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LC–ESI–MS method for the monitoring of Abl 1 tyrosine kinase

Hui Chen, Erwin Adams, Ann Van Schepdael*

Laboratory for Pharmaceutical Analysis, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, O&N 2, PB 923, Herestraat 49, B-3000 Leuven, Belgium

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ABSTRACT

A liquid chromatography–electrospray ionization–mass spectrometric (LC–ESI–MS) method was developed and validated to study Abl 1 tyrosine kinase. An online desalting system was adopted, and a transformation of the ratio of product to substrate instead of a deuterated internal standard was introduced to calculate the concentration of product. In this study, the substrate used was Abltide (KKGEAIYAAPFA-NH_2). The detection was performed by selected ion monitoring (SIM) mode via positive ESI interface. Chromatographic separation was achieved on a C_{18} column using an isocratic mobile phase system. The limit of quantification (LOQ) was 10 nM for the product and 25 nM for the substrate. The simple ratios of product to substrate maintained a linear relationship (R^2 = 0.9997) over the ratio of 0–50% product. Intra- and inter-day precision was less than 10% and accuracy was from −1.6 to +5.3%. The validated method was applied to the Abl 1 kinase kinetic study and the K_m and V_{max} constants obtained for Abltide were 34.78 μM and 5.563 μmol/mg/min and for adenosine triphosphate (ATP) were 43.61 μM and 5.906 μmol/mg/min. The enzymatic reaction of Abl 1 tyrosine kinase belongs to ternary-complex mechanism.

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1. Introduction

Today, protein kinases are regarded as major drug targets and it is estimated that about 30% of all drug discovery projects in the pharmaceutical industry are currently developing protein kinase inhibitors [1]. Phosphorylation mediated by protein kinases is involved in regulating many aspects of the cell cycle including proliferation, differentiation, secretion and apoptosis, which is common in both prokaryotes and eukaryotes [2,3]. Thus, it is not surprising that malfunctions of protein kinases can lead to disease, such as diabetes and leukemia [4]. Recently, the study of protein kinase has become an important aspect in finding new drug targets for proliferative diseases, including cancers, leukemia, psoriasis, restenosis and others.

The rapidly growing interest in targeting kinases has promoted the development of many biochemical and chromatographic assay technologies. In general, biochemical assay technologies can be grouped into three classes: (i) radiometric assays; (ii) phospho-antibody-dependent fluorescence/luminescence assays; and (iii) phospho-antibody-independent fluorescence/luminescence assays [5]. Radiometric assays are considered the gold standard, even though they involve the use of radioisotopes. Fluorescence/luminescence assays offer the advantage that the reagents do not contain radioactive materials. But, these assays are susceptible to fluorescence interference [6].

In recent years, there are several reports on the use of chromatography-based kinase assays for both evaluation of enzyme activity and screening of enzyme inhibitors [1,7–11]. Most of these assays are based on the change of spectroscopic properties during conversion of a substrate to a product. Detection is most frequently performed by using UV–vis absorbance or fluorescence spectroscopy [12]. According to Copeland, natural substrates should be used for screening to ensure that the measured affinities of different inhibitors match the affinities that they will show in vivo [13]. However, most naturally occurring substrates do not exhibit any optical properties. Consequently, it is necessary to introduce those optical groups synthetically into the molecular structure. These modifications might alter the enzyme recognition of the substrate, thus possibly changing the kinetic properties [12].

Therefore, the demand for a label-free, non-radioactive assay scheme is high. Mass spectrometry (MS) has been widely reported for measuring the conversion of substrates to products for enzyme assays. Since MS is independent from the spectroscopic properties of the analyte molecules, no modification is required and the native substrates may be applied for enzyme assays. The development of MS-based detection schemes in enzymatic bioassays focuses on assays employing the so-called soft ionization techniques matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). Several approaches using MALDI–MS for monitoring of the phosphorylation of substrate under the catalysis by protein kinases have been reported [14–17]. Since this

* Corresponding author. Tel.: +32 16 323443; fax: +32 16 323448.
E-mail address: ann.vanschepdael@pharm.kuleuven.be (A. Van Schepdael).

