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Keratanase II Digestion Accompanied with a Liquid Chromatography/Tandem Mass Spectrometry for Urinary Keratan Sulfate Quantitative Analysis

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Purpose: Mucopolysaccharidosis IV (MPS IV) is a disease characterized by deficient activity of N-acetylgalactosamine-6-sulfatase (GALNS) causing excessive lysosomal storage of keratan sulfate (KS). The identification of the relevant disaccharide units of KS after keratanase II digestion followed by liquid chromatography/tandem mass spectrometry detection (LC-MS/MS) is validated and applicable for the preliminary diagnosis of MPS IV.
Methods: A total of 67 urine samples were collected and analyzed from 11 MPS IV patients comprising 10 MPS IVA and one MPS IVB patients, and 56 normal controls. Urinary glycosaminoglycan was first precipitated by the Alcian blue method followed by a digestion of keratanase II. The protonated species of the digested disaccharide products were detected by using multiple reaction monitoring experiment.
Results: One particular disaccharide of KS was selected. The transition mass-to-charge (*m*/*z*) of the parent ion and its daughter ion after collision was 462.0→97.0, whereas the chondrosine used as an internal standard in this assay was *m*/*z* 353.9→73.0. The results corresponded well with the two-dimensional electrophoresis method. The quantities of urinary KS were significantly raised in confirmed MPS IV patients when comparing with those of normal controls (170.2±81.1 *vs*. 4.06±1.92 μg/mL).
Conclusion: The LC–MS/MS method for MPS IVA determination is specific, sensitive, validated, and applicable for urinary KS quantification. This method can be used not only as a first-line biochemistry examination of MPS IVA, but also as an outcome survey after enzyme replacement therapy.

Keywords: Mucopolysaccharidosis IV, Keratan sulfate (KS), Keratanase, Liquid chromatography/tandem mass spectrometry (LC-MS/MS), Two-dimensional electrophoresis

Introduction

Mucopolysaccharidosis type IVA (MPS IVA; Morquio's A syndrome; OMIM#253000) is an autosomal-recessive inheritance that is caused by the deficiency of N-acetylgalactosamine-6-sulfatase (GALNS; EC 3.1.6.4) due to a particular defect of the *GALNS* gene. The *GALNS* gene is located on chromosome 16q24.3 and encodes a 522–amino acid protein and the gene spans approximately 50 kb, and contains 14 exons[1-3]. According to the review published by Morrone et al., a total of 277 unique GALNS alterations associated with Morquio A have been identified[4]. In those, most of the mutations (more than 76.4%) have been identified in the GALNS gene in MPS IVA patients are missense mutations leading to a change of a single side chain residue in the protein. In keratan sulfate (KS) degradation pathway, the GALNS enzyme will first cut the sulfur trioxide bond off at the C6 of the galactose. If the GALNS enzyme activity is deficient, the degradation pathway will be blocked, and the KS will grossly

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accumulate in lysosomes of bone, cartilage, tendons and in the extracellular matrix of tissues, and organs and finally causes symptoms of Morquio A Syndrome. Clinical manifestations include systemic skeletal dysplasia, short stature, joint abnormality, hearing loss, heart valve involvement, and corneal clouding[5-8].

The MPS comprises the great majority of the lysosomal storage diseases (LSD) population in Taiwan. Limited number of studies of the incidence (or prevalence) of MPS have been investigated. The overall incidence of MPS is estimated to be 1 in 25,000–52,000 births in Europe[9-12]. Whereas the combined birth incidence for all MPS cases was 2.04 per 100,000 live births in Taiwan surveyed from January 1984 to December 2004 (n=130)[13]. MPS II (Hunter syndrome) had the highest calculated birth incidence of 1.07 per 100,000 (2.05 per 100,000 male live births), comprising 52% of all MPS cases diagnosed. The birth incidence of MPS IV was 0.33 per 100,000 live births, which accounted for 16%, of all MPS[13]. The enzyme replacement therapy (ERT) (Vimizim; elosulfase alfa) for MPS IVA is commercially available, in which BioMarin Pharmaceutical received the US Food and Drug Administration (FDA) approval for treatment of patients with Morquio A syndrome in February 2014. The availability of ERT for MPS IVA highlights the need for early diagnosis to avoid irreversible disease progression.

