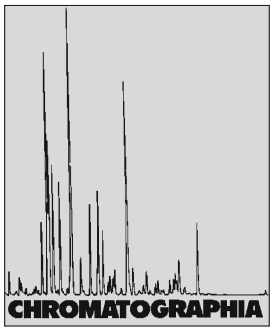
An LC–MS Method for a Hexokinase Inhibitor Study Based on Adenosine 5′-Triphosphate Determination and Application to the Anticancer Mechanism of *Momordica cochinchinensis*



2010, *72*, 425–430

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Received: 1 April 2010 / Revised: 7 June 2010 / Accepted: 7 June 2010
Online publication: 1 July 2010

Abstract

A reliable and validated LC–MS method was established for a hexokinase inhibitor study based on adenosine 5′-triphosphate (ATP) determination. By adding 5 mM ammonium acetate in the aqueous phase, this method enabled the determination of ATP by LC–MS and greatly increased the MS signal of ATP. This method was used in the study of the anticancer mechanism of *Momordica cochinchinensis*, an exact ingredient which had exhibited certain hexokinase inhibitor activity. This might reveal the anticancer mechanism of *Momordica cochinchinensis*.

Keywords

Column liquid chromatography–mass spectrometry
Hexokinase inhibitor
Adenosine 5′-triphosphate
Momordica cochinchinensis

Introduction

The Cucurbitaceae plant *Momordica cochinchinensis* is cultivated as a vegetable in Asian countries. In Chinese, Indian Ayurvedic, and Indonesian Jamu traditional medicines, the dry fruit of this plant is called *Momordica cochinchinensis*, which can help with detumescence and remove toxins for eliminating carbuncles. (Chinese Pharmacopoeia, CHP, 2005). Recent research has indicated that the alcoholic extract of *Momordica cochinchinensis* has certain anticancer activity [1]. However, its mechanism of action and intensity of its activity are unknown.

Hexokinase (EC 2.7.1.1) is the first and pivotal enzyme in the glycolytic pathway of life process. Cancer cells often process increased aerobic glycolysis. This phenomenon—known as the Warburg effect—has been found to be closely associated with hexokinase bound to the mitochondria [2]. Most recently, hexokinase has been regarded as a potential target for preferentially killing cancer cells [3]. Therefore, the identification of hexokinase inhibitors is important for the development of new anticancer drugs.

In general, a kinetic assay of hexokinase mainly involves spectrophotometry using glucose-6-phosphate dehydrogenase as an auxiliary enzyme to produce reduced nicotinamide adenine dinucleotide phosphate, which has a specific ultraviolet (UV) adsorption maximum at 340 nm for detection. However, the spectrophotometric method is not a straightforward way for high-throughput screening of hexokinase inhibitors because the coupled enzyme and addition of substrates make the assay more complex and may generate "off-target hits" [4]. Thus, an easy, fast, and high-throughput LC-UV method was developed to determine the kinetics of hexokinase. Adenosine 5′-triphosphate (ATP), as the major hexokinase substrate, was converted to ADP with the involvement of glucose. An ion-pair chromatography method [5] was used to determine the remaining amount of ATP and the generated ADP at a wavelength of 254 nm after enzyme-mediated reaction so as to calculate its activity. However, owing to the similar structures of ATP and ADP (the only difference is a

Original
DOI: 10.1365/s10337-010-1672-0
0009-5893/10/09

phosphate group), their baseline separation by LC-UV is difficult; further, this method is vulnerable to various factors (column packing material, mobile phase ratio, pH, and column temperature) that cause interference, and thus, accurate quantitative analysis is very difficult. At the same time, since some hexokinase inhibitors are ATP analogues, their chromatographic behavior is similar to that of ATP and ADP, which disturbs the quantitative analysis and determination of their activity, and it even yields false-positive results. Owing to high sensitivity and selectivity, LC–MS has become the first choice for biological analysis. In particular, with the advent of electrospray ionization (ESI) ion source technology, its unique soft ionization technology ionizes the molecules under relatively mild conditions, enabling the detection of moderately polar and thermally instable compounds by MS. In MS, highly polar substances cause a problem; a high proportion of aqueous phase is required to be incorporated in the liquid phase [6–8], which lowers the efficiency of ionization, and polar molecular structures are vulnerable to disruption. ATP and ADP are highly polar compounds with relatively unstable structures, and their decomposition in the ESI source is very complicated [9, 10]. In addition, the common ion-pair reagents are mostly non-volatile buffer salts, which are not suitable for applying to LC–MS; therefore, studies on ATP and ADP analysis using LC–MS are rarely reported. Qian et al. [11] reported the use of a special ion-pair LC–MS method for ATP measurement in cultured cells, but the ion-pair reagent used in this method was very expensive, and thus its fitness is not high.

