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Original Research

The Application of HPLC-FLD and NMR in the Monitoring of Therapy Efficacy in Alpha-Mannosidosis

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Abstract

Background: Alpha-mannosidosis is a rare lysosomal storage disorder, caused by decreased activity of α-D-mannosidase. This enzyme is involved in the hydrolysis of mannosidic linkages in N-linked oligosaccharides. Due to the mannosidase defect, undigested mannose-rich oligosaccharides (Man2GlcNAc - Man9GlcNAc) accumulating in cells are excreted in large quantities in urine. **Methods**: In this work, we determined the levels of urinary mannose-rich oligosaccharides in a patient subjected to novel enzyme replacement therapy. Urinary oligosaccharides were extracted using solid phase extraction (SPE), labeled by fluorescent tag 2-aminobenzamide, and quantified by high-performance liquid chromatography (HPLC) with fluorescence detector (FLD). The identity of peaks was determined by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry. In addition, the levels of urinary mannose-rich oligosaccharides were also quantified by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The data were analyzed using one-tailed paired *t*-test and Pearson's correlation tests. **Results**: Compared to levels before the administration of therapy, an approximately two-folds decrease in total mannose-rich oligosaccharides after one month of treatment was observed by NMR and HPLC. After four months, an approximately ten-folds significant decrease in total urinary mannose-rich oligosaccharides was detected, suggesting therapy effectiveness. A significant decrease in the levels of oligosaccharides with 7–9 mannose units was detected by HPLC. **Conclusions**: The application of both HPLC-FLD and NMR in quantification of oligosaccharide biomarkers is a suitable approach for monitoring of therapy efficacy in alpha-mannosidosis patients.

Keywords: alpha-mannosidosis; HPLC; mass spectrometry; NMR; velmanase alpha

1. Introduction

Alpha-mannosidosis (AM) is an ultra-rare, autosomal recessive lysosomal storage disorder (LSD) [1] with an estimated prevalence between 1:500,000 and 1:1,000,000 [2]. It is caused by a decreased activity of lysosomal enzyme α-mannosidase as a result of heterogeneous mutations in the *MAN2B1* gene. The enzyme catalyzes the hydrolysis of α-(1-2)-, α-(1-3)- and α-(1-6)-mannosidic linkages in the lysosomal N-linked oligosaccharides degradation pathway. The consequence of the enzyme deficiency is the accumulation of mannose-rich oligosaccharides (ManOS) in all tissues [3]. The symptoms include mental retardation, hearing loss, immune defects, skeletal deformities, impairment of motor functions and may result in an early death [1]. Formerly, hematopoietic stem cell transplantation (HSCT) was the sole therapeutic option available for AM promoting mental development and preventing early death [4]. In 2018, European Medicines Agency approved Velmanase alfa, a recombinant human alpha-mannosidase (rhLAMAN), for long-term enzyme replacement therapy (ERT) for AM patients [5]. Administration of ERT, especially after an early diagnosis, is effective in slowing the progression of mild to moderate non-neurological AM, lowering disease biomarkers' levels, and improving the quality of life [6,7].

A diagnostic algorithm for AM was recently proposed [8,9]. Diagnosis of AM is based on measuring α-mannosidase activity in leukocytes or fibroblasts using colorimetry or fluorimetry [10]. Methods detecting urinary oligosaccharides, such as NMR spectroscopy [11], high-performance liquid chromatography (HPLC) [12], thin layer chromatography (TLC), and mass spectrometry (MS) [13] are only suggestive; the diagnosis should be confirmed by gene sequencing [10]. However, these methods may be utilized to monitor disease progression and therapy effectiveness by estimation of ManOS levels, since it was found that the excretion of urinary oligosaccharides is linked to a patient's general endurance, disease severity, and response to treatment [12,13]. Additionally, instrumental methods are a suitable alternative to a traditional TLC screening

method due to their superior sensitivity, resolution, and automation. The ability to reliably and rapidly differentiate between various LSDs based on the unique profile of biomarkers is crucial in early diagnosis and therapy administration [14]. Furthermore, the precise quantification of the level of accumulated oligosaccharides could be useful for the follow-up of the treatment [15].

In the presented work, we have utilized and compared three instrumental approaches to analyze urinary oligosaccharides in AM patients: ^{1}H NMR, HPLC with fluorescence detection, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS. The main goal was to determine changes in biomarker levels during the course of ERT of one pediatric AM patient, as the decrease in oligosaccharides is essential to demonstrate a pharmacological effect of the ERT [5].

2. Materials and Methods

2.1 Samples

All samples were obtained following local ethical and consent guidelines. Urine samples of AM patients and negative controls were obtained from the Center for Inherited Metabolic Disorders of National Institute of Children's Diseases, Bratislava, Slovakia, following standard operating procedures. The experimental study protocol was reviewed and confirmed by the hospital ethics committee. The levels of creatinine (crea) were determined by Vitros 5.1 Fusion Clinical Chemistry Analyzer (Ortho Clinical Diagnostics, Rochester, NY, USA). In total, five samples from four AM patients without the ERT administration (positive controls, males aged 5–20 years) were analyzed in addition to three samples from one patient undergoing the ERT treatment (2 years old female). In order to increase the sample size, two positive control samples from one representative patient were included, based on sample availability. Additionally, analysis of samples over time may provide valuable information about the disease progression and changes in biomarker levels of untreated patients. Diagnosis of all patients was confirmed by enzymatic activity assay and DNA sequencing.

