# Analysis of Enzyme Kinetics Using Electrospray Ionization Mass Spectrometry and Multiple Reaction Monitoring: Fucosyltransferase V[†]

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ABSTRACT: An accurate, rapid, and versatile method for the analysis of enzyme kinetics using electrospray ionization mass spectrometry (ESI-MS) has been developed and demonstrated using fucosyltransferase V. Reactions performed in primary or secondary amine-containing buffers were diluted in an ESI solvent and directly analyzed without purification of the reaction products. Decreased mass resolution was used to maximize instrument sensitivity, and multiple reaction monitoring (MRM), in the tandem mass spectrometric mode, was used to enhance selectivity of detection. The approach allowed simultaneous monitoring of multiple processes, including substrate consumption, product formation, and the intensity of an internal standard. MRM gave an apparent $K_m$ for GDP-L-fucose (GDP-Fuc) of 50.4 ± 5.5 $\mu$M and a $k_{cat}$ of 1.46 ± 0.044 $s^{-1}$. Under the same conditions, the conventional radioactivity-based assay using GDP-[U-$^{14}$C]Fuc as substrate gave virtually identical results: $K_m$ = 54.3 ± 4.6 $\mu$M and $k_{cat}$ = 1.49 ± 0.039 $s^{-1}$. The close correlation of the data showed that ESI-MS coupled to MRM is a valid approach for the analysis of enzyme kinetics. Consequently, this method represents a valuable alternative to existing analytic methods because of the option of simultaneously monitoring multiple species, the high degree of specificity, and rapid analysis times and because it does not rely on the availability of radioactive or chromogenic substrates.

An established role of mass spectrometry (MS)[1] is the characterization of small molecules of biological and nonbiological origin. Matrix-assisted laser desorption ionization (MALDI) (*1*) and electrospray ionization mass spectrometry (ESI-MS) (*2*) have contributed significantly to the expansion of this field into biological and biomedical applications (*3*). This is due to their ability to produce gas-phase ions from thermally unstable and polar compounds of a wide range of molecular weights. In addition to these soft ionization methods, MS allows the direct analysis of mixtures of compounds without the need for radioactive or chromogenic labeling. This is particularly attractive for the analysis of enzyme kinetics, which conventionally requires either radiolabeled or chromogenic substrates. Radioactive substrates are often preferred because of their sensitivity of detection as well as their identical chemical nature to the natural substrates. However, most radioactive substrates must be prepared by multistep syntheses and are therefore not readily available. The use of radioisotopes also creates hazardous radioactive waste. Most importantly, measurement of radioactivity is merely a measure of the radioactive isotope, and it does not provide any information regarding the integrity of the radioactive substrates. Spectroscopic methods are nonradioactive alternatives, yet these methods frequently require substrates that are modified by chromogenic agents. Such substrate modifications often necessitate multistep chemical syntheses and have also been demonstrated to alter enzyme kinetics (*4*). There has been increasing interest in the application of MS for the analysis of enzyme kinetics. In 1995 Henion's group reported the first application of MS in conjunction with liquid chromatography for the real-time analysis of enzyme kinetics (*5*). Siuzdak's group has recently employed ESI-MS using selected ion monitoring (SIM) to follow the progress of enzyme-catalyzed reaction (*6*). Other important developments include the reported ability to measure pre-steady-state kinetics of "time-resolved" ESI-MS by coupling a continuous-flow-mixing capillary directly

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[1] Abbreviations: AWT, MeCN/$H_2O$/$Et_3N$ (35/65/0.2 v/v/v); AWF, MeCN/$H_2O$/HCOOH (50/50/0.1 v/v/v); Bis-Tris, bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; FIA, flow injection analysis; Fuc-T V, fucosyltransferase V; Fuc-Ts, fucosyltransferases; GDP, guanosine 5′-diphosphate; GDP-Fuc, guanosine 5′-diphospho-$\beta$-L-fucopyranose; LacNAc, *N*-acetyllactosamine (Gal$\beta$1−4GlcNAc); Le$^x$, Lewis-X trisaccharide [Gal$\beta$1−4(Fuc$\alpha$1−3)GlcNAc$\beta$1-R]; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MS, mass spectrometry; MRM, multiple reaction monitoring; SIM, selected ion monitoring; Tris, tris(hydroxymethyl)aminomethane.

