Investigation of Ion/Molecule Reactions as a Quantification Method for Phosphorylated Positional Isomers: An FT-ICR Approach

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A rapid and accurate method of quantifying positional isomeric mixtures of phosphorylated hexose and *N*-acetylhexosamine monosacchrides by using gas-phase ion/molecule reactions coupled with FT-ICR mass spectrometry is described. Trimethyl borate, the reagent gas, reacts readily with the singly charged negative ions of phosphorylated monosaccharides to form two stable product ions corresponding to the loss of one or two neutral molecules of methanol from the original adduct. Product distribution in the ion/molecule reaction spectra differs significantly for isomers phosphorylated in either the 1- or the 6-position. As a result, the percents of total ion current of these product ions for a mixture of the two isomers vary with its composition. In order to determine the percentage of each isomer in an unknown mixture, a multicomponent quantification method is utilized in which the percents of total ion current of the two product ions for each pure monosaccharide phosphate and the mixture are used in a two-equation, two-unknown system. The applicability of this method is demonstrated by successfully quantifying mock mixtures of four different isomeric pairs: Glucose-1-phosphate and glucose-6-phosphate; mannose-1-phosphate and mannose-6-phosphate; galactose-1-phosphate and galactose-6-phosphate; *N*-acetylglucosamine-1-phosphate and *N*-acetylglucosamine-6-phosphate. The effects of mixture concentrations and ion/molecule reaction conditions on the quantification are also discussed. Our results demonstrate that this assay is a fast, sensitive, and robust method to quantify isomeric mixtures of phosphorylated monosaccharides. (J Am Soc Mass Spectrom 2003, 14, 916–924) © 2003 American Society for Mass Spectrometry

Phosphorylated carbohydrates are key components in the biosynthesis of oligosaccharides [1], nucleotides and isoprenoids [2], and are involved in several important biological processes such as glycolysis [3] and signal transduction [4]. In particular, the phosphate linkage position of carbohydrates is important to regulate biological function. For example, the conversion between the 6-phosphate and the 1-phosphate isomers of several monosaccharides, which is regulated by the corresponding phosphate mutases, plays an important role in the glycosylation of glycoproteins [5, 6]. Specifically, the deficiency of the enzyme phosphomannomutase, which catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate in the early step of biosynthesis of lipid-linked oligosaccharides, is responsible for a disease called CDG-1a [1], in which glycoproteins with truncated *N*-linked carbohydrates are formed.

Despite their importance, analysis of phosphorylated carbohydrates by traditional methods has been problematic due to their structural variety and non-characteristic UV absorbance. As a result, traditional enzyme kinetic measurements of the phosphate mutases mentioned above require up to three coupling enzymes [7–9]. Recently, Turecek et al. [10] reported an affinity capture and elution/electrospray ionization mass spectrometry assay of phosphomannomutase and phosphomannose isomerase. In their method, the product isomer was subjected to another enzymatic reaction that altered its mass in order to be detected by mass spectrometry. Direct quantitative analysis of phosphorylated glucose has been reported using anion-exchange chromatography combined with a differential refractometer [11] and pulsed amperometric detection [12]. Since these two techniques are rather time-consuming, development of novel techniques to directly identify and quantify different phosphorylated carbohydrate

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isomers would be advantageous in kinetic measurements of the mutases mentioned above.

While the presence of phosphorylation is easily determined by mass spectrometry, differentiation between isomers is significantly more challenging. Until recently, very little information existed for determining phosphorylation sites in carbohydrates because dissociation of underivatized phosphorylated oligosaccharides generates mainly phosphate cleavages [13]. Recently, we developed a method to determine the phosphate position of phosphorylated carbohydrates by using ion/molecule reactions and FT-ICR mass spectrometry [14, 15]. Gas-phase reactions between phosphorylated hexose ions (HexXP, where X = 1 or 6 indicating the linkage of the phosphate to the monosaccharide) and trimethyl borate (TMB) or trimethyl chloro-silane (TMSCl) result in distinguishable mass spectra. Unambiguous differentiation between the 1-phosphate and the 6-phosphate isomers can be further realized by their SORI-CID spectra.