2.2 Chemicals

Acetonitrile (ACN), sodium cyanoborohydride ($NaBH_3CN$), 2-aminobenzamide (2-AB), 2,5-dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA), formic acid, 25% ammonia solution, sodium hydroxide, and glacial acetic acid were purchased from Sigma-Aldrich, Steinheim, Germany. Liquid chromatography-mass spectrometry (LC-MS) water was purchased from Honeywell, Seelze, Germany. Dimethylsulfoxide (DMSO) was purchased from Thermo-Fisher, Eugene, OR, USA. Ethanol was purchased from Slavus, Slovakia. 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (DSS-d_6) and deuterium oxide (D_2O) were purchased from Eurisotop, Saint-Aubin, France. Maltotetraose and maltohexaose were purchased from Elicityl, Crolles, France. Ultrapure water was produced in-house.

2.3 Sample Processing for HPLC-FLD

A volume of urine equivalent of 10 μg of creatinine was diluted to 1 mL with ultrapure water in a triplicate for each urine sample. 10 μL of external standards solutions, maltotetraose and maltohexaose (c = 5 mg/mL) were diluted to 1 mL with ultrapure water and processed analogously to samples. The samples were first purified using 100 mg Supelclean LC-18 solid-phase extraction (SPE) columns (Sigma-Aldrich, Steinheim, Germany). The columns were washed with 1 mL 100% ACN, and 1 mL ultrapure water. The samples were applied to columns, the flowthrough was collected into clean tubes and 52 μL of 100% ACN was added to each tube. The samples were further purified using 100 mg Supelclean ENVI-Carb porous graphitized carbon (PGC) SPE columns (Sigma-Aldrich, Steinheim, Germany). The columns were washed with 1 mL 85% ACN, followed by 1 mL 5% ACN. The samples were applied on the columns and reapplied once. After washing with 1 mL 5% ACN, oligosaccharides were eluted by 1 mL 40% ACN and the eluate was dried under vacuum.

Fluorescent labeling was done using 2-AB according to a previously published method [16]. Briefly, 5 mg 2-AB was dissolved in 100 μL of glacial acetic acid : DMSO mix (3:7, *v/v*) and the mixture was added to 6 mg $NaBH_3CN$. To each sample, 5 μL of labeling solution was added and the samples were incubated at 65 °C for three hours. The excess label was removed by hydrophilic interaction chromatography (HILIC) SPE using a Strata $NH_2$96-well plate (50 mg/well, 55 μm, 70 Å; Phenomenex, Torrance, CA, USA) connected to the vacuum pump. The plate was flushed with 1 mL 70% ethanol, 1 mL ultrapure water, and 1 mL 96% ACN. The samples were diluted with 700 μL ACN and applied in the wells. After two-minute incubation on a shaker, the wells were flushed with 3 mL ACN. Oligosaccharides were eluted twice with 500 μL ultrapure water into a clean collection plate in a centrifuge (70 g, 15 min) after 15-minute incubation on a shaker (100 rpm). The eluate was dried under vacuum and reconstituted with 100 μL ultrapure water.

2.4 HPLC-FLD Analysis

Oligosaccharides were analyzed using Thermo Accela HPLC (Thermo Fisher Scientific, San Jose, CA, USA) on a HILIC ACQUITY UPLC BEH Amide Column (130 Å, 1.7 μm, 2.1 mm $\times$ 150 mm) with ACQUITY UPLC Glycan BEH Amide VanGuard Pre-column (130 Å, 1.7 μm, 2.1 mm $\times$ 5 mm) (Waters Corp., Milford, MA, USA) using a modified technique based on the generic method described in column's manual. To separate the oligosaccharides, a gradient elution of two mobile phases (MP) at 60 °C was used. MP A was 100 mM ammonium formate (pH 4.0) and MP B was 100% ACN. The HPLC gradient conditions are described in Table 1.

Table 1. HPLC gradient conditions used for the analysis of urinary oligosaccharides.

Time (min)	Flow (mL/min)	% MP B
–2	0.4	90
0	0.4	90
2	0.4	80
46	0.4	65
47	0.25	65
48	0.25	0
53	0.25	0
58	0.4	90

The analytes were detected using Dionex UltiMate 3100 fluorescence detector (Dionex Softron, Germering, Germany) with the excitation wavelength set to 250 nm and the emission wavelength set to 430 nm. A calibration ladder of 5, 10, 50, 100, 150, 200, and 250 nM of both standards was measured in triplicate. Chromatograms were processed in Chromeleon v. 7.2.10 (Thermo Fisher Scientific, San Jose, CA, USA). Limits of detection (LOD) and limits of quantification (LOQ) were calculated from obtained calibration curves. The samples were diluted to match the calibration range with a mixture of 70% ACN: 30% MP A before analysis. The concentration of oligosaccharides was calculated from individual peak areas according to calibration curves of maltotetraose and maltohexaose and expressed in μmol/mmol of creatinine as a summary concentration of position isomers.