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approach is tolerant of many buffer salts, reagents and compounds that are typically found in enzyme reaction mixtures, no sample pretreatment is needed. However, the reproducibility and quantitative capabilities of MALDI have always been questionable when compared to ESI methods [14].

A general challenge when using ESI–MS based detection is the sample preparation procedure. ESI–MS analyses are particularly prone to be deteriorated by the presence of matrix components such as salts, non-volatile buffers and biomolecules. Several groups reported utilization of MS-compatible buffer salts for preparation of the enzymatic reaction buffers [18–21]. However, these volatile buffer compounds are not always amenable to the enzymatic reaction. In addition, the presence of a particular cofactor, for example, Mg^{2+}, can also pose a problem, as this salt ion is required for enzymatic reactions and it can suppress the signal in ESI–MS. Many of the ESI–MS based bioassays rely on deuterated internal standards for the quantitation. Unfortunately, the cost of synthesizing a suitable deuterated internal standard is very high.

In this report, a LC–ESI–MS assay method without sample preparation is established to directly measure ratios of substrates and products to monitor Abl 1 tyrosine kinase reactions in vitro. In order to be less labor and time consuming, the enzymatic reaction mixtures were directly analyzed using a LC–ESI–MS method with an online desalting system [22], which protected the ESI interface from the influence of non-volatile salts.

2. Experimental

2.1. Chemicals and reagents

Abl 1 kinase was purchased from proteinkinase.de (specific activity is 33.000 pmol/mg min; Biaffin GmbH & Co KG, Germany). Abltide (KKGEAIYAAPFA-NH_2) was purchased from AnaSpec, Inc (San Jose, CA, U.S.A.). The phosphorylated Abltide [p-Abltide, KKGEAIY (PO_3) AAPFA-NH_2] was synthesized by BACHEM (Bubendorf, Switzerland). Acetonitrile (LC/MS grade) and trifluoroacetic acid (TFA, ULC/MS grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Adenosine 5′-triphosphate disodium salt (ATP), DL-Dithiothreitol (DTT) and anhydrous magnesium chloride were from Sigma–Aldrich (Bornem, Belgium). Tris [(hydroxymethyl) aminomethane] and Tris–HCl were purchased from AppliChem (Darmstadt, Germany). All solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA).

2.2. LC–MS system

High performance liquid chromatography (HPLC) was performed using a P680 HPLC pump from Dionex (Sunnyvale, CA, USA) and an autosampler AS100 Spectra Series with a fixed 20 μL loop from Thermo (San Jose, CA, USA). The HPLC system was coupled to a LCQ ion trap mass spectrometer (Thermo) with an ESI interface. Chromatographic separation was carried out using an ODS HYPERSIL C-18 column (250 × 2.1 mm i.d., particle size 5 μm) (Thermo) at room temperature (23 °C, MS room). Xcalibur 1.3 software (Thermo) was used for instrument control, data acquisition and processing.

The chromatographic separation was performed with an isocratic mobile phase consisting of 0.1% TFA in acetonitrile/0.1%TFA in water (25:75, v/v) at a flow rate of 0.2 mL min^{-1}. The mobile phases were degassed by sparging with helium for 2 min. The total run time was 17 min and in the first 5.2 min the eluent was sent to waste.

The two analytes (Abltide and p-Abltide) were detected by positive ESI ionization mode and qualified by selected ion monitoring (SIM). A voltage of 4.5 kV applied to the ESI needle resulted in a distinct signal. The temperature of the heated capillary was set at 250 °C. The number of ions stored in the ion trap was regulated by auto gain control (ACG). Nitrogen supplied by Air Liquide (Liège, Belgium), was used as sheath and auxiliary gas at a flow rate of 70 arbitrary units (arb) and 5 arb, respectively. Helium (Brussels, Belgium) was used as damping gas and as collision gas. The voltages across the capillary and octapole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. The capillary voltage was set at 25 V and the tube lens offset voltage at −25 V. Octapole 1 offset voltage, octapole 2 offset voltage and the interoctapole lens voltage were set at −3 V, −6 V and −12 V, respectively.

