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Short communication

Characterization of tyrosine kinase and screening enzyme inhibitor by capillary electrophoresis with laser-induced fluoresce detector

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ABSTRACT

An effective, rapid and reliable capillary electrophoresis–laser induced fluorescence (CE–LIF) procedure was built to study the characterization of tyrosine kinase (TK), which was a target for drug screening. In this procedure, CE separated the sample of the TK reaction and LIF selectively detected the fluorescence-labeled polypeptide substrate and product. The precise TK activity was quantitated by introducing the transformation ratio of the substrate (*T*%) to avoid the deviation resulted from the detection sensitivity and the injection amounts in different runs and different capillaries. By measuring the *T*%, the effects of various reaction conditions were optimized. Meanwhile, the progression of the enzyme reaction was monitored. The K_m and V_{max} were calculated for TK under the optimized experimental conditions. In addition, the inhibition effectiveness of two model inhibitors, Staurosporine and SU6656 were evaluated. The results indicated that the screening platform based on electrophoresis was suitable for TK analysis and laid a foundation for the HTS of TK inhibitors.

1. Introduction

The rapid development of modern technologies in synthetic, combinatorial and natural product chemistry has resulted in millions of compounds suitable for pharmaceutical screening. This large number of compounds has put a heave pressure on analytical chemists, who developed a variety of high throughput screening (HTS) methods to meet the demand. Most of the current HTS methods are multi (96, 384, 1536, or more) well plate based assays, which often lack good separation and may generate erroneous results. Therefore, it is advantageous to introduce separation techniques into screening. For example, capillary electrophoresis (CE), like HPLC, has been well-documented in drug analysis [1,2] due to its high separation efficiency, small analyte volume requirement, high analytical speed and ease of automation. Moreover, it is very convenient to assemble multiple capillaries to form parallel CE systems, which are suited for HTS with high resolution.

Previously, CE has shown to be suitable for enzymatic assays, which are often used as the assay models for HTS [3–6]. By comparing with other conventional formats of assays, CE has demonstrated excellent uniformity [7]. Here, we intend to develop a CE-based tyrosine kinase (TK) assay method. TKs are a diverse group of enzymes that catalyze the phosphorylation reaction, in which a phosphate group is transferred from adenosine triphosphate (ATP) onto the tyrosine moiety in a protein or peptide. Tyrosine phosphorylation regulates many cellular functions of proliferation, differentiation, and metabolism in living systems [8]. It can be regarded as the primary or even exclusive indicator of signal transduction in the multicelluar organisms [9]. The enhanced activity of TK may lead to some diseases, such as atherosclerosis, psoriasis, diabetes, inflammatory responses and even many cancers [10–13]. Therefore, inhibition of TK will halt these signaling pathways and thus can be viewed as an effective means of therapeutic intervention. In other words, a compound may be developed into a novel drug if it can inhibit the TK activity.

There are several conventional TK assay methods such as radioactive phosphate transfer assay [14,15], solid-phase radioactive phosphate transfer assay [16], nonisotopic enzyme-linked immunosorbent assay (ELISA) [17,18], microtiter plate-based assay [19] and fluorescence polarization assay [13,20]. The common disadvantages of these methods are labor-intensive, time consuming, high background and low signal to noise ratio. Further, the use of radioactive compounds in some of those approaches generates radioactive waste and is hazardous. Most importantly, the lack of a good separation mechanism often leads to false results. Thus, it is still of interest to develop a fast, simple, user-friendly and reliable enzyme assay.

To the best of our knowledge, there is no report of TK assay by CE. It is expected that CE can separate the non-phosphorylated

Abbreviations: HTS, high throughput screening; CE, capillary electrophoresis; TK, tyrosine kinase; ATP, adenosine triphosphate; ELISA, enzyme-linked immunosorbent assay; LIF, laser-induced fluorescence; MOPS, 3-(N-morpholino)-propanesulfonic acid; ADP, adenosine diphosphate; T, the transformation ratio of the substrate; HD, hydrodynamic; EK, electrokinetic; EOF, electro-osmotic flow.