2.5 MALDI-TOF Mass Spectrometry

Samples prior to HPLC separation, as well as the HPLC eluate, were analyzed by MALDI-TOF MS in order to match the structures to corresponding HPLC peaks. The eluate was collected into empty tubes, dried under vacuum, and reconstituted in 3 μL of LC-MS water. The structures were assigned to corresponding peaks using MALDI-TOF/TOF MS. 0.7 μL of matrix solution (20 mg/mL DHB in 30% ACN with the addition of 0.1% TFA and 1 mM NaOH) and 0.7 μL of sample were spotted onto a MALDI plate and analyzed using MALDI-TOF/TOF mass spectrometer UltrafleXtreme II (Bruker Daltonics, Bremen, Germany) in reflectron positive ion mode within the *m/z* range of 500–3000. Spectra were processed using flexAnalysis v. 3.4 (Bruker Daltonics, Bremen, Germany).

2.6 ^{1}H NMR Spectroscopy

100 μL samples of urine were freeze-dried and redissolved in 250 μL of 0.9 mM DSS-d_6 in D_2O. D_2O was used for magnetic field locking and shimming. DSS-d_6 was used for chemical shift set up (δ = 0 ppm) as well as the internal standard for biomarker quantification. ^{1}H NMR spectra were measured at 60 °C on AVANCE III HDX 600 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with triple resonance inverse CryoProbe TCI H-C/N-D-05-Z using *presat* pulse sequence for residual water signal suppression, with following parameters: acquisition time AQ 3.5 s, relaxation time D1 4 s, digitization mode baseopt, 50 dB power level for *presat*, number of scans ns 128–256. For ^{1}H-^{13}C heterocorrelated HSQC NMR spectra *hsqcedetgpsisp2.3* pulse sequence was used with the following parameters: AQ 0.2 s, D1 1.5 s, ns 300, F1 time domain TD1 256, baseopt digitization mode.

Anomeric H1 signals of all 1,3,6-linked β-mannose units of ManOS, present in the ^{1}H NMR spectrum at δ 4.784–4.747 ppm, were diagnostic for the estimation of a total ManOS concentration in urine samples. ManOS quantity was calculated relative to that of internal standard DSS-d_6, added to a phosphate buffer (pH 7.4) at a known concentration, as follows:

$$[\mathrm{m}] = (9 \times \mathrm{A}_0 \times [\mathrm{A}_{ref}]\,/\mathrm{b} \times \mathrm{A}_{ref}) \times (\mathrm{V_S}/\mathrm{V_U})$$

where [*m*] and [A_{ref}] are the concentrations of the ManOS metabolites and DSS-d_6, respectively; A_0 and A_{ref} is the area of the H1 signal due to the 1,3,6-linked β-mannose units signal and that one of DSS-d_6 signal, respectively, in the ^{1}H NMR spectrum; 9 and *b* are the numbers of protons in DSS-d_6 and in the m metabolite, respectively. V_U is the volume of the lyophilized urine sample; V_S is the volume of phosphate buffer.

2.7 Statistical Methods

Each sample from a monitored patient was analyzed in triplicates by both HPLC-FLD and ^{1}H NMR. Average values, standard deviations (SD), and relative standard deviations (RSD) were calculated. To evaluate the changes in ManOS levels, the data were tested using one-tailed paired *t*-test (α = 0.05). To determine the data correlation between these two methods, Pearson's correlation tests were performed. Pearson's correlation coefficient (r) and *p*-value were calculated. The statistical analyzes were performed in Microsoft Excel v. 2202.

3. Results

3.1 Analysis of the Levels of Total ManOS in Positive Controls Without the ERT Administration

Neither HPLC-FLD, MALDI-TOF, nor ^{1}H NMR-based approach detected signals representing ManOS in negative controls (data not shown). For the HPLC-FLD method, LOD and LOQ of both standards were determined as: LOD (Hex4) = 0.414 nM; LOQ (Hex4) = 1.25 nM; LOD (Hex6) = 0.357 nM; LOQ (Hex6) = 1.08 nM. The detector response was linear throughout the whole calibration range.

For an enhancement of signals' resolution, the ^{1}H-^{13}Cheterocorrelated HSQC spectra were also measured. Obtained NMR data were assigned to individual types of mannose units and GlcNAc by comparison with literature data [17]. Because proton chemical shifts of mannose units were nearly identical for some of them, only the total con-