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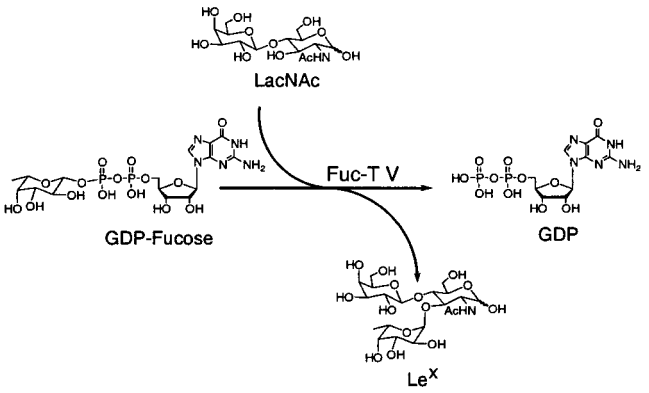


FIGURE 1: Fuc-T V catalyzes the transfer of Fuc from GDP-Fuc to type II acceptor (LacNAc, Gal$\beta$1−4GlcNAc) to form Le$^x$ trisaccharide.

to an ESI source (*7*). The use of MALDI-TOF MS is also rapidly expanding into this field with the use of rapid quench techniques to explore both enzymatic reaction mechanisms and pre-steady-state kinetics (*8*, *9*). Recent reports have also illustrated the usefulness of ESI-MS for rapid screening of libraries of compounds for potential enzyme inhibitors (*10*, *11*). Here, we report that an alternative approach comprised of ESI-MS coupled to flow injection analysis (FIA) and multiple reaction monitoring (MRM) is a rigorous alternative for the analysis of enzyme kinetics. MRM is a technique which has been frequently employed as an analytical tool for quantitative analysis (*12*, *13*). In this technique a gas is introduced into a collision chamber positioned between the two spectrometers of a tandem instrument. A parent ion is preselected for transmission through the first mass spectrometer and allowed to undergo collisionally activated dissociation (CAD) in the collision cell. The fragment ions produced as a result of this process are monitored by the second mass spectrometer. MRM is one method of data acquisition in which the intensities of specific fragment ions derived from specific parent ions are recorded (so-called parent $m/z^* \rightarrow$ fragment $m/z$ transitions). The resulting fragment ion current profile intensities reflect the abundance of the parent ion in the original sample.

α-1,3-Fucosyltransferase V (Fuc-T V) has been chosen as a model enzyme. Fuc-T V catalyzes the transfer of L-fucopyranose (Fuc) from GDP-Fuc to type II (Gal$\beta$1−4GlcNAc$\beta$1-R) acceptors to form the Le$^x$ trisaccharide structure (*14*) (Figure 1). There has been a general interest in designing mechanism-based inhibitors of Fuc-Ts because of their important role in cellular interactions (*15*). Among the known Fuc-Ts, Fuc-T V is the most thoroughly characterized enzyme, which is reported to follow an ordered sequential bi−bi kinetic mechanism (*16*, *17*) with GDP-Fuc binding first and the product GDP being released last.

## MATERIALS AND METHODS

*Chemicals and Reagents.* Soluble recombinant human Fuc-T V, GDP-Fuc (sodium salt), Le$^x$ trisaccharide, and *N*-acetyllactosamine (LacNAc) were purchased from Calbiochem (San Diego, CA.). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Fisher Biotech (Fair Lawn, NJ). Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris), 2-deoxyguanosine 5′-diphosphate (dGDP) (sodium salt), and GDP (sodium salt) were purchased from Sigma Aldrich (St. Louis, MO). GDP-[U-$^{14}$C]Fuc (287 mCi/mmol) was purchased from Amersham Pharmacia Biotech (U.K.). Quartz distilled water (>16 mΩ cm$^{-1}$) was produced in-house, and all other reagents and solvents were of analytical grade.

