Talanta 107 (2013) 88–94

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Study of Abl1 tyrosine kinase inhibitors by liquid chromatography–electrospray ionization-mass spectrometry

Hui Chen, Erwin Adams, Ann Van Schepdael*

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

ARTICLE INFO

Article history:
Received 14 September 2012
Received in revised form
14 December 2012
Accepted 26 December 2012
Available online 2 January 2013

Keywords:
LC–ESI-MS
Abl1
Abltide
Inhibitor study

ABSTRACT

A method to study Abl1tyrosine kinase inhibitors (TKIs) by liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) was developed and validated. Chromatographic separation was achieved on a Symmetry® C-18 column using a gradient. The detection was performed by selected ion monitoring (SIM) mode via positive ESI interface. The limit of quantification (LOQ) was 40.8 nM for p-Abltide [product, KKGEAIpYAAPFA-NH_2] and 26.7 nM for Abltide (substrate, KKGEAIYAAPFA-NH_2). The residual plot of linearity calibration curve indicated a good fit with a linear model. Intra- and inter-day precision was less than 10% and accuracy was from −6.93% to +0.15%. Matrix effect was not significant in this method. The validated method was applied to an Abl1 TKIs study. Imatinib mesylate (IM) and dasatinib were used to evaluate this method and the IC_{50} values were 202.1 nM and 925.1 pM, respectively. Two natural products (−)- epigallocatechingallate (EGCG) and caffeic acid were tested with this model. The IC_{50} value of EGCG was found at 64.03 nM and caffeic acid showed fluctuant inhibitory activity from 26% to 55% in the concentration range from 1 nM to 1 mM. The IC_{50} value of a dimethylpyrrole hydroxylbenzoic acid derivative (MPB) was 1.915 μM.

1. Introduction

Cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries. In 2008, about 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide [1].Cancer is a dynamic process that involves many complex factors [2], which may explain why a "magic bullet" cure has not been found [3]. The lack of such a cure leads to considerable attention being focused on chemoprevention as an alternative approach to the control of cancer [3]. Chronic Myeloid Leukemia (CML) is a type of cancer that starts in the blood-forming cells of the bone marrow and invades the blood. Based on the report of the American Cancer Society, CML accounts for about 20% of all leukemias and occurs at similar frequency in countries around the world. The age-adjusted incidence rate for CML was 1.6 per 100,000 adults [4]. Based on rates from 2000 to 2002, 1 in 619 men and women born today will be diagnosed with CML at some time during their lifetime, which shows the probability for a person to develop CML during his/her lifetime [5]. Both the incidence data and the lifetime risk estimates of CML shown here indicate that the attention paid on this disease is never too high.

CML was the first human malignant disease to be linked to a single, acquired genetic abnormality, which produces a constitutively active Bcr–Abl tyrosine kinase. The hybrid Bcr–Abl gene is formed by fusion between Abelson (Abl) tyrosine kinase gene at chromosome 9 and break-point cluster region (Bcr) gene at chromosome 22 [6]. This chromosomal translocation is called Philadelphia (Ph) chromosome. More than 90% of adults with CML are shown to be Ph chromosome positive (Ph+) [4]. CML is often divided into three phases, namely chronic phase, accelerated phase and blast crisis. Normally, drug treatment has more effect in the chronic phase. In the late 1980s, the Bcr–Abl gene was realized to be the most attractive target for CML therapy. Therefore, attempts to inhibit the tyrosine kinase activity of the constitutively active tyrosine kinase were made and a tyrosine kinase inhibitor, imatinib mesylate (IM), was discovered and developed for the treatment of CML during this process [7]. Now it is still the first-line therapy in patients with CML. However, some people with CML do not respond to IM and even individuals with responsive disease, who must remain on the drug indefinitely, can relapse [8,9]. Therefore, to surmount IM resistance, several other novel tyrosine kinase inhibitors (TKIs) were developed, such as dasatinib, nilotinib and bosutinib [10–12]. However, these agents are not efficacious in a small group of resistant patients [4]. It seems meaningful to further discover and develop more new, specific TKIs.

As the tyrosine kinase activity of the Bcr–Abl proteins is known to be essential to their transforming abilities [13–15],

* Corresponding author. Tel.: +32 1632 3443; fax: +32 1632 3448.
E-mail address: ann.vanschepdael@pharm.kuleuven.be (A. Van Schepdael).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2012.12.046

a specific inhibitor of Abl1 might be useful as a therapy for CML. Additionally, the Abl1 proto-oncogene has been implicated in processes of cell differentiation, cell division, cell adhesion and stress response. Deregulated Abl1is involved in cancer [16]. For these reasons, there is considerable interest in developing inhibitors that are capable of suppressing the activity of Abl1. Hence, in this study, Abl1 was used to investigate the activity of TKIs and potential TKIs in three groups. One group comprised two natural products and two commercial inhibitors were studied as the control group, whereas the last group contained some unknown compound.