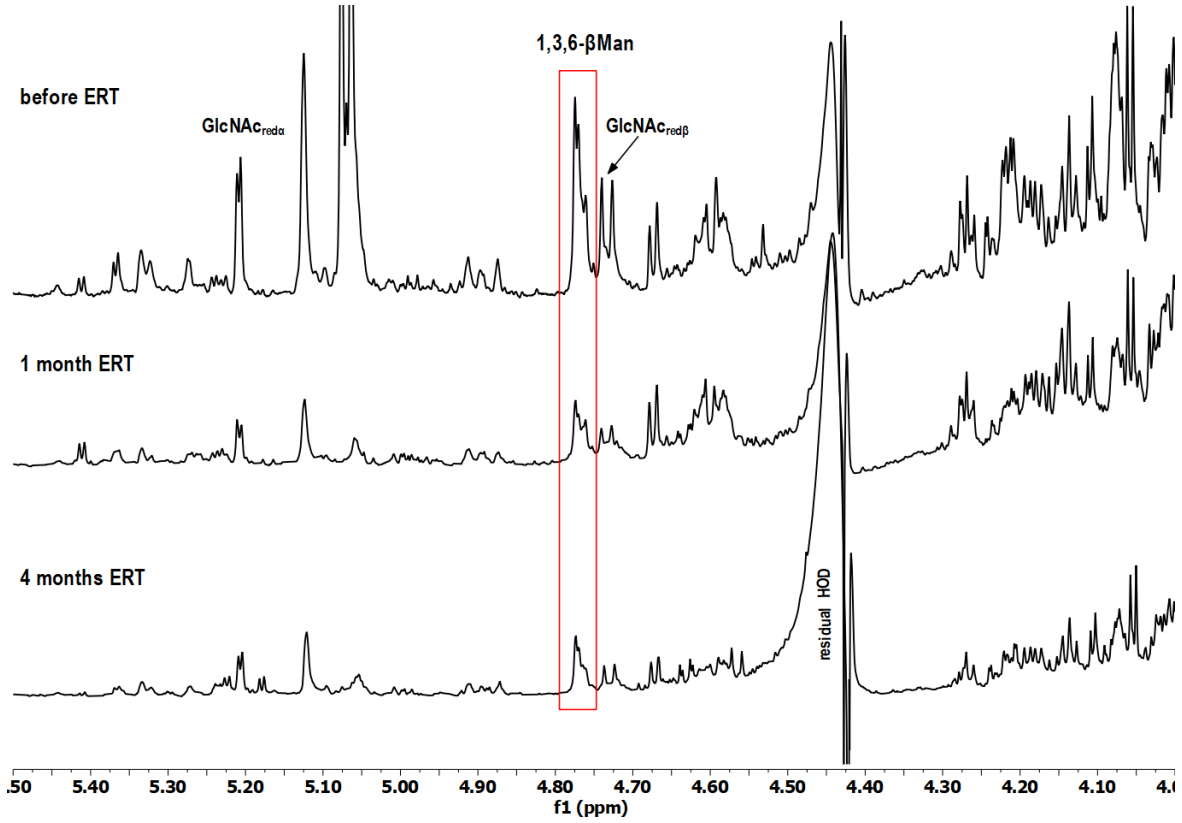

Fig. 1. Selected region of ^{1}H NMR spectra in which anomeric H1 signals of saccharides appear. The diagnostic signal of 1,3,6-linked mannose units in β-configuration (1,3,6-βMan) in mannose-rich oligosaccharides, accumulated in the urine of alpha-mannosidosis patients, is highlighted by red color. GlcNAc$_{red\alpha}$ and GlcNAc$_{red\beta}$ – reducing end glucosamine in α- and β-configuration; Man, mannose; HOD, residual signal of water.

centration of ManOS could be calculated from a well resolved, but overlapped anomeric H1 signals of all 1,3,6-linked β-mannose unit (δ_H 4.784–4.747 ppm) (Fig. 1).

HPLC-FLD and NMR methodology for determination of an overall ManOS content was set up on a set of five urine samples collected from four AM patients not subjected to ERT yet. Obtained values are shown in Table 2. Additionally, samples from one representative patient (9 years old male) were collected twice, with a four-month time lapse, on account of monitoring the patient's status and demonstrating the potential changes in ManOS levels in time.

The different values obtained by both techniques lie in the mode of sample preparation for measurement. While for HPLC-FLD, the derivatization is required for ManOS detection, the urine is used without any treatment for NMR. To compare two different approaches including diverse enrichment methods (sample derivatization for HPLC-FLD analysis), the correlation coefficient was determined. A value of 0.915 of Pearson's correlation coefficient suggested a strong positive correlation between data obtained by NMR and HPLC-FLD, even though each method produced different absolute values of ManOS concentration in the samples.

3.2 HPLC-FLD and MALDI-TOF-Based Overall Evaluation of ERT Efficacy

Previously optimized HPLC-FLD method was used for further analysis of the set of samples from AM patients without ERT, was applied further for the analysis of urine samples of another patient collected (i) prior to ERT onset, (ii) after one month and (iii) after four months of ERT. Representative MALDI-TOF MS spectrum and HPLC-FLD chromatogram of the sample before ERT onset are shown in Fig. 2. All labeled peaks in the HPLC-FLD chromatogram (Fig. 2B) were identified by MALDI-TOF analysis performed on collected single eluates. Oligosaccharides' precise isomeric structures cannot be determined by the utilized methods, therefore individual ManOS were quantified as a sum of position isomers. Due to a severe limitation of the MALDI-TOF approach in the distinction of stereoisomers, the identity of mannose units in detected oligosaccharides was confirmed by NMR.