In terms of differentiating isomers of important biomolecules by mass spectrometry based techniques, several methods have been reported as classified and reviewed by Cooks and co-workers [16, 17] and others [18–28]. For example, chiral recognition based on gas-phase ion/molecule reactions has been investigated by several research groups [18–25]. In work by Dearden et al. [18, 23, 24] and Lebrilla et al. [19, 22, 25], a chiral host was used to form diastereometric adducts with a chiral ligand in the solution phase. The adduct ions were then electrosprayed into the gas phase, and allowed to exchange the chiral ligand through ion/molecule reaction with a neutral gas. Chiral distinction was achieved based on the variation of the exchange rate with the chirality of the analyte incorporated into the adduct ion. Quantification of the amount of each enantiomer was accomplished through the utilization of a calibration curve of the apparent exchange equilibrium constant versus enantiomeric excess [24] or by generating a calibration curve after a constant reaction time between the abundance of the unexchanged reactant and the mole fraction of one enantiomer [25]. In addition, ion/molecule reactions have also been successively used in the differentiation of diasteromeric species [26–28]. Kenttämaa and co-workers used ion/molecule reaction with $(CH_3O)_2B^+$ to distinguish between the *cis*- and *trans*-isomers of cyclic diols [26], and $(CH_3O)_2P^+$ was utilized to do the same between diasteromeric cyclic vicinal diols [27] and *cis*- and *trans*-1,2-diaminocyclohexanes [28].

Another mass spectrometric approach for isomeric recognition is based on unique collision-induced dissociation (CID) spectra of isomeric adducts formed from an analyte and a chiral reference [16]. Our laboratory has developed a multicomponent quantification methodology combined with diagnostic ions in CID spectra to determine the composition of mixtures of up to three isomeric species [29–31]. Using diagnostic product ions in MS^2 and MS^3 spectra, quantification of isomeric mixtures of hexosamines [29], *N*-acetylhexosamines [30], and sulfated disaccharides [31] has been achieved. Compared with the other isomeric quantification methods mentioned above, the unique aspect of this protocol is that neither kinetic constants nor calibration plots are necessary. In addition, using systems of equations based on diagnostic ions for each isomer to calculate mixture composition is very accurate. In the study presented herein, it is demonstrated that quantification of isomeric mixtures of phosphorylated carbohydrates can be achieved using this multicomponent quantification method in combination with ion/molecule reactions with trimethyl borate.

Experimental

Chemicals

The phosphorylated monosaccharides used in this study were purchased from Sigma Chemical Company (St. Louis, MO) and include the following diastereomeric pairs: Glucose-1-phosphate (Glc1P) and glucose-6-phosphate (Glc6P); mannose-1-phosphate (Man1P) and mannose-6-phosphate (Man6P); galactose-1-phosphate (Gal1P) and galactose-6-phosphate (Gal6P); *N*-acetylglucosamine-1-phosphate (GlcNAc1P) and *N*-acetylglucosamine-6-phosphate (GlcNAc6P). Trimethyl borate (TMB, 99.9%) was purchased from Aldrich Chemical Company (Milwaukee, WI). All solvents were of HPLC grade.

Instrumentation

Experiments were performed on an APEX II FT-ICR MS (Bruker Daltonics, Billerica, MA), equipped with a 7 T actively shielded magnet. The normal operational pressure is maintained at 5×10^{-11} mbar. Ions were generated using an Analytica API 100 electrospray ionization (ESI) source in the negative mode. The monosaccharide samples were sprayed from a 1:1 acetonitrile/H_2O solution at a concentration of 20 μM unless specified elsewhere. The ions were accumulated for 2 s in an external hexapole ion guide before transmission to the ICR cell for further analysis. The ion of interest was isolated by correlated sweeps using correlated harmonic excitation fields [32]. The mass range scanned was *m/z* 100 to 600. Each spectrum is composed of 16 transients with 256 k data points. The operating software was XMASS version 5.010. For quantification experiments, trimethyl borate was introduced into the ICR cell region via a pulse valve for 45 ms with a pulse valve reservoir pressure kept at 6.3 mbar. After a delay of 2 s to allow the ICR cell region to return to near baseline vacuum conditions, the ions were detected under high-resolution conditions.

For determination of relative ion/molecule reaction efficiency, trimethyl borate was introduced into the ICR cell at a constant pressure of 2×10^{-9} mbar by using a Varian leak valve. Reaction time was varied by adjust-

ing the delay time between isolation and detection. The experimental rate constant k_{exp} was determined from the decrease of reactant ion abundance over time; the theoretical collision rate constant k_{coll} was calculated based on the average dipole orientation (ADO) theory by Su et al. [33]. The reaction efficiency was calculated by dividing k_{exp} by k_{coll}. Correction for the difference in pressure between the ion gauge and the ICR cell was accomplished by comparison of the measured efficiency of the reaction between TMB and $H_2PO_4^-$ to that reported in the literature [34].