*Enzyme Reactions.* Fuc-T V (0.5 milliunit/$\mu$L) was supplied in a 50% glycerol solution containing 25 mM 2-(*N*-morpholino)propanesulfonic acid (pH 7.2), 100 mM NaCl, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Before use, the enzyme was diluted 5-fold with a solution containing 10 mM DTT, 10 mM Bis-Tris, and 10 mM $MnCl_2$. Each reaction tube contained 20 mM Bis-Tris (pH 6.8), 10 mM $MnCl_2$, 2 mM DTT, 24 mM LacNAc, and GDP-Fuc (0.025, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 mM) in 49.15 $\mu$L. Reactions were started by the addition of 0.085 milliunit of Fuc-T V (0.85 $\mu$L of the diluted enzyme preparation) and incubated at 37 °C for 0, 7, 14, and 21 min. For the control reactions both enzyme and substrate were individually omitted. Reactions were terminated by addition of 10 $\mu$L of the reaction mixture into a tube containing 30 $\mu$L of 70% MeOH and 0.5 nmol of dGDP as an internal standard. Each sample was centrifuged (20000*g*, 10 min), 15 $\mu$L was removed and diluted with 135 $\mu$L of MeCN/$H_2O$/$Et_3N$ (35/65/0.2 v/v/v) (AWT), and aliquots (20 $\mu$L) were analyzed by ESI-MS.

*Standard Curve.* A standard curve was constructed from the data obtained from a series of samples consisting of 2.0, 1.0, 0.67, and 0.05 nmol of GDP placed into tubes containing 30 $\mu$L of 70% MeOH and 0.25 nmol of dGDP. The reaction mixture (10 $\mu$L) devoid of LacNAc was added to each tube, and each sample was centrifuged for ESI-MS analysis as described above.

*ESI-MS.* A Perkin-Elmer Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer was tuned and calibrated in the positive ion mode as previously described (*18*). Under standard resolution conditions the isotopes of the poly(propylene glycol)/$NH_4^+$ singly charged ion at $m/z$ 906 were resolved with 40% valley. Under degraded resolution conditions (to enhance sensitivity) the isotopes at $m/z$ 906 were not resolved from one another. For the analysis of authentic standards and enzyme reaction mixtures in the negative ion mode the polarity of the instrument was reversed, the orifice was set to −75 V, and a stream of AWT was constantly infused into the ion source at 25 $\mu$L/min. Authentic standards (dissolved in AWT at 20 pmol/$\mu$L) and reaction mixture samples were injected into this stream via a 20 $\mu$L injection loop. Normal spectra were recorded by scanning from $m/z$ 300 to $m/z$ 2200 (0.3 Da step size, 6.66 s/scan). Fragment ion spectra of Q1 preselected parent ions were recorded by scanning Q3 from $m/z$ 50 to $m/z$ 600 (0.3 Da step size, 7.43 s/scan). Fragment ion spectra and MRM recordings were made with 10% nitrogen in argon collision gas with a CGT (collision gas thickness) instrument setting of 120 and a rod offset ($R_0-R_2$) of 50 V. In preliminary experiments, the authentic standards were also analyzed in the positive ion mode, in which case the samples were dissolved in MeCN/$H_2O$/HCOOH (50/50/0.1 v/v/v) (AWF).

*Data Processing.* Representative spectra were computed as the average of all of the spectra accrued from each injection of a standard using instrument-supplied software (MacSpec, version 3.3, PE Sciex, Ontario, Canada). For the measurement of SIM and MRM responses, the profiles were smoothed, and the relative peak areas were measured after