Two of the potential inhibitors studied in this case were natural products. Historically, the majority of new drugs have been generated from natural products and from compounds derived from natural products. It seems that natural products represent privileged structures for drug discovery. This suggestion is supported by the fact that a limited number of protein folds are known now and that natural products must bind to some of them in order to be biosynthesized and to fulfill their inherent function in the producing organisms. Therefore, many of them may be structurally favored to bind to enzymes or protein receptors [17]. Polyphenols are a group of chemical substances found in plants that are available as chemoprotective agents against commonly occurring cancers. They are an important part of the human diet and are found in berries, grapes/wine, tea, chocolate/coca, coffee, soybeans and other fruits and vegetables [18]. (−)- epigallocatechingallate (EGCG) and caffeic acid are isolated from green tea and coffee, two important drinks for Eastern and Western populations, respectively. Currently, there is interest in the beneficial health effects of dietary polyphenols, because these compounds may have anti-oxidative, anti-inflammatory and anti-carcinogenic activities. Hence, these two classic polyphenols from diets were chosen as representatives to study their inhibitory activity on Abl1, in order to estimate whether CML could be controlled through dietary modification. EGCG is the most abundant catechin in tea and recent study suggests that it can inhibit xanthine oxidase activity to suppress intracellular ROS in HL-60 human promyelocytic leukemia cells [19]. Caffeic acid is a well-known phenolic acid present in many foods including coffee. A series of pull-down assays revealed that caffeic acid directly binds to Fyn kinase in an ATP non-competitive manner [20].

IM and dasatinib were used in this study as the control group. IM is an ATP-competitive inhibitor of the Abl protein kinase that is able to bind the inactive conformation of Bcr–Abl, preventing ATP from entering its binding pocket [21]. Dasatinib is a second generation tyrosine kinase inhibitor for IM resistant or intolerant Ph+ leukemias. It is a dual specific Src and Abl inhibitor that is able to bind and inhibit both active and inactive conformation of Abl, resulting in 100–300 fold higher activity than that of IM [11,22].

MPB, a derivative of compound 1 [4-(2,5 dimethyl-pyrrol-1-yl) -2-hydroxybenzoic acid], is able to inhibit the interaction of EphA4 with a peptide ligand as well as the natural ephrin ligands. It also inhibits ephrin-induced phosphorylation of EphA4 and EphA2 in cells, without affecting cell viability or the phosphorylation of other receptor tyrosine kinases [23]. Newly synthesized compound 1 does not show any detectable inhibitory activity. However, when left exposed to air at room temperature in a dry room, compound 1 acquires inhibitory activity of ephrin-A5-EphA4 binding. Through some yet unknown physical or chemical effects at room temperature, compound 1 acquires a darker brown color to become active MPB. Surprisingly, proton and carbon NMR spectra show no significant difference between these two compounds and the MS spectrum shows the same molecular weight [24]. The MS and NMR data of MPB can be found in the supplementary data of reference [24].

Robust methods that monitor enzyme activity and inhibitory potency are crucial to drug discovery and development. For high-throughput inhibitor screening, various forms of fluorescence and chemiluminescence readout always dominate the market. However, with the advance in sensitivity, speed and miniaturization of mass spectrometry methods, opportunities to couple mass spectrometry with screening will continue to come to the forefront [25]. Therefore, a LC–ESI-MS method for studying inhibitors of Abl1 is introduced in this report. Under the catalytic action of Abl1, a phosphate group can be transferred from an ATP to the substrate (Abltide) that results in a mass difference (80 Da) between Abltide and the product (p-Abltide). As such, the developed MS-based method can directly measure Abltide and p-Abltide in this case. For natural substrates without the chromophores required for spectrophotometric readouts, this ESI-MS approach dramatically reduces the reagent cost for derivatization and the false positive or false negative results caused by interference with the readout "tag". Thus, the establishment of an ESI-MS-based approach to measure inhibition of Abl1 is significant. However, many of the buffers and salts required for the enzyme reactions interfere with electrospray. Thus an online desalting system assisted by LC separation was applied in this study. By monitoring the conversion of substrate to product (instead of quantification vs. an internal standard), the need to use a costly heavy deuterated internal standard was eliminated. As described above, we developed a sensitive, accurate and cost effective LC–ESI-MS method and applied it to study the inhibitors of Abl1. The objective of this study was to establish a versatile and low cost LC–ESI-MS approach to discover new inhibitors of Abl1.