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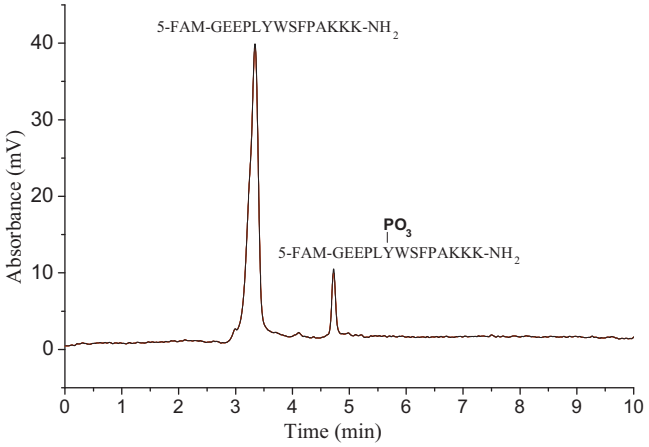

Fig. 1. Typical electropherogram showing the separation of the polypeptide substrate and product in a TK catalyzed reaction. Electrophoretic conditions: 10 kV, 37 cm (27 cm effective separation length) ×75 μm i.d. (375 μm o.d.) uncoated fused silica capillary, 488 nm excitation, 520 nm emission, capillary at 30 °C and the samples at ambient, sample loaded from anode for 5 s hydrodynamically. The running buffer: 20 mmol/L borate buffer at pH 7.4.

polypeptide substrate from the phosphorylated polypeptide product due to the relatively large difference of their electrophoretic mobilities. It is convenient to monitor the increase of the polypeptide product or the decrease of the polypeptide substrate or both for the entire process of the enzymatic reaction. The addition of the heavily negative charged phosphate group made the separation of the product from the substrate relatively easy. Further, laser induced fluorescence (LIF) detection was chosen for avoiding interference in this CE assay. Based on these settings, we evaluated the characteristics of the TK and measured the inhibition effects of two model compounds, i.e. Staurosporine and SU6656.

Instead of using the peak area or height of the individual peak, the transformation ratio of the substrate to product was proposed in order to increase the repeatability and reduce the variation in the assay.

2. Theoretical background

In our study, the TK catalyzes the following enzymatic reaction:

$$\text{5FAM-GEEPLYWSFPAKKK-NH}_2 + \text{ATP} \xrightarrow{\text{Src}} \text{5FAM-GEEPLY}(\text{PO}_3)\text{WSFPAKKK-NH}_2 + \text{ADP}$$

Under the catalysis of TK, the phosphate group is transferred from ATP to the Tyr residue of the polypeptide substrate, 5-FAM-GEEPLYWSFPAKKK-NH_2, resulting in the formation of phosphorylated polypeptide, 5-FAM-GEEPLY(PO_3)WSFPAKKK-NH_2. Meanwhile, ATP is converted to ADP.

Due to the selective detection of fluorescent tag in CE–LIF, the TK, ATP and ADP are not detected leaving only the 5-FAM tagged polypeptide substrate and product detectable in the electropherogram. Since the electrophoretic mobility of the substrate and product is significantly different due to the phosphorylation, they could be easily separated and detected without any interference in the CE–LIF assay (Fig. 1).

On this basic of this separation, it is feasible (1) to monitor the progression of the reaction; (2) to perform the characterization of TK; and (3) to evaluate the perturbation of other parameter, such as inhibition to this reaction. The last will constitute the basis for selecting the best candidate to be further developed into drugs.

The separation of the polypeptide product from the substrate as shown in Fig. 1 provides the basic for quantitative analysis. The formation of the product or the depletion of the substrate can be easily studied by integrating the individual peak areas without any interference. Therefore, it should be straightforward to analyze various activities of TK based on the integrated peak areas.