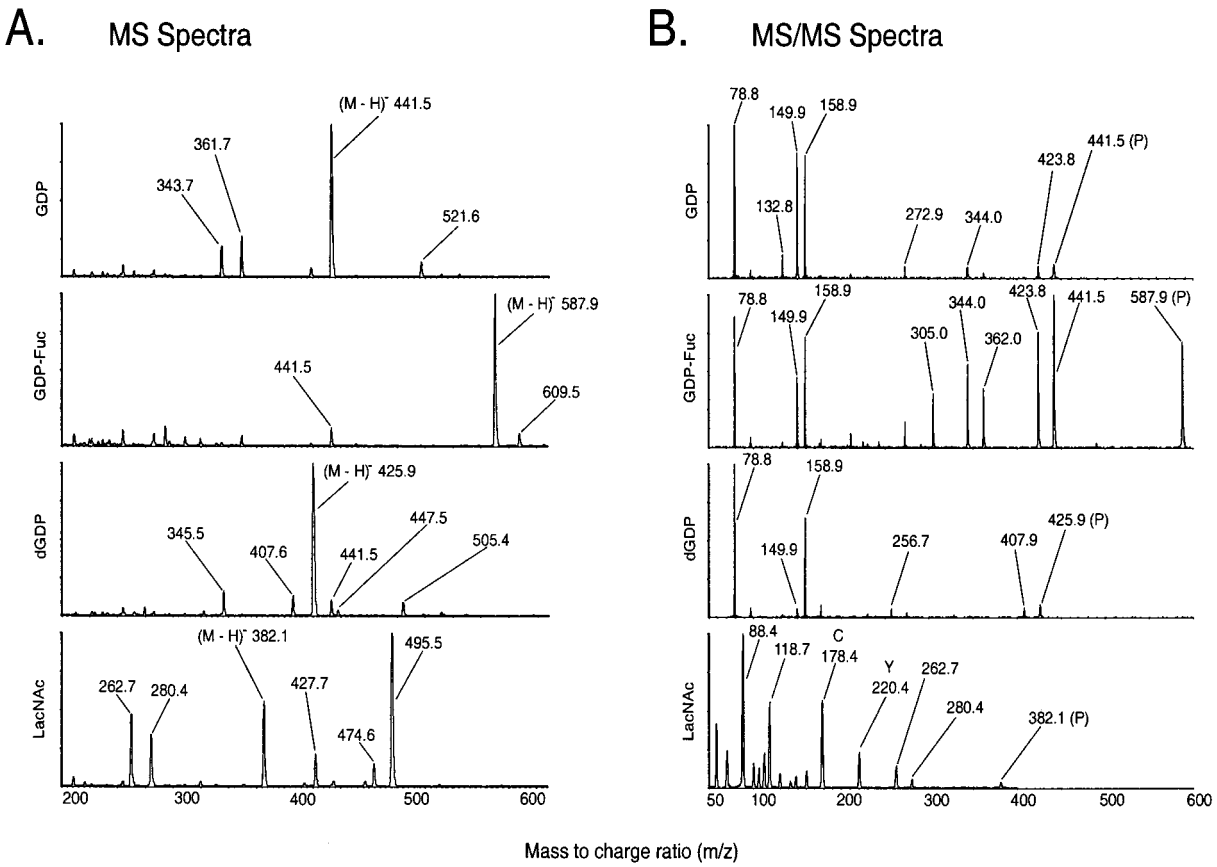


FIGURE 2: (A) Negative ion mode ESI-MS spectra of authentic standards GDP, GDP-Fuc, dGDP, and LacNAc. The use of 0.2% TEA (as AWT) under negative ion mode analysis gave predominantly the singly charged alkali metal-free molecules. Under these conditions, GDP, dGDP, LacNAc, and GDP-Fuc all gave signals with 0 or less than 10% being distributed among counterions. (B) MS/MS spectra of authentic standards GDP, GDP-Fuc, dGDP, and LacNAc. For GDP (*m*/*z* calcd 442, obsd 441.5), intense ion fragments appear at *m*/*z* 78.8, 158.9, and 149.9 which correspond to the fragments $(PO_3)^-$ (calcd 79.0 Da), $(HO_6P_2)^-$ (calcd 158.9 Da), and $(G-H)^-$ (calcd 150.0 Da), respectively. The fragment ion, $(HO_6P_2)^-$, at *m*/*z* 158.9 was arbitrarily chosen to monitor the production of GDP using MRM via the transition *m*/*z* 442 → 158.9. Parent ions are indicated by (P). For the LacNAc spectra, two structurally significant ions are assigned as Y *m*/*z* 220.4 [$(C_8H_{14}NO_6)^-$, calcd 220.1 Da] and C *m*/*z* 178.4 [$(C_6H_{11}O_6)^-$, calcd 179.0 Da] according to the nomenclature of Domon and Costello (ref *19*).