Accurate diagnosis of MPS IVA is achieved generally by performing the urinary glycosaminoglycan (GAG) quantitation, two-dimensional electrophoresis (2-D EP)[14-17], and leukocyte GALNS or beta-galactosidase enzyme activity assay[18,19]. Urinary GAG quantitative analysis can provide a valuable reference for MPS diagnosis, but cannot be used alone for a definite decision of MPS. A conventional 2-D EP is the most ordinary and feasible method used for MPS IVA determination, which is the basis of MPS confirmation. Nevertheless, an overlap or a poor separation of chondroitin sulfate (CS) and KS under an electrophoretic mobility is frequently observed which may make the diagnosis more unreliable. Based on the limitations of MPS first-line screening methods, the establishment of an advanced liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for urinary KS quantification applied for MPS type IVA diagnosis is valuable and innovative.

KS is either of two GAGs (I and II), consisting of repeating disaccharides units of N-acetylglucosamine (GlcNAc-6-sulfate) and galactose in β-1,4 linkage. KS are present in the cornea, cartilage, and bone. KS classes including KS type I and II have been designated on the basis of structural differences in the linkage oligosaccharides that connect KS to the protein core which are originally distributed on the basis of different tissue types. KS I is segregated from corneal which is N-linked to specific asparagine amino acid via N-acetylglucosamine; KS II from skeletal tissue is O-linked to specific serine or threonine amino acids via N-acetyl galactosamine. KS is a linear polymer that consists of repeating disaccharide units of *N*-acetylglucosamine (GlcNAc) and galactose (Gal), but minor difference in monosaccharide content and localization between KS I and KS II[20-22]. Several articles reported that LC-MS/MS method was successfully accomplished to analyze the relevant disaccharides produced from KS by digested each disaccharide by keratanase II (Bacillus sp., EC 3.2.1) for MPS IVA determination[23-25]. The treatment of keratanase II extracted from *Bacillus* sp., which cleaves *N*-acetylglucosamine linkages of the KS chain, releasing Galβ1-4GlcNAc (N-acetylglucosamine) disaccharides with mono-sulfates. In this study, the identification of KS by applied LC-MS/MS method of the predominant disaccharide unit of KS after digestion of keratanase II is proposed and validated for MPS IVA diagnosis, and also can be used as an outcome survey after enzyme replacement therapy.

Materials and Methods

1. Sample precipitation

A total of 67 urine samples were collected and analyzed. In those, 11 confirmed MPS IV patients, comprising 10 MPS IVA and one MPS IVB patients according to their skeletal manifestations and clinical signs, leukocyte enzymatic assays, as well as molecular DNA analysis. Distinct soft tissue and skeletal disease presented in MPS IV patients, but without central nervous system involvement. Fifty-six healthy, non-MPS controls were chosen from out-patient department of MacKay Memorial Hospital, and informed consents were obtained from all normal population and the MPS IVA patients. The study was approved by the ethics committee of Mackay Memorial Hospital, Taipei, Taiwan (14MMHIS279).

Urine samples (approximate 10 mL) were collected in a sterile urine container (polyethylene; Nalge Nunc International, USA). The method of GAG precipitation was described previously[14]. The GAG precipitate was dissolved in water based on the DMB/Creatinine ratio in mg/mmol creatinine. In this study, keratanase II was purchased from the GlycoSyn (Lower Huff, New Zealand). The GAG supernatant (1.5 μL) was subjected to digest with 0.2 unit of keratanase II (0.075 μL), and then, chondrosine (IS; 7.5 μL) and 0.1 M ammonium acetate buffer (NH_4OAc; 65.925 μL) were also added. A total of 75 μL in volume of analyte sample had been proceeded by incubation in 40°C for 2 hours, and the ana-

lyte was ready for LC-MS/MS analysis. The method was referred from the method reported by Oguma et al.[23] and Martell et al.[24]. In the keratanase II digestive process, the KS was then degraded to KS-derived disaccharide of Galβ1–4GlcNAc(6S), and the mass spectrum of LC-MS/MS analysis showed negative ion mode [M–H]$^-$ at the transition mass-to-charge (*m*/*z*) of 462 precursor to the *m*/*z* 97 production for Gal β1→4GlcNAc(6S) disaccharides derived from KS.