Based on the results from HPLC-FLD, prior to ERT onset, the total level of ManOS (calculated as the sum of single Man2GlcNAc - Man9GlcNAc oligosaccharides) was 107.87 $\pm$ 42.64 μmol/mmol crea. After one month, the level of ManOS decreased almost two-folds to 57.42 $\pm$ 4.75 μmol/mmol crea and after four months, a significant

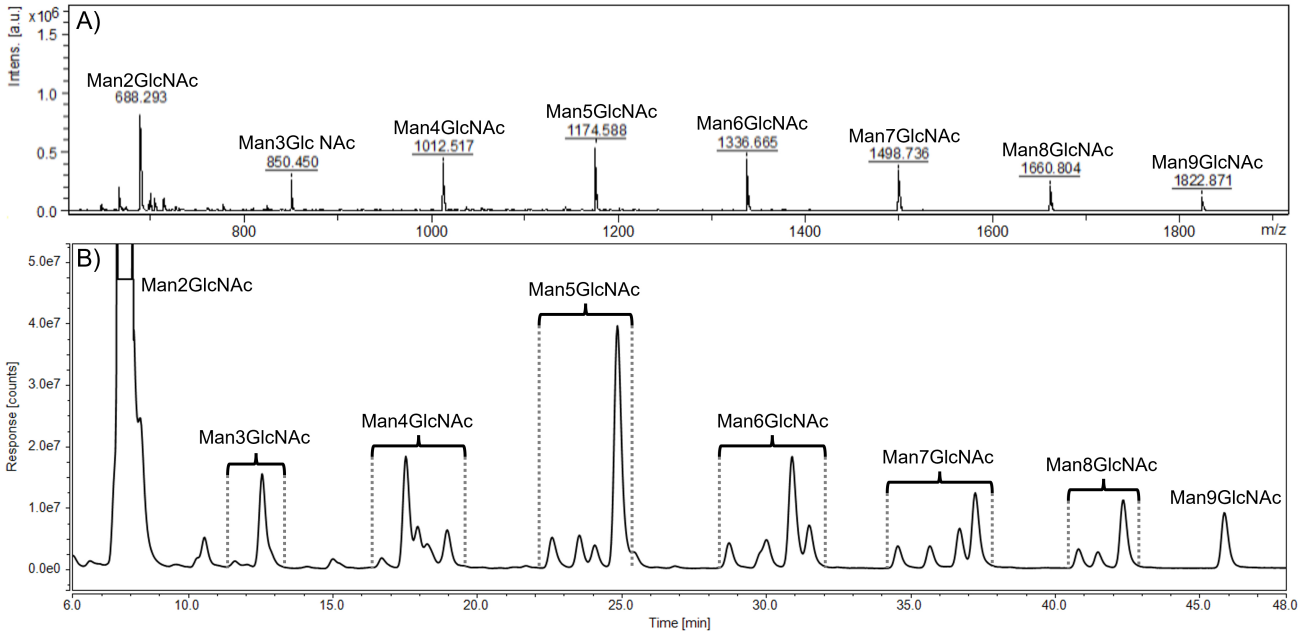

Fig. 2. Representative MALDI-TOF spectrum (A) and HPLC-FLD chromatogram (B) of urinary oligosaccharides in alpha-mannosidosis. Profiles were determined in the alpha-mannosidosis patient sample before the ERT administration. HPLC peaks within the brackets represent position isomers of mannose-rich oligosaccharides (Man2GlcNAc - Man9GlcNAc). GlcNAc, N-acetylglucosamine; Man, mannose.

Table 2. Total levels of mannose-rich oligosaccharides in the urine of four alpha-mannosidosis patients without ERT, determined by ^{1}H NMR and HPLC-FLD (two samples from one representative patient, a 9-year-old male, were collected twice, with a time gap).

AM Patient	ManOS (μmol/mmol crea)	
	^{1}H NMR	HPLC-FLD
14y M	122.6	46.3
20y M	150.1	52.5
5y M	287.0	84.9
9y M	166.0	62.2
9y M	206.7	82.1

Pearson's correlation coefficient was calculated as 0.915. AM, alpha-mannosidosis; crea, creatinine; HPLC-FLD, high-performance liquid chromatography with fluorescence detector; M, male; ManOS, mannose-rich oligosaccharides; NMR, nuclear magnetic resonance.

($p \leq 0.05$) decrease in total ManOS level was detected, represented by the value of 12.35 $\pm$ 2.44 μmol/mmol crea. Changes of ManOS levels during ERT are further discussed in section 3.5.

3.3 ^{1}H NMR Spectroscopy-Based Overall Evaluation of ERT Efficacy

^{1}H NMR urine spectra of the AM patient undergoing ERT are shown in Fig. 1. In the urine of the AM patient under ERT, collected prior to ERT onset, the level of total ManOS was 433.67 $\pm$ 2.36 μmol/mmol crea. After one month, it decreased significantly ($p \leq 0.001$) almost two-folds (222.00 $\pm$ 11.43 μmol/mmol crea). This result is in accordance with the HPLC-FLD data. After four months, an almost twelve-folds significant ($p \leq 0.001$) decrease in total ManOS level (36.00 $\pm$ 0.00 μmol/mmol crea) was observed. Changes in total urinary oligosaccharide levels during ERT determined by ^{1}H NMR were then compared with HPLC data (section 3.5).

A detailed analysis has shown that in the HSQC spectrum (data not shown) of the baseline urine sample (before ERT administration), the cross-peak signals, specific for a trisaccharide Man2GlcNAc, were dominant: the signal of the terminal αMan unit $\delta_{H,C}$ 5.13/104.8, which was (1$\rightarrow$3)-linked to βMan $\delta_{H,C}$ 4.77/102.8 (1,3,6-βMan) which is (1$\rightarrow$6)-linked to the reducing end GlcNAc. Its α anomer was present at $\delta_{H,C}$ 5.21/93.0 and β anomer at $\delta_{H,C}$ 4.73/97.6 ppm. Other cross-peaks present in the spectrum were of low intensity. This finding is in accordance with data obtained by HPLC-FLD. Due to the low specificity of chemical shifts of individual mannose units in other oligosaccharides present in the urine, and low intensities of signals their structures were not identified.