2. Experimental

2.1. Chemicals and reagents

Abl1 was purchased from proteinkinase.de (specific activity is 33.000 pmol/mg min; Biaffin GmbH & Co KG, Kassel, Germany). Abltide (KKGEAIYAAPFA-NH_2) was purchased from AnaSpec, Inc (San Jose, CA, USA). The phosphorylated Abltide [p-Abltide, KKGEAIpYAAPFA-NH_2] was synthesized by BACHEM (Bubendorf, Switzerland). IM was purchased from Cayman Chemical (Europe) and dasatinib was from LC Laboratories (Woburn, MA, USA). EGCG and caffeic acid were purchased from Sigma (St. Louis, MO, USA). MPB was provided by the Laboratory for Neurobiology, KU Leuven. Acetonitrile (LC/MS grade) and trifluoroacetic acid (TFA, ULC/MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands). Dimethyl sulfoxide was from BASF (Antwerp, Belgium). 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 LC 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 LC system was coupled to a LCQ ion trap mass spectrometer (Thermo) with an ESI interface. Chromatographic separation was carried out using a Symmetry® C-18 column (150 × 2.1 mm i.d., particle size 5 μm;Waters, Massachusetts, USA) at room temperature (23 °C, MS room). Xcalibur 1.3 software (Thermo, California, USA) was used for instrument control, data acquisition and processing.

The chromatographic separation was performed with a gradient mobile phase system consisting of mobile phase A (acetonitrile–water containing 0.1% TFA, 25:75, v/v) and mobile phase B (water) at a flow rate of 0.2 mL/min. Gradient program: 0–2 min, 69% of A (isocratic); 2–6 min, 69–80% of A (linear gradient); 6–10 min, 69% of A (isocratic). The mobile phases were degassed by sparging with helium for 2 min. The total run time was 10 min and in the first 5.3 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). The parameters of ESI-MS can be accessed from our previous work [26].

2.3. Method validation

A robust method to monitor inhibitory potency is crucial for successful TKIs discovery and development. To demonstrate the reliability of the method developed for the study of Abl1 TKIs, a method validation process was executed according to FDA and ICH Guidance [27,28]. Here, several quantitative aspects such as matrix effect, quantification limits, linearity, accuracy, and precision were examined. A robustness test was also performed for the LC system to make sure the analytes can be sent to the MS at a fixed time point.

2.3.1. Matrix effect

To determine possible matrix effects, a post-column infusion system was applied [29–31]. For this experiment, a blank enzyme reaction solution containing everything the Abl1 kinase assay contained, except for the analytes (Abltide and p-Abltide), was prepared. The blank enzyme reaction solution was centrifuged (14,100 *g* for 5 min) and then injected during continuous post-column infusion of a 50 μM Abltide solution at a flow of 5 μL/min. The affected area of the chromatographic run was determined by comparing the SIM chromatographic profiles for Abltide with an injection of the blank enzyme reaction solution and an equivalent injection of mobile phase. Each experiment was performed in duplicate. The same procedures were used to determine the matrix effect on 50 μM p-Abltide solution.

2.3.2. Robustness test for LC system

In this study, the effect of small changes of chromatographic parameters on the gradient method was investigated. The parameters examined were the content of TFA in the mobile phase, the flow and age of the mobile phase, and the percentage of mobile phase A at the start and end of the gradient. Their effects on the retention time of p-Abltide were evaluated by means of an experimental design, using Modde 5.0 statistical graphic software (Umetrics AB, Umeå, Sweden). A two-level half factorial design ($2^{k-1}+3$) was used and 19 experiments were performed. The factors and levels investigated in this robustness test are shown in Table 1.

2.3.3. Linearity and limit of quantification (LOQ)

To investigate the linearity, 35 μM total peptide mixtures of Abltide and p-Abltide were determined at known molar ratios. The ratios included 0%, 1%, 2%, 4%, 6%, 8%, 10% and 12% 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 back calculated concentrations of the calibration standards should be within ±15% of the nominal value.

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.

2.3.4. Accuracy and precision

The accuracy and intra- and inter-day precision of the method were evaluated by analyzing peptide mixtures (35 μM) at ratios of 2%, 6% and 10% p-Abltide. To determine intra-day precision and accuracy, six replicates were analyzed at each ratio. The inter-day precision and accuracy were evaluated by analyzing six replicates at each ratio on three separate days. The mean accuracy values should be within 15% of the nominal values and the coefficient of variation (CV) values for precision should not exceed 15%.