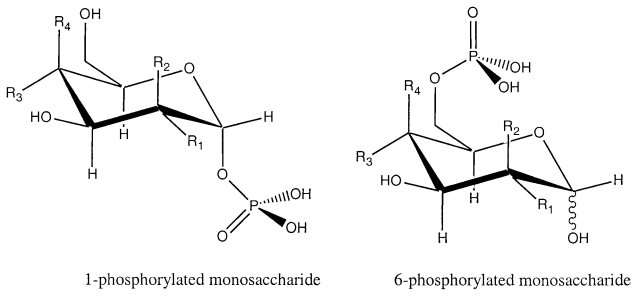
Quantification

The data reported for the reaction of the pure phosphorylated monosaccharide standards with TMB were the result of three replicate measurements. The 1:1 mixtures of the two isomers were also analyzed in triplicate, and each mock mixture was analyzed at least twice with the average values reported. The percent of total ion current for each ion of interest was determined by dividing its current by the total ion current in the corresponding ion/molecule reaction spectrum, and the *m/z* value was reported as the most abundant isotopic ion. The calculated values were used in three different ways: (1) For the pure standard, the averaged abundances are used as constants in a system of eqs as described in the text below; (2) for the 1:1 mixture, the averaged abundances are used to determine the first normalization factor "Norm 1" as described in the text; (3) for every other mock mixture, the abundances are used to determine the composition of the mixture.

Results and Discussion

Differentiation of Phosphorylated Isomers Through the Use of Ion/Molecule Reactions

The structures of phosphorylated monosaccharides investigated in this study are shown in Figure 1, and their corresponding ion/molecule reaction mass spectra are shown in Figure 2. Three ions are present in each spectrum: The reactant and two reaction products. For example, in the spectra for the Glc1P/Glc6P pair as shown in Figure 2a and b, the ion at *m/z* 259 corresponds to the underivatized deprotonated ion $[HexP - H]^-$. The ions at *m/z* 331 and 299 correspond to the reaction products resulting from the addition of TMB to the reactant ion followed by the loss of one methanol ($[HexP/TMB - MeOH - H]^-$) and two methanol ($[HexP/TMB - 2MeOH - H]^-$), respectively. The original adduct ion ($[HexP/TMB - H]^-$) is unstable in the gas phase and is not observed in any spectrum in our experiment. It is noticed that the product distribution in the ion/molecule reaction mass spectra is quite different for the two isomers. For Glc1P, the ion at *m/z* 331 is the most abundant reaction product (Figure 2a), whereas for Glc6P the opposite is observed (Figure 2b). This type of behavior is observed with the Man1P/Man6P (Figure 2c and d) and Gal1P/Gal6P (Figure 2e and f) pairs, except that for Gal6P, the ratio of *m/z* 299 to *m/z* 331 is nearly unity. The same trend is also demonstrated for the GlcNAc1P/GlcNAc6P pair (Figure 2g and h), wherein losing one MeOH molecule from the adduct generates the most predominant ion at *m/z* 372 for the 1-phosphate isomer and losing two molecules generates the most abundant product ion at *m/z* 340 for



Structure of the Monosaccharides Investigated

	R_1	R_2	R_3	R_4
Glc	−OH	−H	−OH	−H
Man	−H	−OH	−OH	−H
Gal	−OH	−H	−H	−OH
GlcNAc	$-CH_3CONH$	−H	−OH	−H

Figure 1. Structures of the phosphorylated monosacchrides investigated.

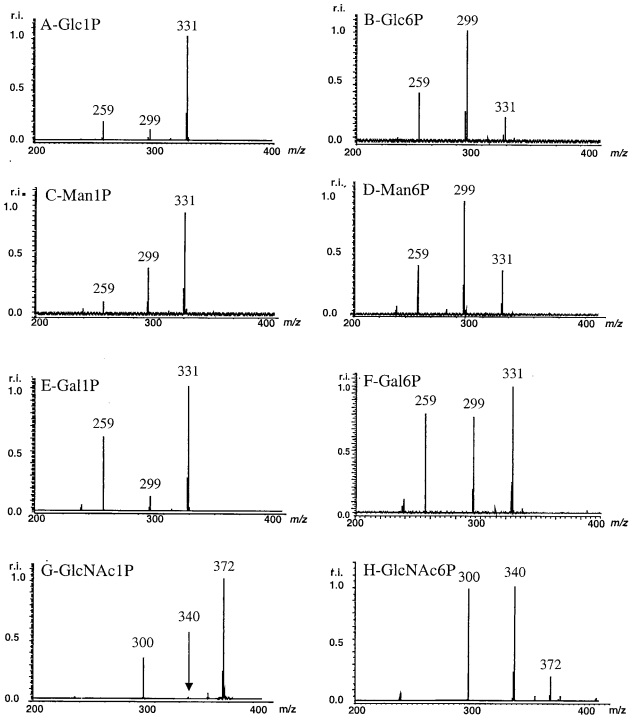


Figure 2. FT-ICR spectra of ion/molecule reactions with TMB for different phosphorylated monosacchrides. (**a**) Glc1P; (**b**) Glc6P; (**c**) Man1P; (**d**) Man6P; (**e**) Gal1P; (**f**) Gal6P; (**g**) GlcNAc1P; (**h**) GlcNAc6P. Pressure in the pulse reservoir was 6.3 mbar; pulsing time was 45 ms.