However, CE is well known to have poorer repeatability as compared to other techniques, such as HPLC. Many factors, such as injection amount, may affect the repeatability of the CE results. In order to obtain more reliable data, we chose to use a relative term called transformation ratio (T%) as defined here:

$$T\% = \frac{n_p}{n_s + n_p} \times 100 \quad (1)$$

where n_s is amount of substrate. n_p is amount of product transformed from the substrate. The sum of n_s and n_p equals to the total amount of substrate and product in the specific reaction. It is worthwhile to point out that n_s and n_p are the amount of materials introduced into the CE run. Therefore, the total amount of n_s and n_p is related to the actual amount of samples injected. Even though the total amount of the substrate plus the product in a specific TK reaction is the same as the starting material, the total of n_s and n_p in individual CE assay may be different in different runs due to injection variation. This variation would result in inaccuracy in monitoring the whole TK reaction course. Since T% is a relative number, it is not related to the absolute amount of the substrate or product because both of them come from the same run and any variation in the injection amount would cancel each other. Therefore, using T% avoids the error of inconsistence in sample injection.

Further, it is well known that the peak area in CE is related to its mobility. Two ions having different mobilities will generate two different sized signal peaks even they have the same concentration and the same chromaphor. The slow moving ion would give a bigger peak. Proper correction of this error is necessary and many approaches have been developed [21–23]. For simplicity, we used the peak area (A)/time (t) ratio as the correction factor to adjust the peak difference between substrate and product [24]. Therefore,

$$T\% = \frac{n_p}{n_p + n_s} \times 100 = \frac{A_p t_s / t_p}{A_s + A_p t_s / t_p} \times 100 = \frac{A_p t_s}{A_s t_p + A_p t_s} \times 100 \quad (2)$$

where A_s, t_s, A_p, and t_p is the peak areas and migration time of the substrate and product, respectively in the same electropherogram.

It is necessary to point out that the above formula has the precondition that both substrate and product have the same chromaphor (fluoresce in this case). Further, the local environment for both substrate and product are the same and the quantum yield of the fluoresce in both the substrate and the product is also the same. It is only under these conditions that the amount of materials can be proportional to the peak areas for both substrate and product.

Eq. (2) indicates that at the early state of the reaction, when the substrate concentration is relatively large and the TK reaction is a pseudo-first-order reaction, the amount of product is directly proportional to the T%.

In addition to the correction of injection variation, using T% also reduces the variations resulted from the detection sensitivity in different runs and different capillaries. Considering that fact that

the *T*% has corrected the variation in mobility, it is expected that the result would be more accurate and reliable because the variance of sample parameter, such as migration times, peak height and peak area have all been considered in the results.

3. Experiment

3.1. Chemicals and reagents

All chemical reagents were of analytical grade, unless otherwise stated, and were from Tianjin Kewei Chemical Co. (Tianjin, China). Buffers for enzymatic reaction and CE operation were prepared by using double distilled water purified by a Nanopure II system (Barnstead, USA).

MOPS buffer (pH 7.0) was used as the incubation buffer for the TK reaction. Borate buffer (20 mmol/L) was prepared with boric acid and titrated to pH 7.4 with 20 mmol/L tetraborate for electrophoresis. All solutions were degassed in an ultrasonic bath (Tianjin Precise Instrument Co., Ltd., Tianjin, China) for 5 min and filtered through a 0.22 μm syringe filter (Ann Arbor, MI) prior to use. The fluorescein-labeled 5FAM-GEEPLYWSFPAKKK-NH_2 substrate at N-terminal was synthesized by Upstate Inc. (Dundee, UK) and purified by RP-HPLC. ATP was from Changsha Oumay Biotechnology Co., Ltd. (Hunan, China). TK was a gift from Upstate. Both the TK and the polypeptide substrate were diluted with 20 mmol/L 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer to the targeted concentrations, and were aliquoted to smaller volumes and stored at −20 °C until use. Staurosporine and SU6656 were obtained from Calbiochem (EMD Biosciences, Inc., USA).

3.2. Instrumentation

All CE experiments were performed on a Beckman (Fullerton, CA, USA) P/ACE 5000 system equipped with a LIF detector. The excitation wavelength for the LIF was at 488 nm and the emission wavelength at 520 nm. An uncoated fused-silica capillary (37/30 cm total/effective length, 75/360 i.d./o.d.) was from Yongnian Co., Ltd. (Hebei, China). Data was collected and processed by the P/ACE station software.