To overcome the previously mentioned deficiencies, a novel LC–MS method for the determination of ATP and ADP was established in our laboratory, in which the LC–MS conditions were investigated and optimized: by adding 5 mM ammonium acetate in the aqueous phase, the MS signal of ATP and ADP was greatly increased, and it had better stability and reproducibility. In our method, which is based on direct determination of the ATP remaining after an enzyme-mediated reaction, the inhibition of screened compounds can be monitored directly from the difference in the area of the ATP peak of the samples with and without inhibitor. This method is convenient and stable with good reproducibility, linearity, accuracy, and recovery; it has higher throughput, specificity, and sensitivity than the LC-UV method. LC–MS has been applied to determine the activity of 3-bromo-2-oxopropionic acid (3-Brpa) [12] (a known inhibitor of hexokinase), and the results of the calculation were matched with those of relevant reports [13, 14]. This method was then used to calculate the inhibitor activity of *Momordica cochinchinensis* extract; the result might help in further study of its anticancer mechanism.

Experimental

Reagents and Chemicals

Hexokinase (EC 2.7.1.1), ATP disodium salt, and 3-BrPA were from Sigma (Steinheim, Germany). HEPES was from Farco Chemical Supplies (Hong Kong, P.R. China). Anhydrous glucose, glycerol, magnesium chloride, as well as all the organic reagents were from Shanghai Chemical Reagent (Shanghai, P.R. China). *Momordica cochinchinensis*s were kindly provided by Mr. Hu Yingkang of Shanghai Baike Pharmaceuticals. The hexokinase solution containing 0.06 U mL^{-1} hexokinase, 3 mM $MgCl_2$, and 5% *w*/*v* glycerol was freshly prepared with 25 mM HEPES buffer (pH 7.5). The substrate and inhibitor solution containing 3 mM $MgCl_2$ and 8 mM glucose in 25 mM HEPES buffer (pH 7.5) was freshly prepared each day.

LC–MS Instrumentation and Conditions

An Agilent (Palo Alto, USA) 1100 MSD single quadrupole mass spectrometer equipped with an ESI source operating in the positive ion mode was used as a detector, which consisted of a G1322A degasser, G1312A binary pump, G1313A plus autosampler, G1316A column compartment with thermostat, and G1946D quadrupole MS detector. Data were collected and analyzed on an Agilent G2710AA analysis workstation. The selected ion monitoring (SIM) mode was used for quantification with *m*/*z* of 508 for ATP and 428 for ADP. The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.0 kV; source temperature, 300 °C; drying gas flow, 10 L min^{-1}; fragment voltage, 100 V; and nebulizer pressure, 30 psi.

The LC separation was performed using an Agilent Extend C-18 column (3.1 × 100 mm, 3 μm). The mobile phase consisted of 5 mM ammonium acetate (pH 7.5) and methanol (60:40, *v*/*v*), and it was delivered at a flow rate of 0.3 mL min^{-1}; the column temperature was set at 20 °C.

Preparation of Standard and Quality Control Solutions

A stock solution was prepared by dissolving 10 mg of ATP powder in 10 mL of substrate solution. The stock solution was freshly prepared on every testing day.

A series of standard solutions was prepared by diluting the stock solution with different amounts of substrate solution to give concentrations of 5, 10, 15, 20, 30, 50, and 60 μg mL^{-1}, which were identified as standard calibration solutions. Meanwhile three typical concentrations (12, 35, and 55 μg mL^{-1}) were prepared as quality control solutions.

Accuracy and Precision

The intra-day accuracy and precision tests were carried out at LLOQ (5 μg mL^{-1}) and three typical concentrations of ATP (12, 35, and 55 μg mL^{-1}) by analysis of five replicate samples; the inter-day test just involved repetition of the test on three successive days.

Extract Preparation of *Momordica cochinchinensis*

Momordica cochinchinensis powder (500 g) was heated by backflow in 500 mL petroleum benzene for fat elimination. Then, the filter residue was extracted for 24 h with 500 mL of ethyl alcohol. The extract was concentrated to 20 mL, and then subjected to preparative liquid chromatography.

The preparation liquid chromatography was carried out with UV detection at 254 nm using an Agilent Extend C18 column. The initial mobile phase gradient (flow rate, 1 mL min^{-1}) consisted of 50% water (A) and 50% methanol (B). The LC gradient was gradually increased from 50% methanol to 100% methanol in 30 min. The ingredient in peak **a** (as shown in Fig. 1) was collected, freeze-dried to a fine powder, and labeled as "MBZA" for hexokinase inhibitor study.