2.4. Time course assay for the phosphorylation of Abltide by Abl1

The reactions by Abl1 (0.2 μg protein/mL, 0.1 μg protein/mL and 0.05 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 (35 μM) and Abltide (35 μM) in a total volume of 1500 μL. The mixture was incubated at 30 °C and an aliquot of the solution (150 μL) was collected at 0, 2, 5, 10, 15, 20, 30, 40, 50 and 60 min. 37.5 μL acetonitrile was added to each aliquot of reaction mixture (150 μL) to stop the reaction. After centrifugation, LC–ESI-MS analysis ($n=3$) was performed.

2.5. Inhibition assay

The inhibition assays 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 (35 μM) and Abltide (35 μM), Abl1 (0.1 μg protein/mL) and inhibitors at 30 °C in a total volume of 150 μL for 15 min. The concentration of the inhibitors was fixed at 1 nM–2 μM (IM) or 10 pM–20 nM (Dasatinib) or 0.5 nM–10 μM (EGCG) or 1 nM–1 mM (caffeic acid) or 10 nM–5 μM (MPB). The reactions were terminated after 15 min at 30 °C by the addition of acetonitrile (37.5 μL). The percent inhibition was estimated by applying Eq. 3 (see below). The assays were repeated three times and averaged. The data processing and calculation of IC_{50} values were performed by using Microsoft Excel and Prism 5.0 (GraphPad software, San Diego, CA).

Table 1
Factors and levels investigated in the robustness test.

Parameter	Units	Limits	Level (−)	Level (+)	Nominal
Flow of mobile phase	μL/min	±10	190	210	200
Concentration of TFA	%	±0.01	0.09	0.11	0.10
Age of mobile phase A	hour	±12	0	24	12
Percentage of mobile phase A at the start of the gradient	%	±1	68	70	69
Percentage of mobile phase A at the end of the gradient	%	±1	79	81	80

2.6. Data analysis

For kinase reaction analysis, the product concentration ([*P*]) is calculated from the following Eqs. (1 and 2) [26] using the chromatographic data.

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

$$[P] = \frac{35Ap}{F \times As + Ap} \quad (2)$$

A normalization factor (*F*) is introduced to eliminate the ionization difference between Abltide and p-Abltide. *Ap* is the cluster area of the p-Abltide at m/z 673.0 ± 1.0, which is the product of the reaction, and *As* is the cluster area of the Abltide at m/z 633.0 ± 1.0. [*s*] and [*p*] represent the concentrations of Abltide and p-Abltide, respectively, in the validation mixture.

For concentration dependent inhibition studies, the percent inhibition (*I*%) is calculated from

$$I\% = \frac{[P]\ without\ inhibitor - [P]\ with\ inhibitor}{[P]\ without\ inhibitor} \times 100 \quad (3)$$

3. Results and discussion

3.1. Method development

The K*m* values of Abltide and ATP for Abl1 were 34.78 μM and 43.61 μM respectively as determined in our previous work [26]. The enzyme reaction conditions were the same in this report. In order for competitive inhibitors to be identified in a competition experiment that measures IC_{50} values, a substrate concentration around or below the *Km* must be used. Hence, 35 μM Abltide was chosen to study the inhibitors of Abl1. In order to provide enough phosphate groups to Abltide and not override the competitive capabilities of inhibitors, the concentration of ATP was also fixed at 35 μM.

For an inhibitor screening method, the shorter analytical time the better. For this purpose, in this study, a gradient mobile phase system was used instead of the isocratic one used in our previous work. When the same ratio of acetonitrile (acetonitrile/reaction mixture 1/3, v/v) was used to quench the enzyme reaction in this case, severe fronting of the p-Abltide peak was observed. Considering the final concentrations of analytes, several tiny modifications in the amount of acetonitrile were investigated. The results showed a ratio of 1/4 (acetonitrile/reaction mixture, v/v) provided a good peak shape and sufficient quenching capability.

3.2. Method validation

While LC–ESI-MS offers much promise for measurement of biological samples, one important issue that must be addressed in method development and validation is matrix effects. To mimic the enzyme reaction under in vivo conditions, special reaction buffers should be prepared to enhance the yield of the product. This kind of special reaction buffers provides optimized physiological conditions for the enzyme, but also the possibility of matrix effects. Matrix effects are the alteration of ionization efficiency by the presence of coeluting substances. Fig. 1a shows that the signal is stable before 5.3 min because no mobile phase flow was directed into the MS analyzer; the signal area from 5.3 to 5.6 min is influenced by the change of mobile phase flow from waste to MS analyzer; the signal becomes stable again after 5.6 min, and its intensity indicates no altered ionization due to injection of mobile phase when p-Abltide and Abltide are eluted out. But, the signal after 5.6 min is higher than the signal before 5.3 min, which might be caused by the mobile phase flow. Therefore, the mobile phase system used here could enhance the ionization of Abltide and p-Abltide, but it enhanced more for p-Abltide compared to Abltide. Fig. 1b and d indeed indicates no altered ionization due to matrix effect for p-Abltide and Abltide. In sum, Fig. 1 shows no significant reduction or enhancement of the ion intensity of the analytes between the injection of mobile phase and blank enzyme reaction solution.