3.3. TK enzymatic reaction

The enzyme and substrate were taken out from a −20 °C freezer and thawed at room temperature for 10–15 min first. Then, a 50 μL substrate stocking solution and a 38 μL (or 39 μL) 20 mmol/L MOPS buffer were mixed in a 0.2 mL vial. Subsequently, 2 μL (or 1 μL) TK and 10 μL 1.0 mmol/L ATP were added to the mixture. The reaction mixture of the 100 μL final volume was gently mixed on a vortex and the enzyme reaction started in a 30 °C water bath. For screening study, 10 μL of each potential inhibitor and 40 μL substrate were mixed first for 5 min at room temperature. Then, other reagents were added to the mixture and started the reaction in a 30 °C water bath. Finally, the samples were directly analyzed by the CE–LIF without further treatment.

3.4. Electrophoretic conditions

Samples were injected from anodic side by a gas pressure (0.5 psi) for a short time (5 s) and separated at 30 °C under a constant voltage of 10 kV. Running buffer was 20 mmol/L borate buffer at pH 7.4. Prior to the assay runs, a sequence of 0.1 mol/L NaOH, distilled water, and the running buffer was used to rinse the capillary for 0.5 min under 20 psi pressure.

4. Results and discussion

4.1. CE separation

4.1.1. Sample injection mode

CE sample can be introduced into capillary either by a hydrodynamic (HD) or an electrokinetic (EK) injection. Each of them has certain advantages and/or limitations. For example, it is hard to perform HD injection in parallel CE without rather sophisticated instrumentation. On the contrast, EK injection has the advantages of instrumental simplicity, convenient operation with precise control, on-line pre-concentration, less zone broadening, and convenience for parallel operation, etc. However, the accuracy and reproducibility of EK injection are poorer as compared with HD. Since a high reproducibility is essential for an enzymatic analysis, it is necessary to choose an injection mode that would generate the highest reproducibility. For this purpose, a mixture of the substrate and product was analyzed in four different capillaries with both EK and HD injection. The results (data not shown) indicated that the repeatability (RSD%) was improved to 13.0% and 2.3% for EK and HD injection, respectively. It was clear that HD injection had better reproducibility than EK injection. In addition, compared with HD, *T*% results, 12.6%, indicated that there was the sampling bias during EK injection. Thus, HD injection was employed in all of subsequent assays.

4.1.2. Optimization of CE–LIF assays

In order to optimize the experimental conditions, the impacts of a series of experimental parameters, such as the pH values of the running buffer, the separation voltage, the concentration of buffer and the temperature of separation channel on the CE separation were evaluated first.

The pH value of the buffer could directly affect the TK activity in three aspects. First, the fluorescent-labelled substrate and product are easy to decompose and loss the fluorescent tags in either strong acidic or strong basic medium. Second, the electro-osmotic flow (EOF) fluctuates with pH. The experimental results indicated that pH 7.4 was preferred for the CE running buffer.

Higher separation voltage should speed up the separation process and provide higher efficiency due to less dispersion. However, too high a voltage would generate too much Joule heat and raise the temperature, which would cause the loss of separation efficiency. Similarly, the buffer concentration and temperature within the capillary also influence the separation speed and efficiency. After optimization, it was concluded that 10 kV, 20 mmol/L borate buffer at pH 7.4, and 30 °C gave the best separation efficiency. All subsequent TK assays were performed under those conditions.

4.2. Characterization of tyrosine kinase

The characteristics of the TK enzyme were evaluated at different incubation pH's, temperatures, incubation time, TK concentrations, etc. The enzymatic reaction was ceased before CE–LIF assay by adding tetrasodium ethylenediamine tetraacetate.