2.3. Method validation

To demonstrate the reliability of the method developed for the determination of Abl 1 tyrosine kinase kinetic parameters, a method validation process was executed according to the Food and Drug Administration (FDA) Guidance [23]. Here, several quantitative aspects such as quantification limits, linearity, accuracy, repeatability and stability were examined.

2.3.1. Linearity and limit of quantification (LOQ)

To investigate the linearity, 150 μM total peptide mixtures of Abltide and p-Abltide were determined at known molar ratios. The ratios included 0, 2.5, 5, 10, 20, 30, 40, and 50% p-Abltide. Replicate samples of each ratio were prepared and analyzed on three separate days. While obtaining peak areas, calibration curves were also constructed by performing linear regression analysis of the calculated p-Abltide concentration (y) vs the true p-Abltide concentration (x).

The LOQ was estimated by means of the baseline noise method. A signal to noise (S/N) ratio of 10 was considered as statistically acceptable for LOQ.

2.3.2. Accuracy and precision of peak area and repeatability of retention time

The accuracy and precision of the peak area and repeatability of the retention time of p-Abltide were evaluated by analyzing peptide mixtures at ratios of 5, 20, 40% p-Abltide. To determine intra-day precision, accuracy and repeatability, six replicates were analyzed at each ratio. The inter-day precision, accuracy and repeatability were evaluated by analyzing six replicates at each ratio on three separate days.

2.3.3. Stability of Abltide and p-Abltide in the working up process of enzyme activity assays

The stability of both peptides was evaluated by comparing two groups of peptide mixtures. Each group had two levels of peptide mixtures (high: 50 μM Abltide and 50 μM p-Abltide; low: 10 μM Abltide and 10 μM p-Abltide), which were prepared using reaction buffer. One group with inactivated Abl 1 tyrosine kinase went through the whole procedure from the start of the enzyme reaction to the analysis by LC–MS. The other one was analyzed directly by LC–MS.

2.4. Abl1 tyrosine kinase assay

2.4.1. Time-course assay for the phosphorylation of Abltide by Abl 1 tyrosine kinase

The reactions by Abl 1 tyrosine kinase (0.5 μg protein/mL, 0.25 μg protein/mL, 0.1 μg protein/mL) were conducted in 15 mM Tris–HCl buffer (pH 7.5) containing DTT (1 mM) and $MgCl_2$ (5 mM) in the presence of ATP (200 μM) and Abltide (200 μM) in a total volume of 1200 μL. The mixture was incubated at 30 °C and an aliquot of solution (120 μL) was collected at 5, 10, 15, 20, 30, 45, 60, 90

and 120 min. 40 μL acetonitrile was added to each aliquot of reaction mixture (120 μL) to stop the reaction. After centrifugation, the LC–ESI–MS analysis ($n = 3$) was performed.

2.4.2. The kinetic constant of Abl 1 tyrosine kinase against Abltide

All the experiments were carried out in 15 mM Tris–HCl buffer (pH 7.5) containing DTT (1 mM) and $MgCl_2$ (5 mM) in the presence of ATP (150 μM), Abltide (150, 100, 75, 50, 30, 10, 5 μM) and Abl 1 kinase (0.25 μg protein/mL) in a total volume of 120 μL. The mixture was incubated at 30 °C for 30 min and the reactions were terminated by the addition of acetonitrile (40 μL). After centrifugation, the LC–ESI–MS analysis ($n = 3$) was performed and kinetic constants were calculated by fitting the data to the Michaelis–Menten equation using Prism 5.0 (GraphPad software, San Diego, CA).