An online desalting system was used to remove salts, introduced from enzyme reaction buffer, to protect the ESI source against deterioration. This system was accomplished by the assistance of a divert valve, which directed the flow of the mobile phase into waste or MS at the set time point (Fig. 2). In order to fix this time point, the retention time of p-Abltide should be fixed, whereas a gradient mobile phase system might cause a small shift of the retention time of p-Abltide. So, a robustness test was performed to investigate the effect of small changes of chromatographic parameters on the gradient method. Fig. 3 shows the effect of five selected variables on the retention time of p-Abltide. It is observed that the retention time of p-Abltide under the examined conditions is principally influenced by the flow of the mobile phase.The content of TFA in the mobile phase and the percentage of mobile phase A at the start of the gradient have no significant effects. The interaction parameters effects on the retention time of p-Abltide are also insignificant. This is because their error lines include 0, indicating that the change of retention time of p-Abltide caused by changing variables is smaller than the experimental error. In summary, this LC system is robust, although attention should be paid to the flow of the mobile phase.

The LOQ corresponding to 10 times the *S*/*N* was 40.8 nM for p-Abltide and 26.7 nM for Abltide.The average p-Abltide concentration calculated according to Eq. (2) was plotted against the known p-Abltide concentrations to produce a regression equation. The residual plot of linearity calibration curve showed a random pattern, which meant a good fit for the linear model. The standard curve had an acceptable coefficient of determination ($R^2 > 0.995$) value and a *Y*-intercept not significantly different from zero. Therefore, the linear model was used to produce the following best fit linear regression equation: $Y = 0.9151X + 0.0883$, with %RSDs across all data points ≤15%. The precision of the method was evaluated by examining both intra- and inter-day variance. The %RSDs across all data points were less than 10.0%. Assay accuracy ranged from −6.93% to +0.15%. These data indicated that the LC–ESI-MS method was reliable and repeatable. All results are summarized in Table 2.

3.3. Inhibition study in vitro

Most inhibitors chosen here, for example, IM, were not soluble in water. So inhibitors were first dissolved in DMSO. The final concentration of DMSO never exceeded 1% (v/v). DMSO compatibility with the assay was determined and the result showed that the influence of 1% of DMSO on the enzyme assay was not significant.

For ideal kinetic measurements, excess substrate would be used with direct quantitation of the product. Typically, one targets the assay so that the product never exceeds 10% of the substrate concentration. By evaluating enzyme reactions at an early stage, the initial rates can be maintained. Therefore, to determine the conditions at which the enzyme reaction maintained an initial rate with sufficient signal, three different concentrations of Abl1 were evaluated through a time-course study. The data suggested that the standard reaction conditions for further inhibitor study should be 0.1 μg of protein/mL of Abl1 for 15 min at 30 °C.