Table 1. Reproducibility of product distribution for phosphorylated monosaccharide standards

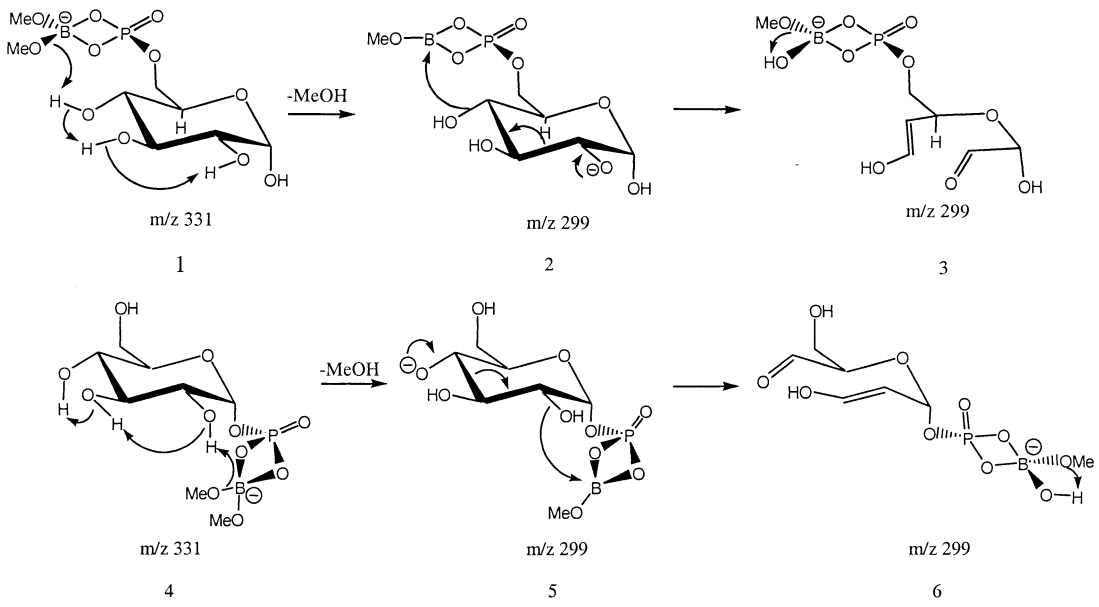
		Percent of total ion current				
Samples		1	2	3	average	stdev
	% *m/z* 259	13.89	13.97	13.92	13.93	0.04
Glc1P	% *m/z* 299	7.67	7.99	7.78	7.82	0.16
	% *m/z* 331	78.44	78.04	78.30	78.26	0.20
	% *m/z* 259	25.64	26.72	26.41	26.26	0.56
Glc6P	% *m/z* 299	60.56	59.91	60.28	60.25	0.33
	% *m/z* 331	13.80	13.37	13.31	13.49	0.27
	% *m/z* 259	8.32	7.68	7.88	7.96	0.33
Man1P	% *m/z* 299	28.82	29.23	28.81	28.95	0.24
	% *m/z* 331	62.86	63.09	63.31	63.09	0.22
	% *m/z* 259	23.11	23.72	23.42	23.42	0.31
Man6P	% *m/z* 299	55.31	54.93	55.12	55.12	0.19
	% *m/z* 331	21.58	21.35	21.46	21.46	0.11
	% *m/z* 259	10.99	10.72	10.81	10.84	0.14
Gal1P	% *m/z* 299	9.82	9.58	9.72	9.71	0.12
	% *m/z* 331	79.19	79.70	79.47	79.45	0.26
	% *m/z* 259	31.03	30.8	30.89	30.91	0.12
Gal6P	% *m/z* 299	29.80	29.83	29.81	29.81	0.02
	% *m/z* 331	39.17	39.37	39.30	39.28	0.10
	% *m/z* 300	24.2	24.91	24.93	24.68	0.42
GlcNAc1P	% *m/z* 340	0.96	1.31	1.16	1.14	0.17
	% *m/z* 372	74.84	73.78	73.91	74.18	0.58
	% *m/z* 300	45.9	45.58	45.89	45.79	0.18
GlcNAc6P	% *m/z* 340	44.37	44.75	44.50	44.54	0.19
	% *m/z* 372	9.73	9.67	9.61	9.67	0.06