2. Experimental parameters of LC-MS/MS analysis

The LC-MS/MS method for KS disaccharide analysis was described previously[23-25]. For GAG disaccharide LC-MS/MS analysis, a negative-ion ([M–H]$^-$) mode of multiple reaction monitoring (MRM) experiment giving a precursor molecular ion (Q1) and a respective fragment ion (Q3) was normally applied (Fig. 1). The quadrupoles, Q1 and Q3, were tuned with unit resolution, and the MS conditions were optimized for maximum signal intensity. The LC-MS/MS analysis was performed on an AB 4000 QTRAP LC-MS/MS System (AB Sciex, Foster City, CA, USA) equipped with a TurboIonSpray (electrospray ionization; ESI), and Agilent 1260 Infinity HPLC pump and autosampler (Agilent Technologies, Santa Clara, CA, USA). Data were acquired and processed using Analyst 1.5.2^{TM} software (AB Sciex). Calibrations of KS standards and internal standards were performed with every batch of samples.

A Hypercarb column (5 micron; 50×2.0 mm) was purchased from Thermo Scientific Inc. (Waltham, MA, USA). The mobile phase A consisted of 10 mM NH_4HCO_3 (pH 8.5) in deionized water and the mobile phase B consisted of 10 mM NH_4HCO_3 in 90% methanol (pH8.6), delivered under a flow rate of 500 μL/min with a programmed linear gradient from 90% buffer A : 10% buffer B (vol/vol) over 0.5 min, then returned to 0% buffer A over 1.5 min; finally, switched to 90% buffer A over 4.5 min, in which the column was equilibrated. The injection volume was 5 μL, and the total run time was 4.5 minutes. The instrument was operated in the negative-ion mode of MRM. The experimental parameters were set as followed: curtain gas at 10 psi, CAD set at medium, IonSpray voltage at –4,500 V, temperature at 550°C, Gas 1 at 50 psi, and Gas 2 at 60 psi, DP at –80, EP at –10, CE at –70, and CXP at –18. Nitrogen was used for the curtain gas, Gas 1, Gas 2, and collision gas. Data were acquired and processed using Analyst 1.5.2^{TM} software. The experimental parameters were set and are clearly described in the following section.

3. Calibration and internal standard preparation

KS calibrator was purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA), as well as the IS (chondrosine; 2-Amino-2-deoxy-3-*O*-β-D-glucopyranurosyl-D-galactose) and keratanase II were purchased from GlycoSyn (Lower Huff, New Zealand). The stock solution of KS standard was prepared in concentration of 1,000 μg/mL. The calibration curve was made by plotting six different concentrations of working standards (S1–S6), and the final concentrations of series dilutions were 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL. The protocol of keratanase II digestion was described as followings. Six individual GAG working standard (1.5 μL) and the precipitated GAG sample (1.5 μL) were placed into tubes separately, and then, keratanase II (0.2 unit; 0.075 μL), IS (5.0 μg/mL; 7.5 μL), and NH_4OAc (0.1M; 65.925 μL) were added to each tube. The total volume of analyte was 75 μL. The samples were vortex mixed and incubated at 40°C for 2 hours, after that, the analytes were ready for LC-MS/MS analysis.