2.4.3. The kinetic constant of Abl 1 tyrosine kinase against ATP

All the experiments were carried out in 15 mM Tris–HCl buffer (pH 7.5) containing DTT (1 mM) and $MgCl_2$ (5 mM) in the presence of Abltide (150 μM), ATP (150, 100, 60, 40, 25, 10, 5 μM) and Abl 1 kinase (0.25 μg protein/mL) in a total volume of 120 μL. The mixture was incubated at 30 °C for 30 min and the reactions were terminated by the addition of acetonitrile (40 μL). After centrifugation, the LC–ESI–MS analysis ($n = 3$) was performed and kinetic constants were calculated by fitting the data to the Michaelis–Menten equation using Prism 5.0.

2.5. Data analysis

For kinase reactions, the increased acidity of the phosphorylated product when compared to the substrate may cause different ionization and detection efficiency between phosphorylated product and substrate [14]. In order to address this issue, a normalization factor (F) [24] is determined from Eq. (1), shown below.

$$F = \frac{Ap \times [s]}{As \times [p]} \quad (1)$$

Ap is the cluster area of the p-Abltide at m/z 673.0 ± 1, which is the product of the reaction, and As is the cluster area of the Abltide at m/z 633.0 ± 1. $[s]$ and $[p]$ represent the concentrations of Abltide and p-Abltide, respectively, in validation mixture. This F value is used for all subsequent calculations of $[P]$ (calculated concentration of p-Abltide) and V (velocity) for the samples analyzed.

For kinase reaction analysis, the product concentration is calculated from Eq. (2), using the chromatographic data.

$$[P] = \frac{N \times Ap}{F \times As + Ap} \quad (2)$$

In the protein kinase reaction, one unit Abltide can convert into one unit p-Abltide. Therefore, the total molar amount (N) of peptide Abltide and p-Abltide is fixed [14]. Once the p-Abltide concentration is calculated, it can be used to calculate the velocity, shown in Eq. (3). Thus the V_{max}, $K_{m,Abltide}$ and $K_{m,ATP}$ can be determined.

$$V = \frac{[P]}{(Tq)[E]} \quad (3)$$

Tq is the incubation time and $[E]$ is the enzyme concentration. The method utilizes the theory background that the initial velocity is equal to the product concentration divided by the time and that the product peak area can be related to concentration through the normalization factor. The initial velocities were plotted vs Abltide concentrations and the kinetic parameters were determined by a direct fit of the data to the Michaelis–Menten equation using Prism 5.0.

3. Results and discussion

3.1. Conditions of LC–MS

In the literature most mobile phases consist of a combination of water and methanol or acetonitrile to which some additives are added to improve chromatography and detection [25]. A better sensitivity and peak shape were observed for both Abltide and p-Abltide when acetonitrile was used instead of methanol. The presence of 0.1% TFA resulted in better peak shape and separation, shorter retention time and no apparent ion suppression.

In this experiment, ESI–MS operation parameters were carefully optimized for determination of Abltide and p-Abltide. It was found that Abltide could be ionized only in positive ESI mode, whereas p-Abltide could be ionized under both positive and negative modes. The response of p-Abltide in positive mode was sensitive enough to perform enzyme assay. Therefore, the positive ion mode was selected for the detection of Abltide and p-Abltide in this experiment. Meanwhile, the abundances of doubly charged ions with m/z 633.0 and 673.0 for Abltide and p-Abltide were higher than for the other ions. Therefore, these two doubly charged ions were used to automatically tune the ionization source and MS parameters. In the subsequent experiments, the doubly charged positive protonated molecules of Abltide at $m/z = 633.0$ $[M+2H]^{2+}$ and p-Abltide at $m/z = 673.0$ $[M+2H]^{2+}$ were used to monitor the enzyme reaction.