the 6-phosphate isomers. Therefore for each pair of isomers, the product ion corresponding to the loss of either one or two MeOH molecules from the original adduct can be taken as the diagnostic ion for the 1- and 6-phosphorylated monosaccharide respectively. Further experiments also indicate that when the same amount of TMB was introduced in the cell for each reaction by keeping a constant pulse reservoir pressure and pulse time, the product distribution of resulting ion/molecule reaction spectra is very reproducible. As shown in Table 1, the standard deviations in percent of total ion current of each diagnostic ion are less than 1% for all isomers investigated.

The difference in the ion/molecule reaction product distributions for the 1- and 6-phosphorylated isomers may be explained from their corresponding reaction mechanisms. Previously, dissociation mechanisms were postulated for these species [35]. Regardless of the phosphate position, the structure of the *m/z* 331 ion was postulated to contain a 4-membered ring structure between the boron and the phosphate atoms with bridging oxygen atoms (Scheme **1**, Structures 1 and 4). Progression from *m/z* 331 to 299 occurs through the elimination of MeOH from *m/z* 331. The first step of this mechanism involves deprotonation of a hydroxyl group adjacent to the P–B Complex (Scheme **1**, Structures 2 and 5). Due to the extra degrees of freedom inherent in the 6-position isomer, it is postulated that this step has a lower energy barrier for the 6-linked hexose phosphate than for the 1-linked isomer. After elimination of MeOH, monosaccharide ring opening occurs, forming Complexes 3 and 6. This difference in the extent of methanol elimination in ion/molecule reactions has also been reported by Kenttämaa and co-workers for stereoisomeric diols with $(CH_3O)_2B^+$ [26] and for cyclic vicinal diols with $(CH_3O)_2P^+$ [27].

Principles of Multicomponent Quantification Using Ion/Molecule Reactions

Using the same condition, we further investigated the ion/molecule reactions with TMB for isomeric mixtures. Several representative spectra for mixtures of Glc1P and Glc6P are shown in Figure 3 with the percentage of Glc6P indicated. As expected, the percent of total ion current of the diagnostic ion for the 6-phosphate at *m/z* 299 increased as the percentage of this isomer increased. In order to quantitatively determine the composition of these isomeric mixtures based on their corresponding ion/molecule reaction spectra, a multicomponent quantification method [29–31] is utilized. Instead of measuring the absolute reaction rate constants, which may be affected by variations in instrument condition, a spectrum at a certain reaction stage (a certain amount of TMB is introduced to the ICR cell) is used to calculate the percent of total ion current for each diagnostic ion. The composition of a binary mixture can thus be determined using the following system of eqs:



Scheme 1

$$a \cdot I_{299,\,6P} + b \cdot I_{299,\,1P} = I_{299}$$

$$a \cdot I_{331,\,6P} + b \cdot I_{331,\,1P} = I_{331}$$

In this system of eqs, the variables a and b represent the percentage for the 6-P isomer and 1-P isomer, respectively, in a given mixture. The constants $I_{299,6P}$ and $I_{299,1P}$ are the percent of total ion current of the m/z 299 ions for a pure 6-P and 1-P standard, respectively. Similarly, $I_{331,6P}$ and $I_{331,1P}$ are the corresponding values of the m/z 331 ions for the pure standards. I_{299} and I_{331} are the corresponding values measured for a mixture of the two isomers. Through this system of eqs, the raw percentage of each isomer, a and b, can be calculated. In order to determine the final composition of a mixture, two additional normalizations are introduced. The first normalization takes into account the bias effect of the instrumental response towards each isomer, which may come from a slight difference in ionization efficiency between them. To correct for this bias effect, a 1:1 mixture is taken, and the calculated values of a and b for this mixture are divided by its actual percentage to obtain the normalization factors for each isomer: $N1_{6P} = a(1{:}1)/0.5$; $N1_{1P} = b(1{:}1)/0.5$. The calculated a and b for other mixtures are then divided by $N1_{6P}$ and $N1_{1P}$ to obtain a' and b', i.e., $a' = a/N1_{6P}$ and $b' = b/N1_{1P}$. The second normalization ensures that %1-P + %6-P = 100%, therefore the final percentages a'' and b'' can be expressed as: $a'' = a'/(a' + b')$; $b'' = b'/(a' + b')$.