3.4 Determination of Changes in the Levels of Individual Oligosaccharides during ERT

The levels of individual ManOS were determined by HPLC-FLD. For the evaluation of relative changes of Man2GlcNAc - Man9GlcNAc over the course of ERT, indi-

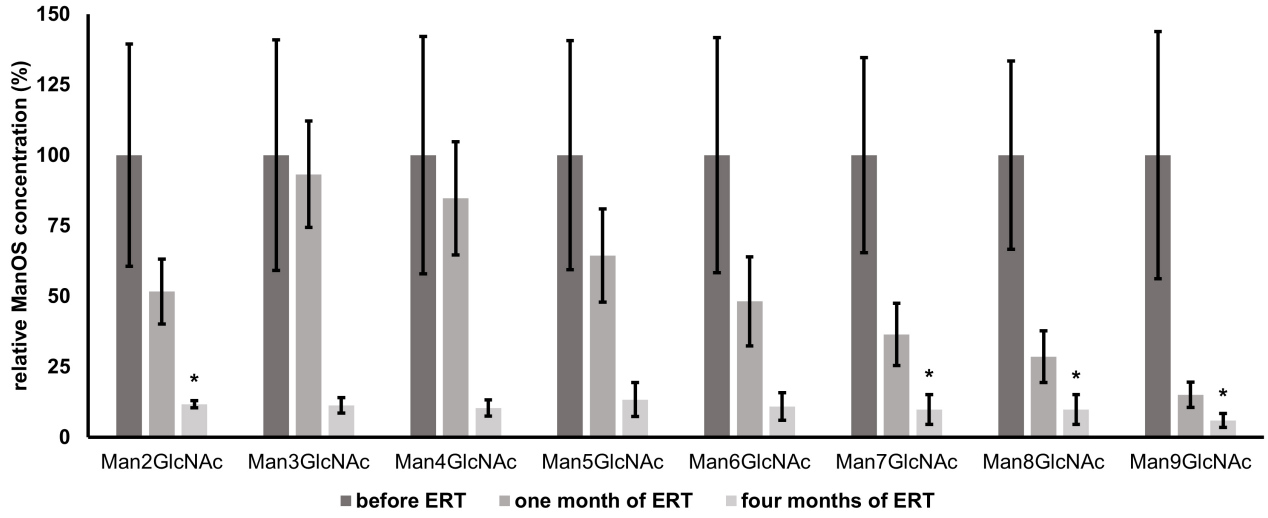

Fig. 3. Mean relative changes from baseline values (prior to ERT administration) of individual urinary oligosaccharides (Man2GlcNAc - Man9GlcNAc) over the course of ERT determined by HPLC-FLD. Individual ManOS concentrations are normalized to their levels prior to therapy administration (baseline values represent 100 %); n = 3, error bars represent ± 1 SD; * = $p \leq 0.05$; GlcNAc, N-acetylglucosamine; Man, mannose; ManOS, mannose-rich oligosaccharides.

vidual levels of oligosaccharides were normalized to those determined in the sample before therapy administration. Mean relative changes from baseline values of individual urinary oligosaccharides over the course of ERT according to HPLC-FLD are shown in Fig. 3. In the baseline sample, collected prior to the ERT, the levels of individual ManOS estimated by HPLC-FLD were following (in μmol/mmol crea ± 1 SD): 70.56 ± 27.84 (Man2GlcNAc); 2.70 ± 1.10 (Man3GlcNAc); 5.62 ± 2.37 (Man4GlcNAc); 10.63 ± 4.32 (Man5GlcNAc); 7.55 ± 3.14 (Man6GlcNAc); 5.27 ± 1.82 (Man7GlcNAc); 3.50 ± 1.17 (Man8GlcNAc) and 2.05 ± 0.90 (Man9GlcNAc). The levels of Man7GlcNAc - Man9GlcNAc decreased rapidly after one month of therapy, however, the levels of smaller ManOS (Man2GlcNAc - Man6GlcNAc) decreased more prominently only after four months. An unambiguous significant decrease of Man2GlcNAc and Man7GlcNAc - Man9GlcNAc was observed after four months of ERT.

3.5 Comparison of Results

After the comparison of ^{1}H NMR and HPLC-FLD data, it was found that ManOS levels estimated by NMR were approximately 2.9–3.9-folds higher than those obtained from HPLC-FLD analysis (summarized in Fig. 4). Nearly the same ratio between NMR and HPLC-FLD ManOS data (from NMR 2.5–3.4-folds higher values) was observed for urine samples collected from positive controls - diagnosed AM patients without ERT therapy (Table 2).

Pearson's correlation coefficient for the data from ERT efficacy monitoring (samples prior to ERT onset, after one month, and after four months of ERT) obtained from two different analytical approaches was calculated as 0.99 ($p < 0.001$), which represents a strong positive correlation, even though each method produced different absolute values of ManOS concentration in the samples (Fig. 4).