exporting the data to the IGOR Pro computer program (version 3, WaveMetrics, Inc., Lake Oswego, OR). For the kinetic data analysis, the peak areas arising from the reaction product transition *m*/*z* 442 [$(GDP-H)^-$] → *m*/*z* 159 [$(P_2O_6H)^-$] were divided by the peak areas arising from the internal standard transition *m*/*z* 426 [$(dGDP-H)^-$] → *m*/*z* 159 [$(P_2O_6H)^-$]. Each normalized peak was then included in a plot of peak area versus time for each substrate concentration. The slope of the line generated from each substrate concentration (units of peak area per minute) was divided by the slope of the normalized standard curve (units of peak area per mole GDP per liter) to obtain a quantitative value of the enzyme velocity expressed in units of concentration of GDP per minute. For nonlinear regression of the data fit to the Michaelis−Menten equation, GraphPad Prism software (San Diego, CA) was used.

*Radioactive Enzyme Assay*. The conditions and procedure were the same as described above with the following exceptions. The concentration of substrate, GDP-[U-$^{14}$C]Fuc (3.3 mCi/mmol), was 0.025, 0.05, 0.1, 0.2, and 0.3 mM. Each 10 $\mu$L time point was terminated in a tube containing 20 $\mu$L of MeOH, diluted with 500 $\mu$L of water, applied to a 1 mL Dowex 1-X8 column ($Cl^-$), and washed with $H_2O$ (3 × 0.4 mL) as described (*17*). The flow-through and washings were collected in 10 mL of ScintiVerse I scintillation cocktail (Fisher, Los Angeles, CA), and the radioactivity was measured by an ISOCAP/300 liquid scintillation counter (Searle Analytic Inc., Des Plaines, IL).

## RESULTS AND DISCUSSION

*ESI-MS of GDP, GDP-Fuc, dGDP, LacAc, and Le$^x$*. With AWT as a solvent the negative ion ESI spectra of GDP, GDP-Fuc, dGDP, and LacNAc revealed base peaks corresponding to the singly charged $(M-H)^-$ ions (GDP, *m*/*z* 441.5, calcd 442.0 Da; GDP-Fuc, *m*/*z* 587.9, calcd 588.1 Da; dGDP, *m*/*z* 425.9, calcd 426.0 Da; LacNAc, *m*/*z* 382.1, calcd 382.1 Da). (Figure 2A). In the negative ion mode the formation of alkali metal adducts was either not detected (GDP, LacNAc) or less than 10% of the base peaks (dGDP, GDP-Fuc). In contrast, the positive ion ESI spectra collected from AWF solutions were more complex with significant signals for the $(M+H)^+$, $(M+Na)^+$, and $(M+K)^+$ and for their formate adducts $(M+45)^+$ (data not shown). At the same concentration (20 pmol/$\mu$L), the molecular ion $(M-H)^-$ for the Le$^x$ trisaccharide (the product of Fuc-T V reaction) was not detected in the negative ion mode, while in the positive mode weak ions were observed at *m*/*z* 530.3 [$(M+H)^+$, calcd 530.2 Da] and 552.2 [$(M+Na)^+$, calcd 552.2 Da]. Because the signals for all the nucleotides and LacNAc were principally confined to the single $(M-H)^-$

species, the negative ion mode was selected to eliminate the possible problem arising due to counterion heterogeneity for the analysis of enzyme reaction mixtures.

The negative ion MS/MS spectra of GDP, GDP-Fuc, dGDP, and LacNAc, recorded with identical collisionally activated dissociation conditions, all contained structurally diagnostic fragment ions suitable for MRM (Figure 2B). GDP produced intense fragment ions of $(PO_3)^-$ at *m*/*z* 78.8 (calcd 79.0 Da), $(G-H)^-$ at *m*/*z* 149.9 (calcd 150.0 Da for $C_5H_4N_5O$), and $(HO_6P_2)^-$ at *m*/*z* 158.9 (calcd 158.9 Da). Both GDP-Fuc and dGDP also produced the same three prominent fragment ions. In addition, GDP-Fuc produced other intense fragment ions, two of which resulted from loss of the fucosyl moiety with both retention and loss of the glycosidic oxygen (obsd *m*/*z* 441.5, calcd 442.0 Da for $C_{10}H_{14}N_5O_{11}P_2$, and obsd *m*/*z* 423.8, calcd 424.0 Da for $C_{10}H_{12}N_5O_{10}P_2$, respectively). Domon and Costello have described the principal ion types formed during MS/MS of oligosaccharides as arising by charge retention on the nonreducing (A, B, and C) and reducing (X, Y, and Z) termini, following cross-ring sugar cleavage (A and X), cleavage on the nonreducing side of the linking oxygen (B and Y), and cleavage on the reducing side of the linking oxygen (C and Z) (*19*). The negative ion MS/MS spectrum of LacNAc shows a characteristic Y fragment at *m*/*z* 220.4 [$(C_8H_{14}NO_6)^-$, calcd 220.1 Da] and a C fragment at *m*/*z* 178.4 [$(C_6H_{11}O_6)^-$, calcd 179.0 Da] in addition to other fragment ions.

