 ± 0.1 (0.5–1.0) (14.4%)	0.26 ± 0.1 (0.2–0.5) (34.4%)	0.77 ± 0.2 (0.4–1.1) (33.7%)	1.77
Difference vs control		−67.1%	+2.5%	+124.0%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	
GalN/GlcN ratio (AUC)	0.96 ± 0.1 (0.6–1.3) (14.5%)	0.33 ± 0.1 (0.2–0.6) (30.1%)	0.82 ± 0.2 (0.5–1.1) (26.9%)	2.93
Difference vs control		−65.6%	−14.6%	+205.2%
ANOVA/student		$P < 0.005$/$P < 0.000$	P = NS/P = NS	

The results are the mean of three different analyses. Data are reported as μg/mg creatinine (CR) ± standard deviation. Minimum and maximum values are illustrated in parentheses along with the coefficient of variation%. Differences vs control and significance determined by analysis of variance (ANOVA) and by the Student–Newman–Keuls test are also indicated. NS, nonsignificant.

icant differences were found for the GalN/GlcN ratio calculated as μg/mg CR using specific calibration curves or by a ratio between the two Hexs analytical peak areas, making this parameter useful for a direct and simple quantification. It is worth noting that agarose-gel electrophoresis, a common analytical technique useful to measure urinary GAGs [18,19,32,33], mainly detects ∼85–90% CS and ∼10–15% HS. However, this approach is unable to determine GAG fragments having molecular mass lower than ∼1500 [18]. As a consequence, by considering the results discussed above, we can suppose that in normal urine CS is mainly present as a high-molecular-mass polysaccharide greater than ∼1500 contrary to HS, which is mainly present in the form of oligomers lower than ∼1500.

MPS I is caused by a deficient activity of α-L-iduronidase, the lysosomal enzyme which selectively cleaves L-iduronic acid from GalNAc in DS and GlcNAc in HS molecules [1,2] leading to widespread accumulation mainly of these two GAGs. MPS I is characterized by a wide clinical spectrum ranging from the severe, Hurler syndrome, which occurs in infancy and is characterized by relentless cognitive decline, to the attenuated Hurler-Scheie and Scheie syndromes, occurring in childhood or later with slower progression and moderate-to-absent central nervous system involvement [2]. In our patients, we observed an increase in total Hexs urinary concentration, in particular in more severe forms due in particular to a strong increase in GalN content rather than GlcN. As a consequence, the total content of Hex and the GalN/GlcN ratio may be used as a mass screening marker of MPS I disease. To date, no analytical data have ever been found to correlate with clinical diagnosis and the severity of MPS signs and symptoms. Very interestingly, the proposed analytical procedure was able to distinguish between mild and severe MPS I forms useful for the application of appropriate possible therapeutic interventions. Further larger study would be required to define this aspect.

X-linked Hunter syndrome (MPS II), due to the deficient activity of L-iduronate-2-sulfatase, is presently thought to exist in two clinically and genetically distinct forms, mild and severe, which are biochemically indistinguishable. However, we were able to correlate the clinical spectrum and severity of the two forms with the total content of Hexs and the GalN/GlcN ratio. Even if the increase in the total content of Hexs was found to be nonsignificant compared to the controls, the mild form of MPS II showed a significant increase in the ratio, useful as a screening marker. On the other hand, the severe form of MPS II produced a strong increase in total Hexs and the GalN/GlcN ratio, due in particular to the significantly high amount of GalN (as observed for the mild form). As for the severe form of MPS I, this analytical approach may be useful for distinguishing between mild and severe forms of MPS II for more appropriate possible interventions. It is noteworthy that the defect of the two enzymes responsible for MPS I and II produces the accumulation of the same GAGs, i.e., DS and HS, and we observed similar trends and modifications in the total content of Hexs and related ratio for the two MPS syndromes and also in the comparison between their mild and severe forms. This is more important by considering that MPS I and II have many similar clinical features [34,35].

MPS III, or Sanfilippo syndrome, results from the deficiency or absence of four different enzymes that are necessary to specifically degrade HS [1,3]. Each enzyme deficiency defines a different form of Sanfilippo syndrome, namely type IIIA (Sanfilippo A), type IIIB (Sanfilippo B), type IIIC (Sanfilippo C), and type IIID (Sanfilippo D). We analyzed MPS III patients belonging to the subtype A, B, and C, but no significant differences were found among these. On the contrary, the total content of Hexs was found to be strongly increased compared to the controls and, very significantly, the GalN/GlcN ratio was calculated as being very low due in particular to the high content of HS. These parameters may be utilized for a possible neonatal mass screening, also considering that the Sanfilippo syndrome is considered to be the most common of MPS disorders [1,3].

In Morquio syndrome (MPS IV), the degradation of KS is defective because of a deficiency of either *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS gene) in type IVA or β-galactosidase (GLB1 gene) in type IVB. Moreover, defective GALNS also affects the catabolism of CS [1,3]. No significant variations in total Hexs and/or ratio were observed versus the controls or comparing subtypes IVA and IVB with each other. However, two patients affected by Morquio type IVA showed very high values of total Hexs and in particular of GalN consistent with a strong increase in KS and/or CS (or DS). Deeper and more specific analytical approaches are in progress to bring to light possible anomalous galactosaminoglycans types and structures to obtain new information and eventually to outline a different subtype.

MPS VI (Maroteaux-Lamy syndrome), which is inherited as an autosomal recessive trait, results from the deficiency of *N*-acetylgalactosamine-4-sulfatase (arylsulfatase B) activity and the lysosomal accumulation of DS. As a consequence, we observed a strong urinary level of Hexs in particular related to the presence of high amounts of GalN derived from DS producing a very high increase in the GalN/GlcN ratio. After a more extensive and exhaustive clinical and laboratory trial, these data may be useful for a biochemical diagnosis of this syndrome.

In conclusion, a new, previously unapplied, analytical approach based on the determination of the main Hexs forming GAGs, i.e., GalN and GlcN, produced important differences compared to the control group when applied to the urine of patients affected by various MPS syndromes. In particular, the total content of Hexs is indicative of an abnormal urinary excretion of GAGs and the specific quantification of GalN and GlcN and the related ratio are markers of different MPS syndromes in both their mild and severe forms. Furthermore, on the basis of the obtained data, we can suppose that the severity of the syndrome may be ascribed to the quantity of total GAGs, as high-molecular-mass polymers and fragments, accumulated in cells and directly excreted in the urine. This is a previously unassumed supposition and, up to now, the urinary GAG level is not considered as a reliable indicator of MPS severity. Finally, due to the high-throughput nature of this approach and to the equipment commonly available in analytical laboratories, these methods are suitable for newborn screenings in the early detection of disorders and their treatment, as well as for a more accurate follow-up of therapeutic interventions. Large clinical and analytical trials are expected to increase our knowledge and the limits of this new frontier of analytical science applied to severe disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.12.016.

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