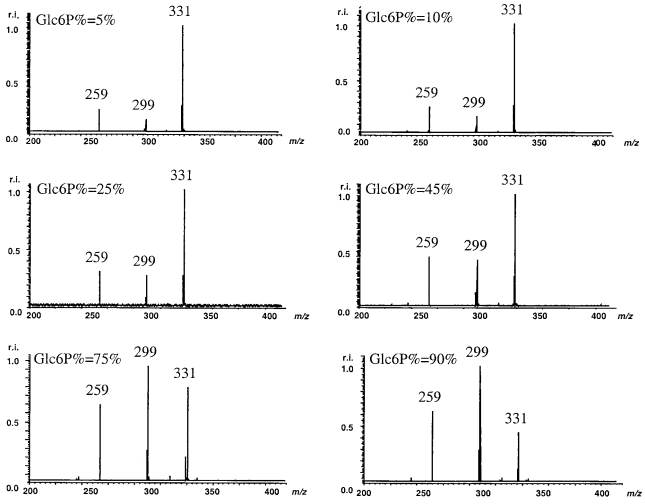


Figure 3. FT-ICR spectra of ion/molecule reactions with TMB for different Glc1P/Glc6P mixtures. The composition of the mixtures was indicated for each spectrum. Instrumental conditions were the same as in Figure 2.

Quantification of Mock Isomeric Mixtures

Mock mixtures of varying isomeric composition were used to evaluate the applicability of this method. Ion/molecule reaction spectrum for each mixture was taken under the same condition as for the pure isomers, and the percent of total ion current of the diagnostic ions at m/z 299 and 331 was measured and input in the above eqs. Percentages of each isomer were calculated as described above and tabulated as shown in Table 2. Here, the influence of the bias effect can be observed. The raw percentage, a and b under "Raw %" indicate that the percentage of the 6-phosphate isomer is underestimated without further corrections. After the two normalizations, the final percentages for a binary mixture correlate quite well with the actual values, and the deviation is less than 2%.

The same methodology can be applied to the other three isomeric pairs Man1P/Man6P, Gal1P/Gal6P, and GlcNAc1P/GlcNAc6P, where different ion/molecule

Table 2. Quantification result for two component mixtures of Glc1P/Glc6P

Actual composition of Glc6P: Glc1P	Ion contributions		Raw %		% after norm. 1		% after norm.2		Diff. (calc.-actual)
	m/z 299	*m/z* 331	*a*	*b*	*a′*	*b′*	*a″*	*b″*	
5:95	9.24	74.86	3.0	95.1	3.9	78.5	4.7	95.3	−0.3
10:90	10.82	72.25	6.1	91.3	7.9	75.3	9.5	90.5	−0.5
25:75	16.73	64.21	17.5	79.0	22.7	65.2	25.8	74.2	0.8
45:55	25.72	55.91	31.5	60.9	40.7	50.3	44.8	55.2	−0.2
55:45	30.13	45.74	38.3	51.8	49.6	42.8	53.7	46.3	−1.3
75:25	40.30	32.84	62.9	31.1	81.4	25.7	76.0	24.0	1.0
90:10	49.25	20.75	80.1	12.7	103.7	10.5	90.8	9.2	0.8
95:5	52.75	16.12	86.8	5.6	112.4	4.6	96.0	4.0	1.0

reaction product ions, *m/z* 299 and 331 or *m/z* 340 and 372, are utilized as diagnostic ions. Following the measurement of these ion abundances, quantification can be accomplished according to the above procedure. The quantification results are shown in Table 3. For the Man1P/Man6P pair and the GlcNAc1P/GlcNAc6P pair, where the difference in percent of total ion current of the two diagnostic ions for the two isomers is apparent, the same accuracy was obtained with an average deviation of less than 2%. However, for the Gal1P/Gal6P pair, the error is larger. This is not surprising since the difference in the percent of total ion current of the two diagnostic ions (*m/z* at 331 and 299) between Gal1P and Gal6P is much smaller than that between the other three pairs. As a result, small errors in the measured ion abundances greatly influence the calculated percentage of each isomer.