The calibration curve was made by plotting the standards with

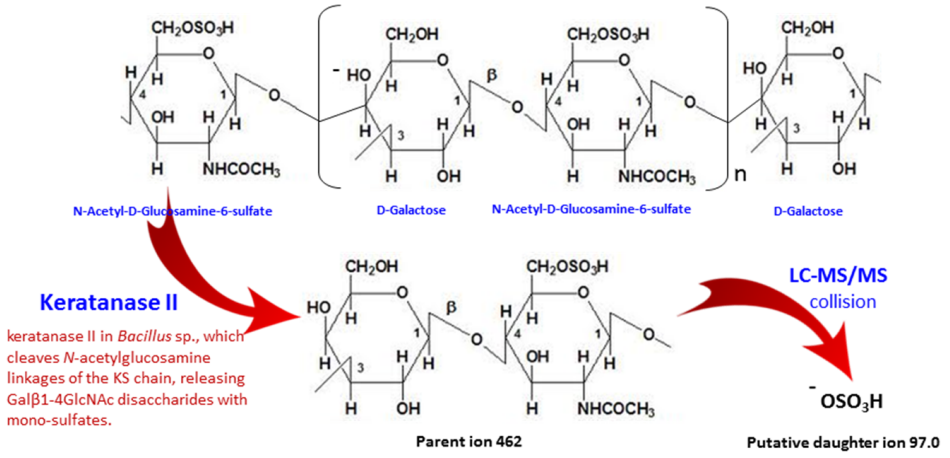

Fig. 1. Principle of the tandem mass spectrometry assay for urinary KS. In the multiple reaction monitoring (MRM) mode, the mass spectrometer detected ions by monitoring the decay of the *m*/*z* 462 precursor to the *m*/*z* 97 production for Gal β1→4GlcNAc(6S) disaccharides derived from KS.

six concentrations (X-axis) and their integrated areas (Y-axis) after MS/MS measurements. The linear range was set from 31.25 to 1,000 μg/mL. The "linear through zero" regression was excellent (r=0.000891×±0.00502; *r*=0.9992). The intra-assay and inter-assay precisions were determined by performing three different known concentrations of samples in triplicate over a period of six days, designated as low (5 μg/mL), medium (50 μg/mL), and high (150 μg/mL) levels. In this study, the triplicate results in low, medium, and high levels from three runs were used to estimate intra-assay and inter-assay precisions.

Results

1. Transition mass-to-charge ratio of KS standard and internal standards

The retention time of LC–MS/MS method for KS and chondrosine (IS) eluted was 2.98 min (±0.05) under the apparent pH of the mobile phase being applied. The endohydrolysis of (1→4)-beta-D-galactosidic linkages in keratan sulfate was proceeded by keratanase II, which cleaved the (*β*1-4)-glycosidic bond between Gal and GlcNAc residues, and the latter must be 6-*O*-sulphated[24,25]. One particular disaccharide of KS was selected. The *m*/*z* of the parent ion and its daughter ion after collision was 462.0→97.0, whereas the *m*/*z* of chondrosine was 353.9→73.0. Quantitations were achieved using peak areas (counts) and peak height (cps) that were processed using Analyst 1.5.2™ software (AB Sciex) according to the calibration curve for determining the KS concentration in an unknown urine sample. The MRM mass spectrum of normal controls and MPS IV patient are illustrated in Fig. 2A and Fig. 2B.

2. Precision and recovery analyses of the tandem mass spectrometry assay

The precisions of intra-assay and inter-assay were determined by an assay of 15 samples in low, medium, and high concentrations of KS using the method described earlier. All 15 samples were run in six consecutive replicates for intra-assay, and a duplicate analysis of each sample was performed on six different days sequentially. The averaged intra- and inter-assay precision values (CVs) of estimated Galβ1→4GlcNAc(6S) for the control urine were less than 7.26% and 9.78% (low concentration), 6.17% and 8.67% (medium concentration), as well as 5.73% and 8.87% (high concentration), respectively. The averaged accuracy (%) of LC-MS/MS analysis for varied calibrators was 103.8% (±9.81%) when comparing with the calibrators of definite concentrations. The analytical recovery was also investigated by spiked standard with known concentration of KS (10 μg/mL) into pooled normal urine samples as in the precision studies. The recoveries of this LC-MS/MS assay were 94.2%. The linearity study of this LC-MS/MS method was carried out with concentrations ranging from 31.25 to 1,000 μg/mL and each concentration was performed in triplicate. Linearity of KS was calculated individually and the correlation coefficients (r) were 0.9992.