3.2. Desalting system for LC–MS

Normally, sample pretreatment and preparation are necessary due to the large discrepancy between biochemical reactions and ESI–MS. Biochemical reactions need additives which increase the solubilization and stabilization of proteins, reduce oxidation damage and protease release, prevent nonspecific binding to surfaces and mimic physiological conditions [26]. These additives are mostly non-volatile substances, which are not compatible with ESI–MS. In order to determine enzymatic reaction products by ESI–MS without performing sample pretreatment, an online desalting system was adopted in this study. In this system, the flow could be directed by the assistance of a divert valve. When the divert valve was turned to waste position, the eluent containing non-volatile additives was sent to the waste. Whereas, the eluent containing analytes entered into the MS when the divert valve was in detection position. The early eluent (before 5.2 min, as shown in Fig. 1) was sent to the waste to remove non-volatile additives, such as Tris–HCl, $MgCl_2$ and DTT. In order to remove these additives completely, liquid chromatography-contactless conductivity detection (LC–C^4D) and liquid chromatography-pulsed electrochemical detection (LC–PED) were used to find the exact retention times of the targets and additives (data not shown).

3.3. Method validation

Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the FDA Guidance. The accuracy and precision of intra- and inter-day analyses should not exceed 15% of the relative error (RE%) and 15% of the coefficient of variation (CV%), respectively.

3.3.1. Linearity and LOQ

The average p-Abltide concentration calculated according to Eq. (2) was plotted against the known p-Abltide concentrations to produce the following best fit linear regression equation: $y = 0.8895x + 0.0443$; $R^2 = 0.9997$.

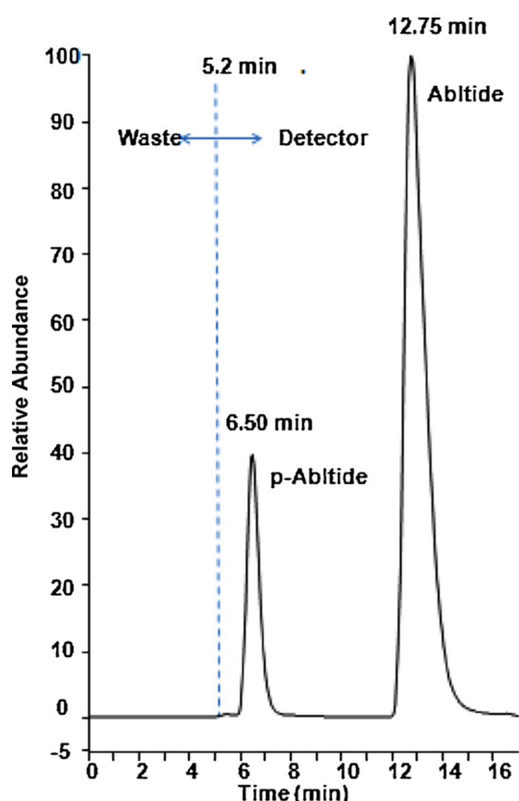

Fig. 1. Chromatogram of reaction mixture after incubation at 30 °C for 30 min. The non-volatile additives for stability of kinase and enzymatic reaction were eluted before 5.2 min and sent to the waste. After that the target substances were delivered to the MS detector for analysis.

The LOQ corresponding to 10 times the S/N was 10 nM for p-Abltide and 25 nM for Abltide.

3.3.2. Accuracy, precision and repeatability

The repeatability of the method was evaluated by examining both intra-day and inter-day variance. The %RSDs across all data points were less than 10.0%. Assay accuracy ranged from −1.6 to +5.3%. These data indicated that the LC–ESI–MS method was reliable and repeatable. All results are summarized in Table 1.

The repeatability of retention time of p-Abltide is a key point in the established method. This method adopted an online desalting system, which requires an accurate time point to switch the divert valve from the waste point to the detection point. To estimate this time point, six different reaction mixtures were analysed by LC–ESI–MS. The data are shown in Table 1 and the typical chromatogram in Fig. 1.

3.3.3. Stability of peptides in the process of enzyme activity assay

In the evaluation of the enzyme assay, the stability of the measured peptides is obviously of great importance [27]. During the enzyme activity assay, the p-Abltide might be unstable or both of the peptides could be lost. There was no significant difference between the peptides experiencing the enzyme activity assay procedure and the peptides without this procedure ($p > 0.05$). Therefore, both of the peptides were stable enough in this study.