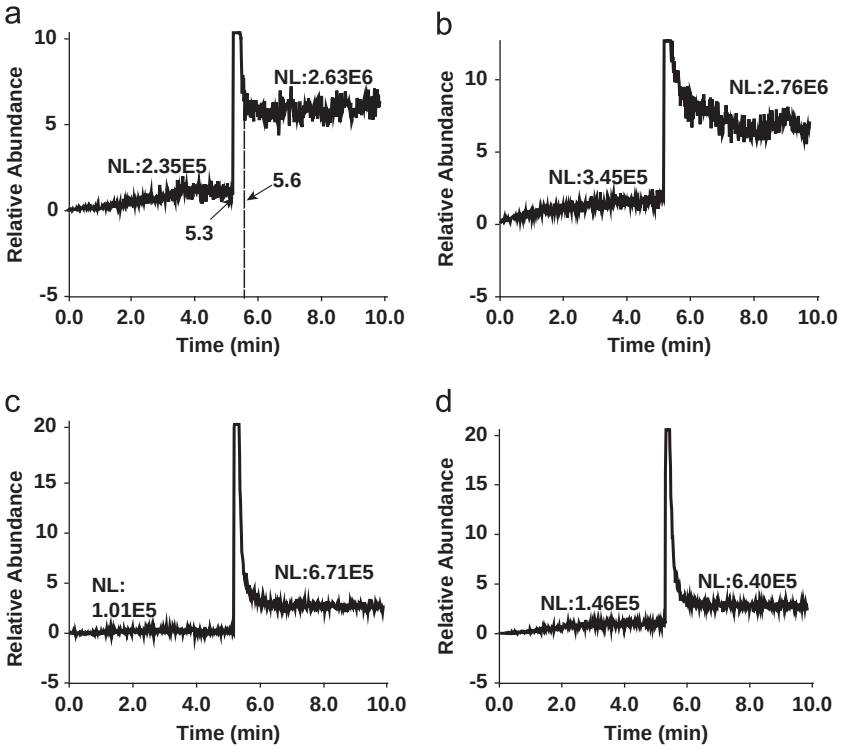

Fig. 1. Post-column infusion chromatograms performed as described under section of matrix effect (2.3.1). p-Abltide is shown in a, b and Abltide in c, d for LC–ESI-MS analysis of blank mobile phase injection (a, c) and blank enzyme reaction buffer with inactivated Abl1 (b, d). Before 5.3 min, the mobile phase flow was directed into waste; after 5.3 min, the mobile phase flow was directed into the MS analyzer, causing the peak-like signal change. NL: intensity.

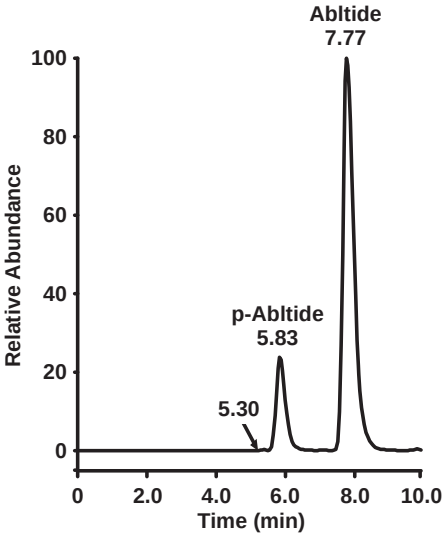

Fig. 2. Chromatogram of reaction mixture after incubation at 30 °C for 15 min. The non-volatile additives used for stability of Abl1 and for the enzymatic reaction were eluted before 5.3 min and sent to the waste. After that the target substances were delivered to the MS analyzer for analysis.

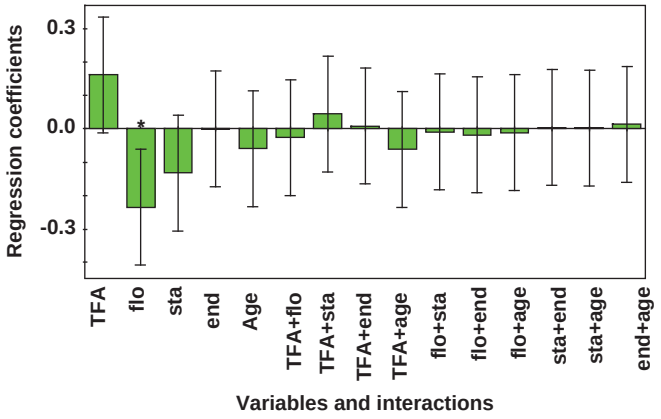

Fig. 3. Effect of five selected variables (factors) on retention time of p-Abltide. flo: flow of mobile phase; sta: percentage mobile phase A (%A) in the mobile phase at the start of the gradient; end: %A in the mobile phase at the end of the gradient; age: age of mobile phases A and B *: significant effect.

Table 2
Summary of precision and accuracy.

Ratio of p-Abltide (%)	Intra-day ($n=6$)		Inter-day ($n=18$, 3days)	
	Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)
2	9.7	−2.04	9.9	−3.33
6	4.4	−3.88	8.9	0.15
10	7.8	−1.51	5.0	−6.93

To demonstrate the feasibility of this simple assay in the characterization of inhibitors in vitro, IM and dasatinib were tested using the established inhibition assay. The dose–response curves were obtained by plotting the percentage of inhibition vs. the logarithm of the concentrations of inhibitors. Fig. 4a shows the inhibition curve for dasatinib inhibition of Abl1. The IC_{50} values of IM and dasatinib against Abl1 were determined to be 202.1 nM and 925.1 pM, respectively, thus indicating that the inhibitory activity of dasatinib is 200-fold higher than that of IM. This result is consistent with the fact that dasatinib is 100 to 300-fold more inhibitory than IM [11,32,33] and the IC_{50} values estimated herein are in good agreement with the data reported