3. Normal KS values and the MPS IV patients

In this study, a total of 67 urine samples including 11 MPS IVA patients and 56 normal controls were collected and analyzed. As shown in Fig. 3, normal samples could be simply distinguished from MPS patients based on the cut-off point (7.90 μg/mL) that determined from overall urinary KS measurements. In the control group, the main GAGs that could be identified in urine was CS and with very little amount or absence of KS were found. The reference values of normal controls were ranged from 0.22 to 7.9 μg/mL (4.06±1.92 μg/mL). For the MPS IVA patients, there were primarily accumulations of KS resulting in an enhanced excretion in urine. The urinary KS values found in confirmed MPS

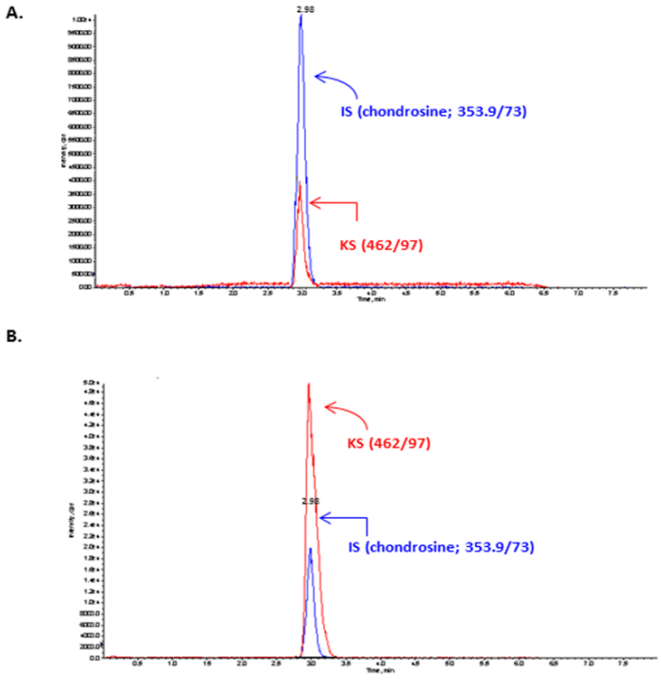

Fig. 2. Mass spectrum of KS and chondrosine (IS). (A) Normal control; (B) A confirmed MPS IVA patient. *Note*: IS is labeled as BLUE and urinary KS is labeled in RED.

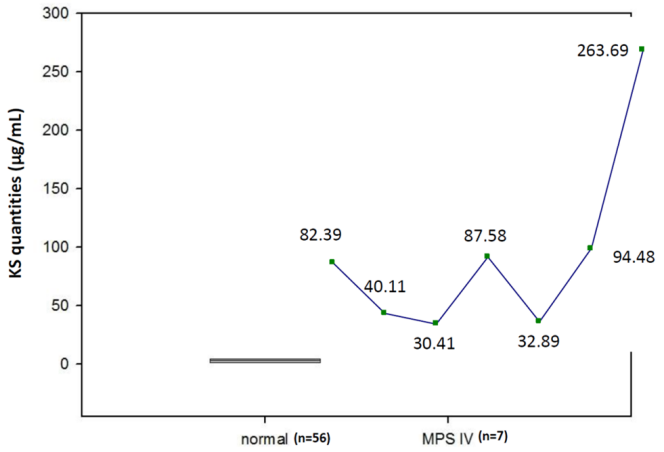

Fig. 3. Urinary KS of MPS IVA patients and the normal controls. The reference range (±2 SD) of normal control was less than 7.90 μg/mL (n=56) or less than <8.78 μg/mg creatinine. The urinary KS values of the confirmed MPS IVA cases (n=7) were elevated significantly.

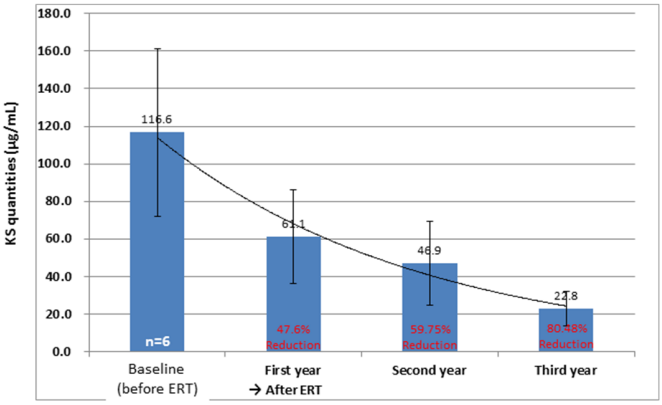

Fig. 4. Averaged UKS quantities of confirmed MPS IVA patients before and after ERT, and the % reduction. The evaluations of the effectiveness of ERT in MPS IVA patients (n=6). The % reductions of urinary KS after ERT were significant, ranged from 47.6% in the first year, 59.75% in the second year, to 80.48% in the third year when comparing with the baseline (without ERT).