4. Discussion

No detected presence of ManOS in healthy controls is in accordance with literature data [15] and our previous results, even though traces of low molecular mass ManOS (Man2GlcNAc - Man4GlcNAc) were reported in some healthy subjects [18]. Despite a higher concentration of lower molecular mass mannose-rich oligosaccharides identified in the urine of AM patients, ManOS with seven to nine mannose units are more specific for alpha-mannosidosis as they were not reported for other LSDs [18,19].

Urine is a key carrier of hydrophilic waste products, including carbohydrates and their conjugates [20]. Complete chromatographic separation of complex mixtures of urinary oligosaccharides and their isomers is challenging due to frequently occurring overlapping peaks, as was the case of Man4GlcNAc in our study (Fig. 1B). The selected HILIC-based column provides better separation of polar and small hydrophilic molecules as well as glycosidic linkage isomers when compared to reverse-phase columns. This fact makes it suitable for the analysis of free urinary oligosaccharides. For a detailed description of HILIC advantages, we refer to two comprehensive reviews published on this topic [21,22].

An exceptional capacity of NMR to resolve thousands of peaks in complex metabolite mixtures such as urine is probably what led NMR to be the technology of choice to initially develop the field of metabolomics [20,23–25]. In

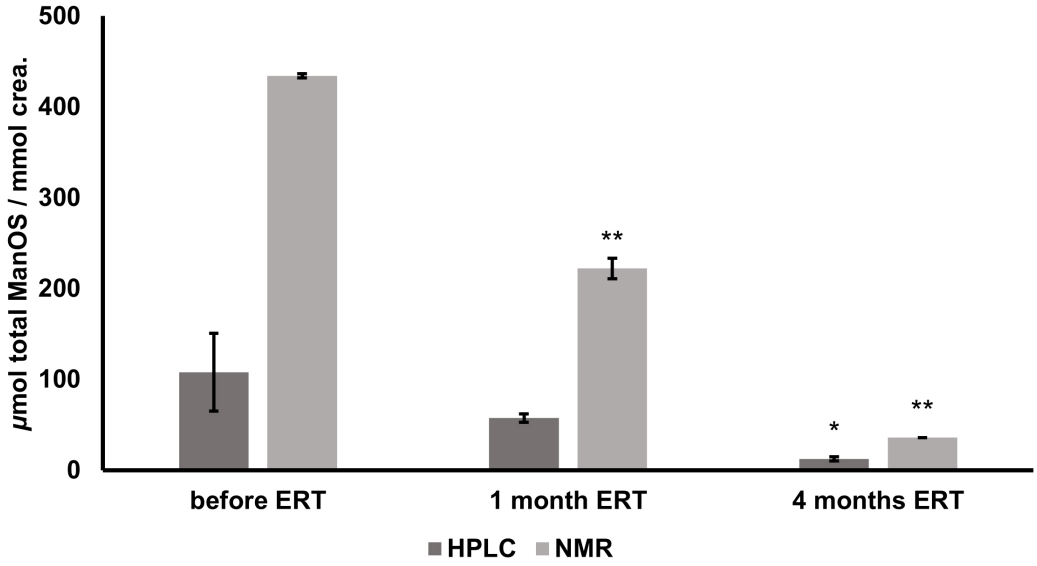

Fig. 4. Changes in total urinary oligosaccharide levels of alpha mannosidosis patient during ERT determined by HPLC-FLD and 1H NMR. Expressed as μmol/mmol creatinine; n = 3, error bars represent $\pm$ 1 SD; * = $p \leq 0.05$, ** = $p \leq 0.001$.

addition, NMR does not require preselection of analysis conditions, sample preparation is straightforward [26,27] and each separate resonance observed in an NMR spectrum is specifically assigned to an individual compound, while simultaneously providing comprehensive structural information. The main disadvantage of the application of NMR spectroscopy in the monitoring of ERT efficacy in alpha-mannosidosis is its inability to distinguish individual ManOS and their respective levels in urine. However, the combination of both HPLC-FLD and ^{1}H NMR methods is the approach with the most precise value.

4.1 Comparison of NMR and HPLC

Contrary to HPLC-FLD analysis, ^{1}H NMR spectroscopy does not require sample derivatization or SPE clean-up. MS-based techniques usually require sample preparation steps, which can cause metabolite losses [28]. High reproducibility is one of the fundamental advantages of NMR spectroscopy. However, peak overlaps from multiple metabolites pose major challenges for NMR spectroscopy [29]. Furthermore, NMR is nondestructive, thereby allowing further analysis of the same sample, if needed [30].

When compared with other published literature concerning the absolute quantification of ManOS by HPLC-FLD [12], LC-ESI-MS/MS [31], and HPLC-PAD [32], the quantities determined in our study by HPLC-FLD are in better accordance than those estimated by NMR (Fig. 4). However, the absolute quantification of urinary ManOS by LC-MS/MS is problematic due to a lack of external and internal standards and, in the case of HPLC-FLD, the need for labeling and SPE, which can cause the loss of analytes [13]. Nevertheless, monitoring of ERT efficacy based on the determination of ManOS levels is a unique approach providing complementary information, as usually only the clinical parameters and unspecific improvement of a patient's subjective condition are monitored. Furthermore, the precise determination of ManOS levels might become an essential addition to the management of AM patients, because it is speculated that high levels of circulating oligosaccharides (in addition to intracellular accumulation) may contribute to the immunodeficiency common in AM patients, since they bind to interleukin-2 (IL-2) receptors, disturbing IL-2-dependent responses [33,34].