4.2.1. Effect of incubation pH

It is well known that enzymatic activity is highly dependent on the incubation pH. By keeping all reaction conditions the same and only varying pH, the *T*% at different pH values was individually calculated within 20 min of reaction time. The experimental results (data not shown) manifested that the *T*% increased dramatically to 18.0% from 0.9%, when incubation pH was adjusted gradually from 8.0 to 7.0. The *T*% slowly decreased in the pH range of 7.0–6.0. This result indicated that the proper incubation pH of the TK was at pH 7.0, which was consistent with the literature [25].

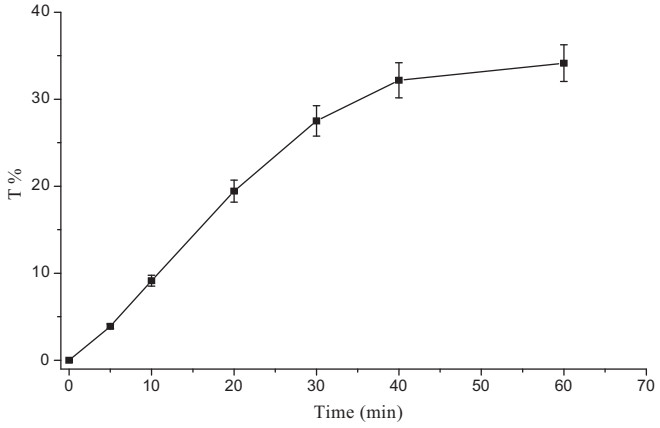

Fig. 2. Monitor of process of TK reaction. Experimental conditions: all experimental conditions remained the same as indicated in Fig. 1 except the enzymatic incubation time.

Therefore, the incubation pH was fixed at 7.0 in all of subsequent assays.

4.2.2. Effect of incubation temperature

Incubation temperature is also important to enzyme activity. The high incubation temperature could inhibit enzymatic activity and even cause deterioration because the enzyme is also a protein. For optimizing the temperature for TK assay, it was found that the *T*% at a fixed 40 min reaction time was <12% when the temperature was below 28.0 °C or above 36.0 °C, data not shown. Among the 28.0–36.0 °C temperature evaluated, 30.0 °C gave the highest value *T*% of 30.0%. Thus, 30.0 °C was chosen as the standard temperature.

4.2.3. Characterization of TK

After optimizing to experimental conditions, it is feasible to characterize the TK enzyme by constructing a Michaelis–Menton equation. This needs to obtain a series of reaction curve first. Since CE consumes only nano-liter volumes of samples each time, it is feasible to make continuous injections from even a small volume of reaction mixture without affecting the characteristics of the reaction. For TK analysis, samples were taken out of the reaction mixture and injected consecutively into CE. From the plot of *T*% vs. incubation time (Fig. 2), a linear relationship was observed for at least 20 min. The slope of this linear curve corresponds to the initial reaction rate. Subsequent process of similar runs at different concentrations gave a series of initial run rates, which can be used to construct the M–M plot. By filling the data into the M–M equation, we obtained the K_m and V_{max} for TK under the optimized experimental conditions as 328.22 nmol/L and 43.85 nmol/(L min), respectively.

4.2.4. TK activity standard curve

To validate the quantitation results, an external TK enzyme actively standard curve was established. For this purpose, a 500 nmol/L substrate was added into a series of tubes containing various concentrations of TK ranging from 0.1 to 1×10^{-8} mol/L and incubated at 30.0 °C for 15 min. A perfect linear relationship (data not shown) was obtained when TK concentration was $<0.5 \times 10^{-8}$ mol/L. Beyond that concentration, the increase slowed down and implied the change of the enzymatic kinetics. No apparent saturation effect was observed in the whole TK concentration range. Finally, a 0.3×10^{-8} mol/L TK concentration was used as the final concentration in order to avoid misleading and ensure proper detection of the polypeptide product.

4.3. Reproducibility

Good repeatability is crucial to the successful application of this CE based enzyme assay in a high throughput screening setting. Under the optimal experimental conditions, the repeatability (RSD%) of the CE method was evaluated for retention time, peak height, peak area, and the substrate conversion rate for inter- and intro- day using a series of replicate injections ($n = 6$) of the analytes (Table 1). The reproducibility of peak area and migration time was <4.1% and 1.2% for inter- and intro-day, respectively. However, the RSD% of the *T*% was <2.0% for the corresponding parameters.