Concentration Effects on Quantification

All of the above mock mixtures were made to the same concentration of 20 μM. However, in analysis of real mixtures, the total concentration of an analyte is either unknown or intentionally varied. For example, in enzyme kinetic measurements, the concentration of the substrate is varied in a range wide enough to determine the value of the Michaelis-Menten constant, K_m. Therefore it is important to test whether the assay described above can also be applied to mixtures with different total concentrations. In order to apply the multicomponent quantification method to mixtures with different concentrations, it is necessary to first demonstrate that the product distribution for each sample is independent of its concentration. Table 4 lists the percent of total ion current of *m/z* 299 and 331 ions for a pure Man1P

Table 3. Quantification result for two component mixtures of Man1P/Man6P, Gal1P/Gal6P and GlcNAc1P/GlcNAc6P

Sample	Actual % of the 6-phosphate isomer	Raw %		% After normalization		Diff. (calc.-actual)
		a	*b*	*a′*	*b″*	
Man6P:Man1P	5	5.6	94.6	5.5	94.5	0.5
	10	9.7	90.3	9.5	90.5	−0.5
	25	23.9	75.9	23.6	76.4	−1.4
	45	46.2	53.6	45.9	54.1	0.9
	55	55.0	44.7	54.7	45.3	−0.3
	75	76.3	23.8	75.9	24.1	0.9
	90	90.8	9.4	90.5	9.5	0.5
	95	95.4	4.3	95.6	4.4	0.6
Gal6P:Gal1P	5	0.3	99.4	0.7	99.3	−4.3
	10	2.8	96.8	5.9	94.1	−4.1
	25	10.9	89.0	20.8	79.2	−4.2
	45	28.0	72.5	45.4	54.6	0.4
	55	37.2	62.1	56.3	43.7	1.3
	75	60.1	39.5	76.6	23.4	1.6
	90	84.6	15.2	92.3	7.7	2.3
	95	92.3	7.6	96.3	3.7	1.3
GlcNAc6P: GlcNAc1P	5	1.8	97.1	4.4	95.6	−0.6
	10	3.9	94.4	9.3	90.7	−0.7
	25	11.9	85.6	25.5	74.5	0.5
	45	24.3	75.6	44.2	55.8	−0.8
	55	32.6	62.2	56.3	43.7	1.3
	75	53.8	40.3	76.7	23.3	1.7
	90	73.2	18.5	90.7	9.3	0.7
	95	82.2	9.5	95.5	4.5	0.5

Table 4. Quantification result for different concentrations

Sample	Concentration (μM)	Percent of total ion current *m/z* 299	Percent of total ion current *m/z* 331	Calculated % of Man6P
Pure Man1P	1	28.78	62.91	
	5	28.92	62.73	
	10	28.93	62.93	
	20	28.75	62.99	
	S.D.	0.14	0.17	
10:90 mixture of Man6P: Man1P	1	31.70	58.35	10.7
	5	31.87	58.52	10.9
	10	31.49	59.07	9.5
	20	31.79	58.44	10.8
	50	31.37	57.61	10.9
	S.D.	0.21	0.52	0.6
	av.diff			0.2

standard and a 10:90 mixture of Man6P and Man1P with the concentration varied from 1 to 50 μM. The results here show that if TMB is delivered in excess at a constant pressure and pulse time, the abundance for each diagnostic ion remains constant at different concentrations. Also tabulated in Table 4 are the calculated percentages for different 10:90 mixtures. In this concentration range, the calculated percentages of Man6P have a standard deviation of less than 0.6% and an average difference from the actual percentage of 0.2%. The quantification method is thus demonstrated to be unaffected by the concentration of the mixture under optimized reaction conditions.

Effects of Ion/Molecule Reaction Conditions on Quantification

Comparing the ion/molecule reaction spectra of the phosphorylated isomers in Figure 2, it is also noticeable that under identical reaction conditions, more reactant ions of the 1-phosphate convert to product ions than that of the 6-phosphate isomer. For example in Figure 2a and b, the percent of total ion current of the reactant ion at *m/z* 259 is 14% for Glc1P compared with 26% for Glc6P, indicating that the ion/molecule reaction efficiency is different for the two isomers. To determine the relative ion/molecule reaction efficiency, several ion/molecule reaction spectra were taken at different reaction times while a constant pressure of TMB was introduced into the ICR cell. Kinetic plots were generated by plotting the natural logarithm of the percent of total ion current of the reactant ion and the product ions as a function of reaction time. Figure 4a and b are the kinetic plots for Glc1P and Glc6P, respectively. The reaction efficiency, k_{exp}/k_{coll}, calculated as described in the experimental section, is 0.54 and 0.36 for Glc1P and Glc6P with TMB, respectively, which demonstrates that the 1-phosphate isomer reacts with TMB faster than the 6-phosphate isomer. This trend of greater reaction efficiency for the 1-isomer was also found for the other three pairs, as listed in Table 5.