3.4. Abl 1 tyrosine kinase assay

The activity assay of Abl 1 tyrosine kinase was based on the quantitative determination of Abltide and p-Abltide simultaneously. The enzyme catalyzed reaction was stopped by addition of 40 μL acetonitrile to 120 μL reaction mixture. Normally, the volume ratio of added organic reagent to the reaction mixture should be equal to or larger than 1:1. However, after adding 120 μL acetonitrile to 120 μL reaction mixture, the p-Abltide peak would broaden a lot. So, 40 μL, 80 μL and 120 μL acetonitrile were tried as well as boiling 1 min to stop the reaction, and then the data were evaluated by student's *t*-test (Two-Sample Assuming Unequal Variances). The *p* values were larger than 0.05, which meant there was no significant difference. Considering the peak shape and ease of operation, 40 μL acetonitrile was used to stop the reaction.

To select the proper enzyme concentration and reaction time within the linear range of the reaction, a time course at various Abl 1 tyrosine kinase concentrations was evaluated. The reaction was linear until 30 min, for a concentration of 0.25 μg protein/mL. To evaluate the total balance of both peptides, the concentration of Abltide and p-Abltide at different time points in the time course assay were calculated. The sum of concentrations of Abltide and p-Abltide was in the range of 95.2–101.2% of the theoretical quantity (data not shown).

In this study, a two-substrate enzymatic reaction was studied by LC–ESI–MS. One substrate is Abltide, the other one is ATP. To determine the Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) for Abltide, the concentration of this substrate was varied while the other substrate, ATP, was kept at a fixed saturating concentration. One or two runs were carried out using a limited number of Abltide concentrations to obtain a rough estimate of K_m and V_{max}. Improved estimates then were obtained by narrowing the Abltide concentration range between 0.25 and 5.0 times K_m to yield velocities of 20–80% of V_{max} [28]. Six different Abltide concentrations ranging from 5 to 150 μM were used to determine the kinetic parameters for Abltide. The concentration range of substrates used here was sufficient to determine the K_m value, as it covered the substrate concentration range of $1/3K_m$ to $3K_m$ [29]. Data for each substrate concentration point were measured in triplicate. Fig. 2 is a plot of velocity vs substrate concentration obtained from the MS data of the reaction of Abltide with ATP catalyzed by Abl 1 tyrosine kinase. The inset shows the Eadie–Hofstee plot, which is the linear transformation of the primary data. The K_m was calculated by non linear regression as 34.78 μM and the V_{max} value was 5.563 μmol/mg/min.

Fig. 3 and inset show the Michaelis–Menten plot and corresponding Eadie–Hofstee plot for ATP. From these data, the K_m and V_{max} were 43.61 μM and 5.906 μmol/mg/min, respectively.

Table 1
Accuracy, precision and repeatability.

Ratio of p-Abltide (%)	Intra-day ($n = 6$)			Inter-day ($n = 18$, 3days)		
	Precision[a] (RSD%)	Accuracy[a] (RE%)	Retention time (mean ± sd, min)	Precision[a] (RSD%)	Accuracy[a] (RE%)	Retention time (mean ± sd, min)
5	4.2	2.6	6.41 ± 0.03	9.2	5.3	6.43 ± 0.08
20	2.2	−1.6	6.49 ± 0.02	9.8	2.1	6.47 ± 0.07
40	1.6	−0.2	6.46 ± 0.02	1.6	0.6	6.47 ± 0.05

[a] Data from the calculated concentration of p-Abltide.