*Fuc-T V Reaction Conditions.* Organic buffers containing primary or secondary amines, such as Tris or Bis-Tris, were selected for use in the enzyme reactions because at the pH of the ESI solvent (pH ~9) they would be electronically neutral and therefore would not be expected to contribute significantly to the total ESI ion current. This was demonstrated experimentally in a comparison with buffers containing sulfate, phosphate, and carboxylate functional groups. Suppression of nucleotide signal was observed when the enzyme reactions were done with cacodylate, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and phosphate buffers, but significantly less suppression of signal occurred with Tris and Bis-Tris buffers. A pH rate profile for Fuc-T V in both Tris and Bis-Tris (pH 6.2−7.5) showed that optimal activity occurred using Bis-Tris at pH 6.8 (see Supporting Information).

*SIM and MRM Analysis of Fuc-T V Reaction*. SIM represented the simplest mode of data acquisition involving measurement of the temporal intensity change of substrate and product molecular ions during the progress of the enzymatic reaction. However, at the relatively low *m*/*z* values of these ions, the transient appearance of contaminant ions of the same or similar *m*/*z* seriously confounded the reliability of this approach, resulting in the inability to monitor some analytes and at times misleading kinetic data (our case). The likelihood of this interference should be reduced by using LC-SIM in the case where such contaminants arise from the sample. LC will be of little help, however, if the contamination is within the solvent or the instrument itself. For example, the most persistent contaminant ions appeared to originate within the mass spectrometer as residual signals from previous unrelated analyses, and they could not be removed simply by washing or replacing the infusion capillary used to deliver the sample to the ESI source. Occasional contaminants were also found to arise from solvent or buffer preparations. The contaminant ions were of variable intensity but were occasionally up to 10 times more intense than the analyte ions of interest. This problem may be more serious on a general-purpose instrument such as the one used here on which a wide variety of compound types and solvent conditions were routinely used. These inherent problems that were encountered using SIM precluded its use as a means of reliably and accurately monitoring the progress of the enzymatic reaction, thus necessitating the use of another mode of analysis (MRM).

MRM, under MS/MS conditions, was used successfully to monitor the temporal progress of the enzymatic reactions without being confounded by contaminating ions. MRM ion current profiles were used to monitor GDP-Fuc consumption (*m*/*z* 588 → 442, 424) and GDP production (*m*/*z* 442 → 159, 150) relative to the intensity of the internal standard, dGDP (*m*/*z* 426 → 159). The lower analyte concentrations encountered in these experiments, which, for GDP-Fuc for example, were down to 0.83 pmol/$\mu$L when injected into the ESI source, were at the lower limit of detection for the conventional Ionspray source used. Significant augmentations of signal intensities (10−100-fold) were obtained using slightly reduced mass resolution conditions for both Q1 and Q3 (see Materials and Methods) without compromising the specificity of detection. Under these conditions strong ion currents were routinely obtained for all transitions measured (Figure 3).