Table 5. Reaction efficiency with trimethyl borate[a]

Reactant	k_{exp}	k_{coll}[b]	Efficiency
Glc1P	4.57	8.49	0.54
Glc6P	3.08	8.49	0.36
Man1P	5.88	8.49	0.69
Man6P	3.27	8.49	0.39
Gal1P	2.47	8.49	0.29
Gal6P	1.92	8.49	0.23
GlcNAc1P	3.08	8.32	0.37
GlcNAc6P	1.76	8.32	0.21

[a]Rate constants in units of 10^{-10} cm^3/molecule/sec.
[b]Collision rates were calculated using ADO theory

The difference in the reaction efficiency for the positional isomers can affect the accuracy and precision of the multicomponent quantification method. For a particular amount of TMB, the population of 1-phosphate isomer will react at a faster rate than that of the 6-phosphate isomer, producing a product distribution more similar to that of the 1-phosphate isomer than what should be observed for the actual ratio of isomers. Consequently, the multicomponent quantification method might result in an overestimation of the amount of the 1-phosphate isomer in the mixture. This bias

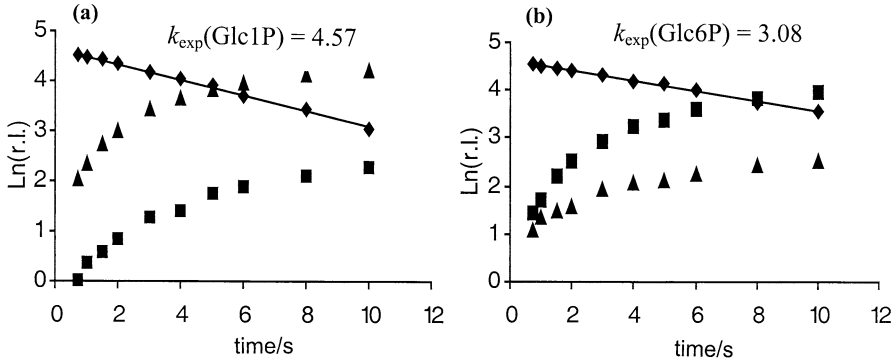


Figure 4. Kinetic plots of ion/molecule reaction between Glc1P (**a**) and Glc6P (**b**) with TMB (filled diamond) *m/z* 259; (filled square) *m/z* 299; (filled triangle) *m/z* 331. The ordinates are natural logarithm of ion abundance normalized to the total ion current. Unit of k_{exp} is 10^{-10} cm^3/molecule/s.

towards the more reactive isomer is most prevalent when small amounts of TMB are introduced and only a small fraction of the reactant ion (*m/z* 259) is converted to products. To test the influence of this bias on the quantification method, a series of mass spectra were measured following pulses of TMB that resulted in less than fifty percent conversion of the reactant ion. The calculated percentages were within experimental error (data not shown), suggesting that the quantification of the binary mixture is unaffected by the difference in reaction efficiency under the experimental conditions as outlined herein. Even for reactions involving the isomeric pair with the greatest disparity in reaction efficiency (Man1P/Man6P), the measured error in the quantification was still less than two percent (data not shown). Despite the relative insensitivity of the method towards differences in reaction efficiency of the isomers, the potential errors in quantification can be minimized by operating under conditions where most of the reactant ions are converted to products.

While the quantification method is robust over relatively long periods of time, it is recommended that the two pure standards and a 1:1 mixture are run each day of analysis to generate new coefficients for the system of eqs used for quantification. Our experiments demonstrate that accurate determination of the mixture composition (less than 2%, data not shown) could be obtained using different constants and different normalization factors as long as the same conditions used for standards are applied to the samples. Therefore, for quantification of any unknown mixtures, only three additional samples beside the unknown need to be run.

Conclusions

Through the use of gas-phase ion/molecule reactions and multicomponent quantification method, quantification for positional isomeric mixtures of phosphorylated monosaccharides has been demonstrated using an FT-ICR mass spectrometer. After reaction with trimethyl borate (TMB), different product distributions are observed in the resulting mass spectra for isomers with different phosphate position. The percents of total ion current of the diagnostic ions were utilized in the multicomponent quantification method to determine the percentage of each isomer in the mixture. The difference between the calculated compositions and the actual values is less than 2%, and this quantification method is demonstrated to be applicable to mixtures with different total concentrations. This is a rapid method in which the isomeric percentage from 5 to 95% can be accurately determined. In addition, the gas-phase reaction obviates the need for solution-phase derivatization, which can result in sample loss, a major problem for the analysis of compounds isolated in limited quantities from biological systems. Possible applications of this assay include percentage determination of the above isomers in biological samples and enzyme kinetic measurements for phosphate mutases, such as phosphoglucose mutase, phosphomannomutase, and phosphoacetyglucosamine mutase.

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