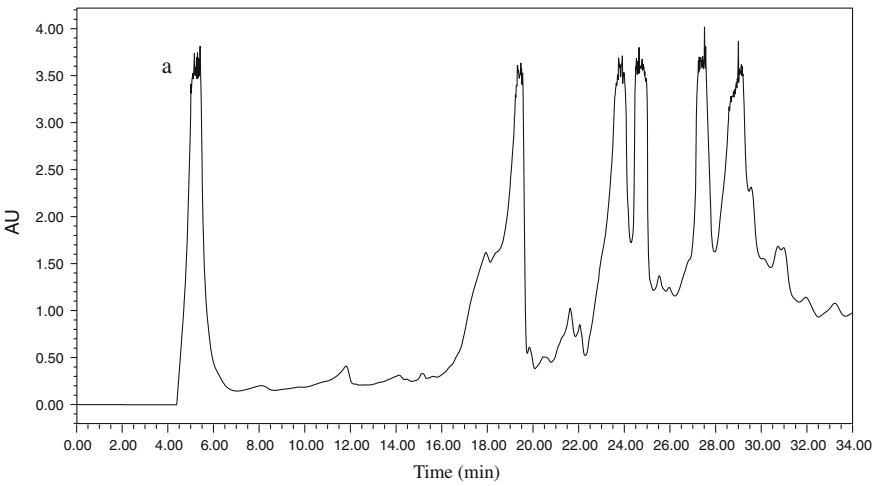

Fig. 1. A typical half preparation liquid chromatograph

Preparation of Series of Inhibitor Solutions

The 3-BrPA and MBZA stock solutions were obtained by dissolving 10 mg of fine powder in 10 mL of inhibitor solution. Then, a series of standard solutions was prepared by diluting the two stock solutions with different amounts of substrate solution to give concentrations of 10, 15, 20, 25, and 30 μg mL^{-1}.

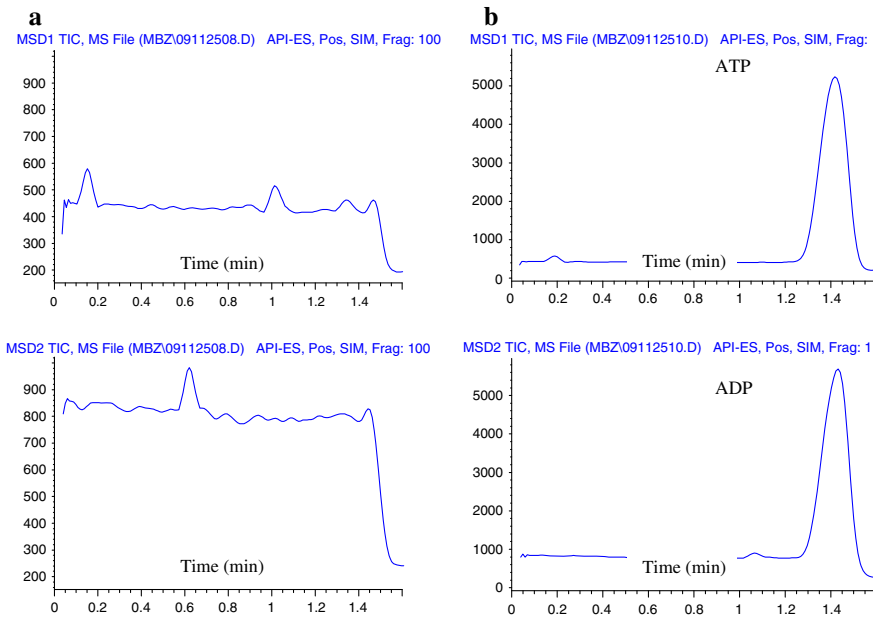

Fig. 2. **a** Blank substrate solution, **b** ATP spiked sample (30 μg mL^{-1})

Enzyme-Mediated Reaction

Each inhibitor solution of the series was treated according to the following steps: first, 100 μL ATP sample (30 μg mL^{-1}) was added to each series solution and mixed in an Eppendorf tube. Then, 100 μL hexokinase solution was added to initiate the enzyme-mediated reaction, the total enzymatic reaction time was 1 min. Finally, 5 μL of the reaction residue was injected into the LC–MS for inhibitor study. Additionally, a blank ATP sample (30 μg mL^{-1}) and a standard control sample (30 μg mL^{-1} ATP mixed with hexokinase solution without any inhibitor) were injected.