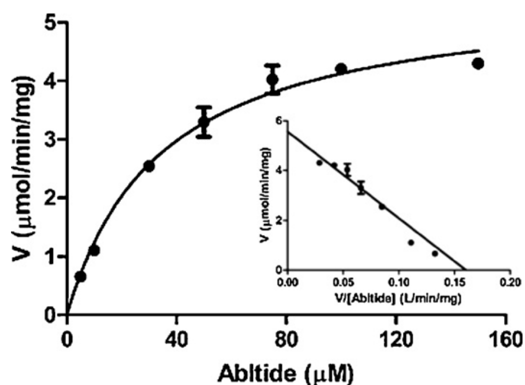

Fig. 2. The Michaelis–Menten plot and corresponding Eadie–Hofstee plot (inset) for the enzymatic reaction of Abl 1 tyrosine kinase at a concentration of 0.25 μg protein/mL, incubation time 30 min, fixed ATP at 150 μM and varying Abltide from 5 to 150 μM. The error bars represent the estimated standard deviation on the mean of three analyses.

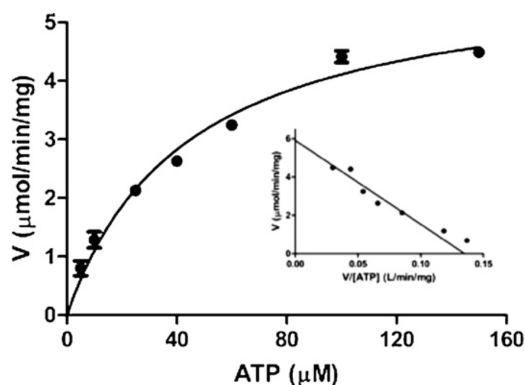

Fig. 3. The Michaelis–Menten plot and corresponding Eadie–Hofstee plot (inset) for the enzymatic reaction of Abl 1 tyrosine kinase at a concentration of 0.25 μg protein/mL, incubation time 30 min, fixed Abltide at 150 μM and varying ATP from 5 to 150 μM. The error bars represent the estimated standard deviation on the mean of three analyses.

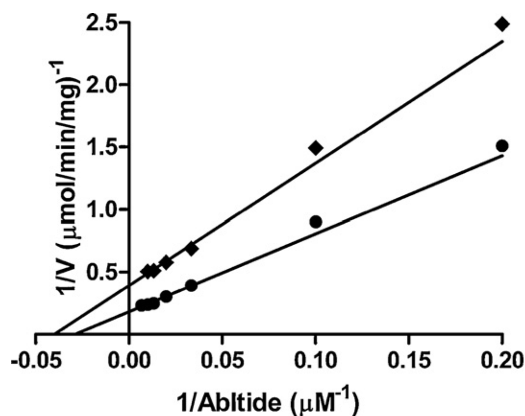

Fig. 4. Overlapped Lineweaver–Burk plots for the enzymatic reaction of Abl 1 tyrosine kinase at a concentration of 0.25 μg protein/mL, incubation time 30 min. The line (■) is the plot for fixed ATP at 100 μM and varying Abltide from 5 μM to 100 μM. The line (●) is the plot for fixed ATP at 150 μM and varying Abltide from 5 μM to 150 μM. The two sets of lines intersect together, which points to a ternary-complex mechanism for Abl 1 tyrosine kinase.

After the incubation of Abl 1 tyrosine kinase, ATP and Abltide, two products (ADP and p-Abltide) should be produced. However, in this kinetic study, only the concentration of p-Abltide was utilized to evaluate the velocity of the enzyme in both data analyses for K_m of Abltide and ATP. Fig. 4 clearly suggests that the reaction of Abl 1 tyrosine kinase and these two substrates behaves according to a ternary-complex mechanism. Indeed it shows that Abltide and ATP bind to Abl 1 tyrosine kinase at the same time to produce an Abltide-Abl 1-ATP ternary complex.

4. Conclusions

A simple, accurate, universal and cost effective LC–ESI–MS method was developed and validated for the study of Abl 1 tyrosine kinase. With the online desalting system, this method not only utilized a classical non-volatile reaction buffer, but also avoided sample pretreatment. And using the transformation of the ratio of product to substrate instead of an internal standard to calculate the concentration of product makes this method simple and cost effective. This method has been successfully applied to the Abl 1 tyrosine kinase kinetics study. This technique can also be applied to characterize enzymes in which no simple spectrophotometric or spectrofluorimetric assay is feasible.

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