*Analysis of Enzyme Kinetics by ESI-MS Coupled to MRM*. The analysis of the Fuc-T V dependent conversion of GDP-Fuc to GDP via MRM was carried out by direct injection of the reaction mixture without any purification. The apparent $K_m$ for GDP-Fuc was determined to be 50.4 ± 5.5 $\mu$M, and the $k_{cat}$ value was 1.46 ± 0.044 $s^{-1}$ through nonlinear regression of the data fit to the Michaelis−Menten equation: $\chi^2 = 1.99 \times 10^{-14}$, $R^2 = 0.994$ (*20*). The conventional radioactivity-based assay was also carried out under the same conditions except for the use of GDP-[U-$^{14}$C]Fuc, which yielded the apparent $K_m$ of 54.3 ± 5.5 $\mu$M for GDP-Fuc and a $k_{cat}$ of 1. 49 ± 0.039 $s^{-1}$: $\chi^2 = 7.15 \times 10^{-15}$, $R^2 = 0.997$. Superimposition of the Michaelis−Menten plots for the two measurements shows essentially identical estimates of reaction kinetics that were produced by the two different methods run under the same conditions (Figure 4). The $K_m$ value for GDP-Fuc thus obtained is in good agreement with the literature value of 78 ± 32 $\mu$M (*17*). The $k_{cat}$ is significantly higher than the previously reported value of $k_{cat} = 40$ $min^{-1}$ (or 0.67 $s^{-1}$) (*17*). However, in the absence of DTT a lower $k_{cat}$ of 45.5 ± 2.07 $min^{-1}$ (or 0.76 ± 0.035 $s^{-1}$) was recorded which is comparable with the previously reported $k_{cat}$ value which also did not include DTT in the enzyme reaction mixture. Sensitivity to a thiol reducing agent is consistent with what is known about the cysteine dependent activity of Fuc-T V and other α-1,3-Fuc-Ts (*21*, *22*), although a possible role for a cysteine has not been speculated in either of the published catalytic models of this enzyme (*16*, *23*).

*Conclusion.* The employment of ESI-MS in conjunction with MRM provides several important advantages for the analysis of enzyme kinetics. First, the method is adequately sensitive for microscale reactions. For example, as little as 0.83 pmol/$\mu$L GDP-Fuc and ~80−100 fmol/$\mu$L GDP could be detected, with signal-to-noise ratios in excess of 700/1 and 10/1, respectively, when injected into a standard ESI source. Second, MRM is specific for the analyte(s) of interest,