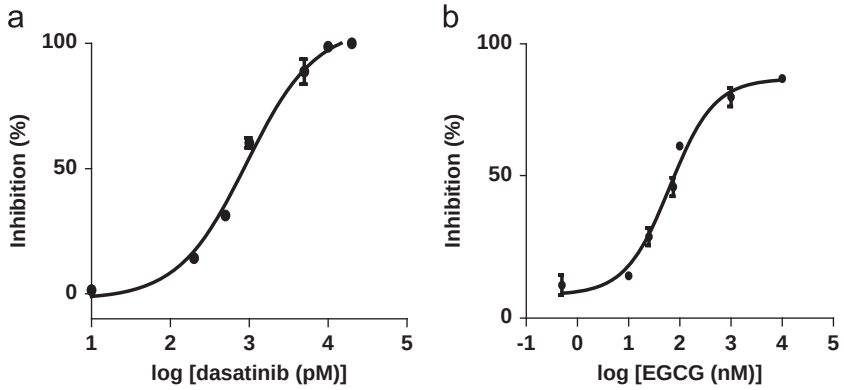

Fig. 4. Inhibition curves for dasatinib (a) and EGCG (b) inhibition of Abl1 Concentrations: dasatinib, 10 pM–20 nM; EGCG, 0.5 nM–10 μM; Abl1, 0.1 μg protein/mL; ATP, 35 μM; Abltide, 35 μM; IC_{50} of dasatinib: 925.1 pM; IC_{50} of EGCG: 64.03 nM.

previously (IC_{50} of IM: 260–678 nM [34,35]; IC_{50} of dasatinib: 0.8–1.8 nM [35]) which demonstrates that the present assay is a promising alternative to conventional methods.

Being two important ingredients of two common drinks for humans, the Abl1 inhibitory activity of EGCG and caffeic acid was determined and compared with the two commercial drugs, IM and dasatinib, to simply investigate the effect of a healthy diet on the prevention of cancer. The IC_{50} of EGCG was found at 64.03 nM (Fig. 4b), which meant the inhibitory activity of EGCG for Abl1 was higher than that of IM, but lower than that of dasatinib. On the other hand, for a concentration range of caffeic acid from 1 nM to 1 mM, the inhibition fluctuated from 26% to 55%. So, the inhibition potency of polyphenols to Abl1 does exist, which indicates that their presence in diet is beneficial for human health. In view of the results reported here, more polyphenols should be studied or modified to obtain more effective Abl1TKIs to cure CML in the future.

The specific inhibitory activity of MPB to EphA4 and EphA2 is confirmed [23,24]. So its inhibitory activity toward non-receptor tyrosine kinase was tested in this study. The IC_{50} value of MPB was determined as 1.915 μM, which suggested that the inhibitory activity of MPB was not better than that of IM. However, this result indicated that MPB could inhibit not only receptor tyrosine kinase, but also non-receptor tyrosine kinase.

4. Conclusion

A simple, accurate, universal and cost effective LC–ESI-MS method was developed and validated for the study of Abl1 TKIs. The method developed here contains several advantages. First, classic non-volatile enzyme reaction buffer was used in this report, yet it was possible to analyze the product directly by ESI-MS directly after quenching the enzyme reaction. Second, the SIM mode was used for quantification, enabling this method to determine natural substrates with or without optical properties. Third, it is cost effective because there is no need for an expensive deuterated internal standard, no sample pre-treatment, a low flow of mobile phase and low consumption of enzyme protein (μg), substrate (mg) and inhibitors (μg). Fourth, the product and substrate can be determined simultaneously and both of their detected signals are involved in calculating IC_{50} values, which reduces the rate of false results. This method was successfully used to study Abl1 TKIs and the inhibition potency ranking is dasatinib > EGCG > IM > MPB > caffeic acid. The LC–ESI-MS based inhibition assay developed in this report has proved to be useful and straightforward to discovering new TKIs.

Acknowledgments

We thank the Laboratory for Neurobiology, KU Leuven, which donated the MPB. Hui Chen acknowledges the China Scholarship Council (CSC) for a scholarship.