The main advantage of the HPLC-based approach is its relatively adequate accessibility in laboratories in healthcare facilities. MS instruments are also much more commonly found in clinical centers and hospitals compared with NMR spectrometers [23]. However, more sample preparation steps are required, and even if the method can be almost fully automated, the HPLC- based approach is more expensive, reagents- and time-consuming than NMR. On the other hand, hospitals usually do not have access to highly sophisticated NMR instruments, which require advanced maintenance and operation. Thus the collaboration between the scientific community and clinicians is an essential tool to understand the molecular basis of diseases and the biochemical mechanisms of their respective therapies.

4.2 Evaluation of ERT Efficacy

Relative intensities of ManOS signals in the MALDI-TOF spectra are in accordance with published data [15]. Man2GlcNAc trisaccharide was identified as the most abundant structure in all AM samples in accordance with literature data [19]. Previous studies have linked the ManOS levels to AM severity [12] and treatment response either by HSCT [35] or ERT [13]. During Phase I-II of clinical testing of rhLAMAN, Borgwardt *et al.* [31] observed an over-

all 54.1% decrease in urinary ManOS in 10 patients over 12 months. Santoro *et al*. [13] reported a 67% decrease in urinary ManOS in a 7-month-old infant after two months of ERT. In our work, we observed an 88.6% decrease in total urinary ManOS after four months of ERT. This difference in ERT efficacy can be related to age, phenotypic variation, residual activity of α-mannosidase, accumulation of ManOS in tissues, immune response to treatment, and progression of the disease.

A more rapid decrease of urinary ManOS of higher molecular weights in comparison with smaller ManOS was observed. This can be attributed to the conversion of higher to smaller ManOS due to therapeutically increased enzyme activity. These truncated structures then might contribute to the pool of structures of lower molecular weights. Therefore, after just one month, the decrease of levels of Man3GlcNAc - Man5GlcNAc is less significant, even though the overall ManOS decrease is approximately 50%. The full effect of ERT is the most remarkable after a longer period. Early biochemical effects of velmanase alfa in a 7-month-old infant with alpha-mannosidosis were determined recently in the study mentioned above [13], where the OS containing 2, 3, and 4 mannose residues decreased while the other ManOS with higher molecular weight were already undetectable by TLC after 8 weeks of treatment. However, the overall efficacy of ERT might vary from person to person. Immune response and the antibodies against the foreign infused enzyme are important issues of the ERT and play a pivotal role in the patients' safety as well as the efficacy and success of the treatment. Furthermore, the biodistribution of the enzyme into the difficult sites of pathology (especially into the central nervous system, bones, etc.) still remains a striking challenge [36]. Nevertheless, the treatment should be initiated as early as possible to prevent further development and consequences of the disease as the full impact of this therapy will be fully understood when it is used on a cohort of patients identified soon after birth [37].

5. Conclusions

The application of both HPLC-FLD and NMR methodologies for quantification of mannose-rich oligosaccharide biomarkers is a suitable approach for monitoring of the enzyme replacement therapy efficacy in alpha-mannosidosis patients. Despite the fact that the data obtained from these different approaches differ in their absolute values, the change of biomarker level during the therapy administration was consistent for both methods. The main limitation of this study is the sample size, which is a typical phenomenon of rare diseases. Future perspectives to generally evaluate the therapy efficacy based on the monitoring of specific glycobiomarkers include adding other patients undergoing ERT into the study since the therapy effectiveness can vary from person to person.

Abbreviations

2-AB, 2-aminobenzamide; ACN, acetonitrile; AM, alpha-mannosidosis; crea, creatinine; D_2O, deuterium oxide; DHB, 2,5-dihydroxybenzoic acid; DMSO, dimethyl sulfoxide; DSS-d_6, 3-(trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt; ERT, enzyme replacement therapy; FLD, fluorescence detector; GlcNAc, N-acetylglucosamine; Hex, hexose; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; HSCT, hematopoietic stem cell transplantation; IL-2, interleukin-2; LC-ESI, liquid chromatography-electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; LSD, lysosomal storage disorders; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; Man, mannose; ManOS, mannose-rich oligosaccharides; MP, mobile phase; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; PGC, porous graphitized carbon; RSD, relative standard deviation; SD, standard deviation; SPE, solid phase extraction; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

Availability of Data and Materials

The datasets are available upon request.

Author Contributions

MK, RK, MN, JK and ZP performed the HPLC-FLD and MALDI-TOF experiments and data interpretation. IU and MM performed NMR experiments and respective data interpretation. AŠ, AH and KJ collected the samples and clinical data and reviewed the manuscript. MK, MN, ZP, MM conceptualised and prepared the original draft. PB, JM and JK critically reviewed and edited the manuscript. JM, ZP and MN were responsible for funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All samples were obtained following local ethical and consent guidelines. The experimental study protocol was reviewed and confirmed by the Ethics Committee of National Institute of Children Diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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