IVA patients (n=11) were varied, from 25.0 to 263.69 μg/mL (170.2±81.1 μg/mL), and the data showed significantly elevated when comparing with those of normal controls (P<0.01).

4. Percentage of reduction of urinary KS after enzyme replacement therapy

The averaged urinary KS quantities of confirmed MPS IVA patients before and after ERT and the percentage (%) of reduction were illustrated in Fig. 4. The Evaluations of the effectiveness of ERT in MPS IVA patients (n=6) were compared the values of urinary KS detected without ERT (baseline) and with ERT started either from October, 2011 or April, 2012. The comparisons of % reductions of urinary KS before and after ERT were significant (P<0.01), ranged from 47.6% in the first year, 59.75% in the second year, and 80.48% in the third year (Fig. 4).

Disscussion

The aim of this study is to develop an urinary KS LC-MS/MS quantitative assay for MPS IVA determination instead of the conventional 2-D EP method. The data showed that the specificity and sensitivity of the urinary KS LC-MS/MS assay according to false positives and false negatives in relation with true positives and true negatives were all excellent, about 100% respectively. It is encouraging to see that the results of this study are successful and can be used to distinguish MPS type IV from the others.

In this study, the DMB/Creatinine ratios of confirmed MPS IVA patients (n=11) showed slightly intermediate increase levels (20.6±2.5 mg/mM creatinine; ranged from 10.4 to 30.8 mg/mM creatinine) when comparing with those of the normal controls (<2 Yrs: 44.6±23.7; 2-17 Yrs: 15.3±13.0; 18–42 Yrs: 5.2±2.5 mg/mM creatinine), and far lower than those of MPS I and MPS II patients that might be associated with the ages of disease onset and the disease being diagnosed. The ages of the MPS IVA patients were ranged from 1.4 years to 29.4 years (21.1±10.5 years old). For the results obtained from 2-D EP, roughly distinct KS pattern were observed in all confirmed MPS IVA patients; however, the interpretation of EP pattern was ambiguous and subjective that could make the diagnosis more difficult and unreliable. So, the establishment of a LC-MS/MS method for predominant disaccharide units derived from KS is particularly important. The LC-MS/MS assay can provide quantitative urinary KS values that are very useful and valuable for MPS IVA determination and for disease follow-up evaluation, particularly the patients receiving ERT.

The basic modification of this LC-MS/MS assay for relevant disaccharide unit derived from KS is the preparation of urine sample. According to the method previously reported by Martell et al., a small amount of the urine sample (100 μL) is directly treat with keratanase II which cleaves N-acetylglucosamine linkages of the KS chain, releasing Galβ1-4GlcNAc disaccharides with mono-sulfates[24]. By doing it this way, few amounts of GAG analytes could be collected and even measurable, but the experimental bias was frequently observed due to too small amounts of GAG to be detectable, that might raise the possibility in erroneous analysis and cause the alteration in result stability. Thus, performing the GAG precipitated procedure that made the GAGs more con-

centrated is necessary and can enhance the detectability of the KS in the urine. Without a doubt, the addition of internal standard, chondrosine, is required to orientate the individual peak position correctly due to the same retention time of elution from the LC system (2.98±0.05 minutes); besides, the known concentration of IS can be applied to calculate the relative amounts of urinary KS according to the integration of peak area and the peak high by using Analyst 1.5.2™ software (AB Sciex). The mass spectrums of urinary KS and chondrosine are demonstrated in Fig. 2A and 2B. From the mass spectrum obtained from the normal control, the KS is relatively smaller than that of IS; on the contrary, the peak of KS analyzed from the confirmed MPS IVA patient is dramatic bigger than that of IS. This is a relative concentration when comparing with the IS with the known concentration. The retention time of KS and IS elution are 2.98 (±0.05) minutes which reveals good retention and elution of analyte from the LC system.