4.4. Inhibition study

Aforementioned method has well established a model method for screening candidate drug. Normally, there are multiple ways of screening enzyme inhibitors. The most accurate way is to evaluate the K_i value of each individual inhibitor. However, this method is not broadly used due to its complexity and experimental difficulty. The most common method is to run the assay at different concentrations of the inhibitors and determine their IC_{50}, i.e. the concentration that inhibits 50% of the enzyme activity at a given experimental condition. If the purpose is only to rank the relative effectiveness of different inhibitors, it is possible to use an even simpler method by only comparing the effectiveness of these different inhibitors under specific conditions. In the TK assay, we only intend to demonstrate the feasibility of this method for drug screening and therefore chose the simplest method.

The first thing is to choose a proper concentration of substrate for the TK reaction. Ideally, the substrate concentration should be on the flat part of the Michaelis–Menton plot to maintain a stable reaction rate without concerning the small change in substrate concentration may have on reaction rate. At the same time, this is the highest rate available and can delivery the fastest results. Unfortunately, it is not always feasible to conduct the experiment at such a high concentration of substrate due to various reasons, such as solubility. For this reason, we evaluated the substrate in a concentration range from 0 to 1000 nmol/L while the TK was remained at constant 0.5×10^{-8} mol/L along with sufficient ATP. After reaction 20 min, the *T*% varied with the substrate concentration (Fig. 3). As expected this plot is very similar to the M–M plot with typical three phases: typical linear phase <200 nmol/L, a flat phase exceeding 300 nmol/L, and a mixed phase in between. Thus, the 500 nmol/L substrate was chosen for drug screening.

4.4.1. Staurosporine inhibitor

Staurosporine is a broad protein kinase inhibitor whose inhibitory effect has been validated extensively. It was reported that the binding site of Staurosporine could cover the ATP-binding domain and reduce the protein kinase activity [26]. The inhibition effect on the TK was studied by CE–LIF at different concentrations of Staurosporine. As shown in Fig. 4, the *T*% apparently decreased

Table 1
Reproducibility ($n = 6$) of migration time, peak height, and peak area for inter- and intro-day.

Analytes	RSD inter-day (%) ($n = 6$)			RSD intro-day (%) ($n = 6$)		
	Peak area	Peak height	Migration time	Peak area	Peak height	Migration time
Substrate	2.99	7.12	0.46	4.14	9.62	1.01
Product	2.76	6.42	0.38	3.67	9.12	1.23

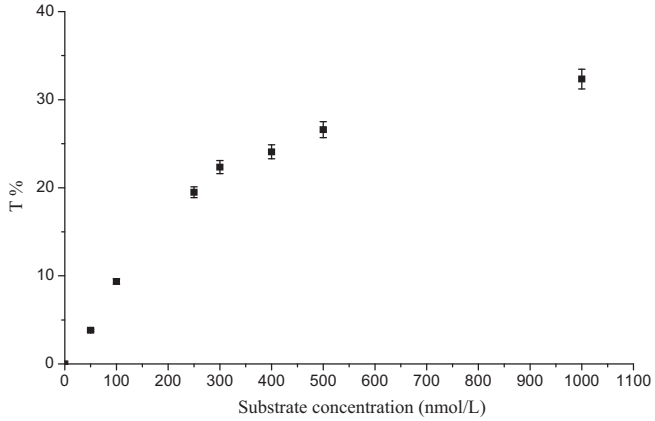

Fig. 3. Effect of substrate concentration. Experimental conditions: all experimental conditions remained the same as indicated in Fig. 1 except the substrate concentration.

along with the increase of Staurosporine concentration. The inhibition effect of Staurosporine against the TK activity was verified based on the CE–LIF results. Comparing to other methods, such as fluorescence polarization, this CE–LIF assay provided more precise, unambiguous and quantitative information. At the same time, this data also proves that CE–LIF is suitable for future HTS assay.