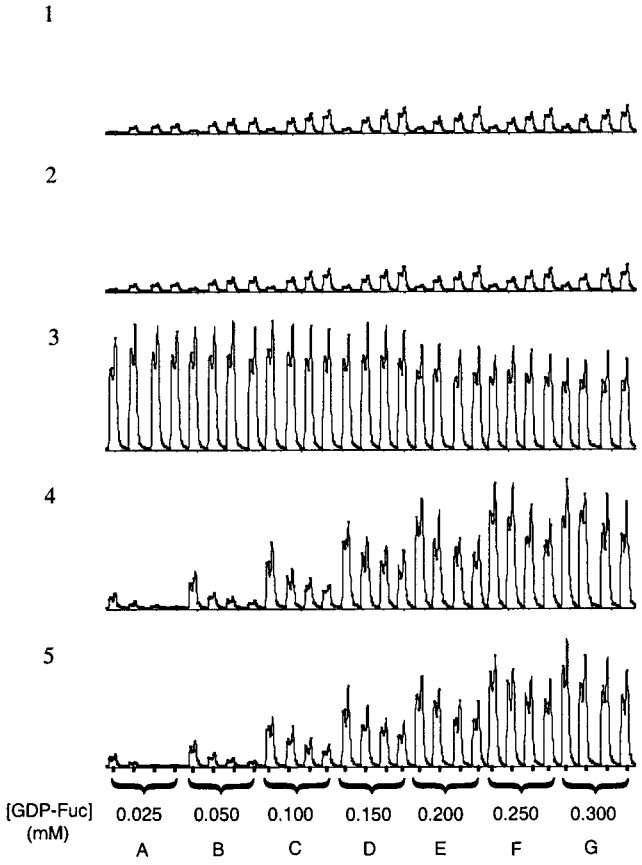


FIGURE 3: MRM analysis of the Fuc-T V reaction. GDP, dGDP, and GDP-Fuc were monitored simutaneously for each injection. The specific transitions were generated by collisionally activated dissociation of the respective parent ions. Rows: 1, GDP [442 $(GDP-H)^- \rightarrow 159\ (P_2O_6H)^-$]; 2, GDP [442 $(GDP-H)^- \rightarrow 150\ (G-H)^-$]; 3, dGDP [426 $(dGDP-H)^- \rightarrow 159\ (P_2O_6H)^-$]; 4, GDP-Fuc [588 $(GDP\text{-}Fuc-H)^- \rightarrow 442\ (GDP-H)^-$]; and 5, GDP-Fuc [588 $(GDP\text{-}Fuc-H)^- \rightarrow 424\ (GDP-H-H_2O)^-$]. The concentrations of GDP-Fuc used were A, 0.025; B, 0.050; C, 0.100; D, 0.150; E, 0.200; F, 0.250; and G, 0.300 mM, respectively. For each concentration, the reaction was analyzed at four different time points (0, 7, 14, 21 min, from left to right within each bracket). In the present investigation the transition of GDP (row 1) was monitored with reference to the transition of the internal standard dGDP (row 3). The transitions of GDP (row 2) and GDP-Fuc (rows 5 and 6) also produced identical kinetic profiles (data not shown).

allowing the analysis of a selected reactant or product from a crude reaction mixture, even in the presence of contaminant ions with the same *m*/*z* values. In addition, MRM provides structural information of the analyte being monitored. Thus, unlike the use of radioactive substrates, there is little ambiguity as to the identity of the signal being measured. Third, with MRM it is feasible to analyze multiple processes essentially simultaneously, e.g., consumption of substrates and formation of products as well as an internal standard. In the case of the Fuc-T V reaction, three of the four compounds (GDP-Fuc, GDP, and LacNAc), each giving strong $(M - H)^-$ signals in the negative ion mode, can be monitored simultaneously. This ability provides versatility particularly to the investigation of dynamic processes such as enzyme reactions. ESI-MS has proven useful in the identification and characterization of covalently linked enzyme−substrate intermediates (*24*−*26*). Direct monitoring of enzyme−substrate interactions will facilitate our understanding of enzyme mechanisms.

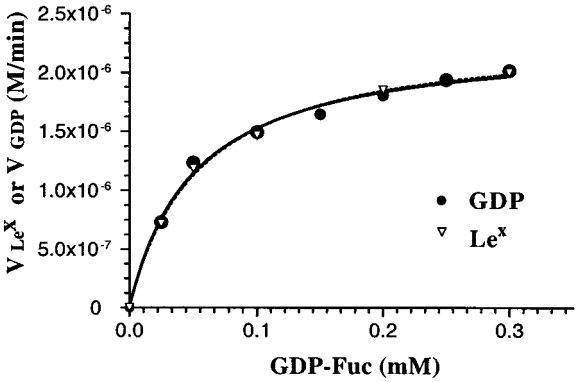


FIGURE 4: Overlay plot showing nonlinear regression of the data fit to the Michaelis−Menten equation for the MRM and the radioactivity-based assay results. The former monitored the accumulation of GDP (a solid line with closed circles), and the latter measured the accumulation of the radioactive trisaccharide $Le^x$ (a dotted line with open triangles). Each plot was used to determine $K_m$ and $V_{max}$ as reported in the text.

As has been demonstrated, MRM is tolerant to impure samples and contaminating ions, and when coupled to FIA, it allowed (our case) the most rapid rate of sample analysis. Although not necessary for the work reported here, LC might prove useful in cases of extreme salt contamination. In such cases, MRM could be combined with LC to provide an even higher degree of specificity in the analysis. Also, the minimum requirement of the ESI-MS method is the production of an adequate ion current from the analyte of interest. In the present study the production of the $Le^x$ trisaccharide could not be monitored because it did not produce any detectable ions in the negative ion mode. The use of other ion sources, e.g., atmospheric pressure chemical ionization (*27*), could provide suitable alternatives in such cases.

We have applied ESI-MS and MRM for the kinetic characterization of a known competitive inhibitor for Fuc-T V (*28*) and are currently extending this methodology to the analysis of a series of synthetic inhibitors (*29*, *30*).

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## SUPPORTING INFORMATION AVAILABLE

A pH rate profile for Fuc-T V in both Tris and Bis-Tris (pH 6.2−7.5). This material is available free of charge via the Internet at http://pubs.acs.org.

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