Methods of KS calculation can be concluded into the following 2 ways, including the use of urinary CS as a standard for urine KS calculation, and the expression of urinary KS in μg/mg creatinine. The major GAG in mammals is CS, and the rest five separate GAGs like hyaluronic acid (HA), dermatan sulfates (DS), KS, heparan sulfates (HS) and heparin are relatively with little amounts. In healthy, non-MPS individual, the only GAG that can be found (or detectable) in urine is CS, and the rest of GAGs is rare or undetectable. By using CS as a standard, it can objectively reveal the actual status of urinary KS without considering many negative influence factors like ages, the dramatic changes of urine creatinine values, and other pathophysiological conditions. The results by using LC-MS/MS assay can discriminate well between the MPS IVA patients and the normal population, and also can be used to evaluate the effectiveness after ERT. However, the display of the urinary KS value does not conform very much to the common expression of urinary KS in μg/mg creatinine. At present, the most common expression of urinary KS is in μg per mg urine creatinine. It indicates that how many μg of KS quantity is contained in an mg of urine creatinine. The urinary KS quantity is variable according to the difference of urine creatinine level, which is proportional to the age but inversely proportional to the KS value. Urinary KS quantity is calculated and determined based on the value (μg/mL) detected by LC-MS/MS assay, urine creatinine in mg/dL, and the dilution factor under the experimental processes.

Based on the results of this study, the LC-MS/MS assay used for affected GAGs like urinary KS seen in MPS IVA are feasibly and reliably. The LC-MS/MS method can thus be used as a major reference for MPS IVA determination instead of the 2-D EP. A comparison of the results obtained from the 2-D EP and LC-MS/MS assay showed very good consistency, and they all correspond well with the confirmative leukocyte enzymatic assay. For the urinary KS of MPS IVA patients and the normal control, the reference range (±2 standard deviation) of normal control was less than 7.90 μg/mL (n=56) or less than <8.78 μg/mg creatinine. The urinary KS values of the confirmed MPS IVA cases (n=11; mean±SD: 170.2±81.1 μg/mL) were elevated significantly. By using this method we could clearly discriminate the MPS IVA patients from the non-MPS healthy population. The modified LC-MS/MS assay used for MPS IVA determination has many advantages including low analytic cost, high sensitivity, high specificity, high throughput, high reliability, and analytic automation. The LC-MS/MS method for the discrimination of urinary relevant disaccharides derived from KS is creative, attractive, and practicable.

Treatment for MPS IVA involves supportive and symptom-based treatment, as well as disease-specific treatments that address the underlying cause of the disease. Disease-specific treatments to replace GALNS enzyme activity for the non-neurologic manifestations of MPS IVA patients has been available since 2014. de Ru et al.[26] reported that ERT can result in good clearance of GAGs from many tissues and can significantly ameliorate several symptoms. The effectiveness of ERT for patients with MPS IVA is significant according to the consequences reported by literatures[27-31]. In general, the improvement of symptoms or physiological activities is one major indicator used to evaluate the effectiveness of ERT, as is, the clearance of GAGs, particularly KS, from urine. Notably, the DMB/Cre. ratio significantly ameliorated and declined around 55% to 65% of the original value when MPS patients had received ERT for more than three to six months. The DMB/Cre. ratio can give useful information to evaluate the effectiveness of ERT; however, it cannot directly reflect the efficiency of KS degradation after ERT. By using keratanase II digestion accompanied with LC-MS/MS assay for urinary KS quantification, we can clearly understand the status of the percentage reduction of urinary KS. Form the results we obtained, the %reduction of urinary KS is significant, ranged from 47.6%, 59.75%, to 80.48% in the sequencing 3-year effectiveness assessment after ERT. This means that ERT is effective, and thus, the urinary KS quantitative assay would be one of the evaluating criteria for the effectiveness evaluation after ERT.

Conclusions

The initial diagnosis of MPS IVA is achieved by clinical manifestations, primarily the skeletal disorder with soft tissue involve-

ments. Keratanase II digestion accompanied with LC-MS/MS assay applied for urinary KS quantification is validated, reliable, and feasible. The LC-MS/MS method can also be used to evaluate the effectiveness and the progression while the MPS IVA patients have received ERT.

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