4.4.2. SU6656 inhibitor

SU6656 is a small molecule having a MW of 371.5. Preliminary experiments have verified that SU6656 could be a potential inhibitor of the Scr family. However, its inhibition effect to TK was unknown. Different SU6656 concentration from 0 to 1×10^{-5} mol/L was added to study the inhibitory effect. As shown in Fig. 5, in the presence of SU6656, the substrate was scarcely transformed to form product in the first 10 min, which indicated SU6656 strongly inhibited the enzymatic activity. Thus, it clearly indicates that SU6656 is an inhibitor to the TK. The results also implied that SU6656 could be a strong inhibitor to the TK, especially when compared with Staurosporine (Fig. 4).

5. Conclusions

A CE–LIF assay was built to study the characterization of tyrosine kinase and screen the potential drug. In characterization study,

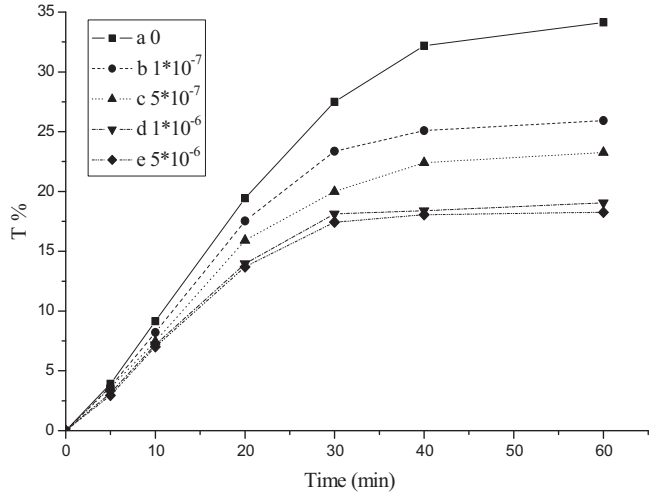

Fig. 4. Effect of Staurosprine concentrations on the enzymatic activity. Experimental conditions, all experimental conditions remained the same as indicated in Fig. 1 except with or without Staurosprine.

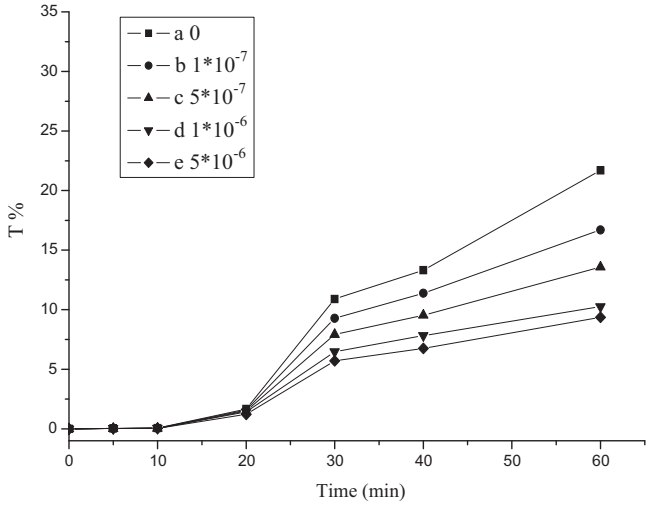

Fig. 5. Effect of SU6656 concentrations on the enzymatic activity. Experimental conditions: all conditions remained the same as indicated in Fig. 1 except with or without SU6656.

the progress of TK was monitored and K_m and V_{max} were calculated after optimizing the effects of various enzymatic reaction conditions, such as incubation pH values, temperatures. Under the optimization condition, the inhibition effects of two model inhibitors, Staurosporine and SU6656 were quantitated and compared. The results indicated that the effect of the SU6656 was better than Staurosporine under the same concentration. Compared with other traditional analysis method, this assay offers several advantages, such as quicker assay, lower sample consuming, higher sensitivity, more precise, unambiguous and quantitative results. The research results have laid a foundation for the HTS of TK inhibitors and opened up a novel idea for the study of other enzymes and their inhibitor screening.

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