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, CA Cancer J. Clin 61 (2011) 69–90.
[2] P.C. Nowell, Cancer Res. 46 (1986) 2203–2207.
[3] M.B. Sporn, N. Suh, Nat. Rev. Cancer 2 (2002) 537–543.
[4] X. An, A.K. Tiwari, Y. Sun, P.R. Ding, C.R. Ashby Jr, Z.S. Chen, Leuk. Res. 34 (2010) 1255–1268.
[5] CML Statistics by the American Cancer Society (2006) 〈http://leukemia.emedtv.com/cml/cml-statistics.html〉 Last updated 18.10.2006.
[6] J.D. Rowley, Nature 243 (1973) 290–293.
[7] E. Buchdunger, J. Zimmermann, H. Mett, T. Meyer, M. Müller, B.J. Druker, N.B. Lydon, Cancer Res. 56 (1996) 100–104.
[8] M. Baccarani, G. Saglio, J. Goldman, A. Hochhaus, B. Simonsson, F. Appelbaum, J. Apperley, F. Cervantes, J. Cortes, M. Deininger, A. Gratwohl, F. Guilhot, M. Horowitz, T. Hughes, H. Kantarjian, R. Larson, D. Niederwieser, R. Silver, R. Hehlmann, European LeukemiaNet, Blood 108 (2006) 1809–1820.
[9] P. Ramirez, J.F. DiPersio, Oncologist. 13 (2008) 424–434.
[10] E Weisberg, P.W Manley, W Breitenstein, J Brüggen, S.W Cowan-Jacob, A. Ray, B. Huntly, D Fabbro, G Fendrich, E Hall-Meyers, A.L Kung, J Mestan, G.Q Daley, L Callahan, L Catley, C Cavazza, M Azam, D Neuberg, R.D Wright, D.G Gilliland, J.D Griffin, Cancer Cell 7 (2005) 129–141.
[11] N.P Shah, C Tran, F.Y Lee, P Chen, D Norris, C.L Sawyers, Science 305 (2004) 399–401.
[12] J.M Golas, K. Arndt, C. Etienne, J Lucas, D. Nardin, J Gibbons, P Frost, F Ye, D.H Boschelli, F. Boschelli, Cancer Res. 63 (2003) 375–381.
[13] B.J. Druker, S. Tamura, E. Buchdunger, S. Ohno, G.M. Segal, S. Fanning, J. Zimmermann, N.B. Lydon, Nat. Med 2 (1996) 561–566.
[14] T. Oda, S. Tamura, T. Matsuguchi, J.D. Griffin, B.J. Druker, Leukemia 9 (1995) 295–301.
[15] T.G Lugo, A.M Pendergast, A.J Muller, O.N Mitte, Science 247 (1990) 1079–1082.
[16] G Saglio, D Cilloni, Cell Mol. Life Sci. 61 (2004) 2897–2911.
[17] J.W Li, J.C Vederas, Science 325 (2009) 161–165.
[18] C Manach, A Scalbert, C Morand, C Rémésy, L Jiménez, Am. J. Clin . Nutr. 79 (2004) 727–747.
[19] J.K Lin, P.C Chen, C.T Ho, S.Y. Lin-Shiau, J. Agric. Food Chem. 48 (2000) 2736–2743.
[20] N.J. Kang, K.W. Lee, B.J. Shin, S.K. Jung, M.K. Hwang, A.M. Bode, Y.S. Heo, H.J. Lee, Z. Dong, Carcinogenesis 30 (2009) 321–330.
[21] T Maekawa, E Ashihara, S Kimura, Int. J. Clin. Oncol. 12 (2007) 327–340.
[22] J.M Golas, K Arndt, C Etienne, J Lucas, D Nardin, J Gibbons, P Frost, F Ye, D.H Boschelli, F Boschelli, Cancer Res. 63 (2003) 375–381.
[23] R. Noberini, M. Koolpe, S Peddibhotla, R. Dahl, Y. Su, N.D. Cosford, G.P. Roth, E.B. Pasquale, J. Biol. Chem. 283 (2008) 29461–29472.
[24] R. Noberini, S.K. De, Z Zhang, B Wu, D Raveendra-Panickar, V Chen, J. Vazquez, H. Qin, J Song, N.D Cosford, M Pellecchia, E.B Pasquale, Chem. Biol. Drug Des. 78 (2011) 667–678.
[25] K.D Greis, Mass Spectrom. Rev. 26 (2007) 324–339.
[26] H Chen, E Adams, A Van Schepdael, J Chromatogr., B Analyt., Technol. Biomed. Life Sci. 897 (2012) 17–21.
[27] U.S. FDA, Guidance for Industry—Bioanalytical Method Validation, 2001.

[28] ICH Harmonised Tripartite Guideline. Validation of analytical procedures: text and methodology Q2 (R2), in: proceedings of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005.
[29] R Bonfiglio, R.C King, T.V Olah, K Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[30] P.J Taylor, Clin. Biochem. 38 (2005) 328–334.
[31] R Dams, M.A Huestis, W.E Lambert, C.M Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290–1294.
[32] J.S. Tokarski, J.A. Newitt, C.Y. Chang, J.D. Cheng, M. Wittekind, S.E Kiefer, K. Kish, F.Y. Lee, R Borzillerri, L.J Lombardo, D. Xie, Y. Zhang, H.E. Klei, Cancer Res. 66 (2006) 5790–5797.
[33] S. Redaelli, R. Piazza, R. Rostagno, V Magistroni, P Perini, M Marega, C Gambacorti-Passerini, F Boschelli, J. Clin. Oncol. 27 (2009) 469–471.
[34] N. Kondo, S. Nishimura, Chem. Eur. J 15 (2009) 1413–1421.
[35] S. Soverini, G. Rosti, I. Iacobucci, M. Baccarani, G. Martinelli, Oncologist 16 (2011) 868–876.