Results

Specificity

LC–MS chromatograms of a blank substrate sample and a sample spiked with 30 μg mL^{-1} of ATP is shown in Fig. 2. The chromatograms exhibited no additional peaks because of endogenous substances or other metabolites that could interfere with the detection of ATP and ADP at the same retention time.

Linearity and LLOQ

The calibration curve was constructed by plotting the area under the ATP curve as the ordinate and the concentration of ATP solution (μg mL^{-1}) as the abscissa, resulting in the equation $Y = 3022.6X + 1902.9$ ($r^2 = 0.998$), while the regression equation was generated by using the least square method. LLOQ was defined as the lowest concentration of the analytes that could be determined with an

Table 1. Result showing that assay meets the method validation regulation

Nominal C (μg mL^{-1})	Intra-day ($n = 5$)		Inter-day ($n = 3$)	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
5	102.32	4.32	101.45	6.02
12	100.16	2.30	102.31	4.21
35	99.84	1.90	99.22	3.34
55	100.72	2.40	100.75	5.44

accuracy of 80–120% and precision below 20% over five analytical runs, and was obtained from the sample solution spiked with 5 μg mL^{-1} ATP.

Accuracy and Precision

The results obtained for accuracy and precision are shown in Table 1. The intra-day accuracy ranged from 99.84 to 100.72% with a relative standard deviation (RSD) of 1.90–2.30%, and the inter-day accuracy ranged from 99.22 to 102.31% with an RSD of 3.34–5.44%. This reported method showed good accuracy and precision and is promising for application to the quantification of ATP.

Hexokinase Inhibitor Kinetic Calculation

After the enzyme-mediated reaction, the inhibition of enzyme activity could be directly determined if the peak area of ATP was higher compared to the standard control sample without any inhibitor. A typical chromatogram for inhibitor assay is shown in Fig. 3. The inhibition percentage could be calculated using the following formula:

$$I\% = \left(1 - \frac{A_0 - A_i}{A_0 - A_1}\right) \times 100\%,$$

where $I\%$ is the inhibition percentage; A_0, the peak area of ATP in the blank sample; A_i, the peak area of ATP in the presence of an inhibitor; and A_1, the peak area of ATP in the standard control sample.

We plotted the area ATP as, respectively, ordinate versus the concentration of 3-BrPA and MBZA (μg mL^{-1}) as abscissa in Fig. 4, which showed the description of their inhibition behavior. The value at each inhibitor concentration was measured in triplicate, and the average value was used to construct the plots. Further, the $I\%$ of 30 μg mL^{-1} 3-BrPA was calculated as 82%, while the $I\%$ of 30 μg mL^{-1} MBZA was calculated as 15%.

Discussion

Optimization of LC Conditions

LC conditions were optimized with respect to (i) the methanol proportion, (ii) ammonium acetate amount, and (iii) pH modifiers. The effect of methanol on the retention time and response signal of the analytes was investigated by varying its content from 0 to 60% in the mobile phase. As the proportion of methanol increased, the area of the ATP and ADP peaks increased greatly; on the other hand, this resulted in high analyte retention and thus, long analysis times (Fig. 5). Because of the high polarity of ATP and ADP, the high proportion of aqueous solvent used would increase the retention time but lower the efficiency of nebulization. A more organic solvent such as methanol would be good for the nebulization efficiency (Fig. 5). Considering the retention time and nebulization efficiency, 40% methanol could be appropriate. Under these conditions, all analyte peaks were baseline separated, and the response signals of ATP and ADP were stronger.

The amount of ammonium acetate added affected the response signal of ATP and ADP remarkably. Ammonium acetate is a fine LC–MS additive, which could help in the ionization of molecules, especially polar molecules [15–17]. A minimum amount of it in the mobile phase would stabilize polar molecules in the ESI source, which then would convert to more [+H] molecular ions, so as to achieve stronger response signal, fine peak shape, and more accuracy in quantification. Most important is that the nucleotide groups of ATP and ADP and ammonium acetate form a compound through noncovalent bonds [18], and the structure of this compound is very stable, which could prevent the decomposition of ATP and ADP in the ESI source. The amount of ammonium acetate added, ranging from 1 to 15 mM, was studied by comparing the chromatographic peak area of ATP at every proportion. As shown in Fig. 6, the ATP peak area increased as the amount of ammonium acetate increased from 1 to 5 mM. However, a sharp drop was observed when more ammonium acetate was added. This is because too much additive would restrain the volatilization of the mobile phase in the ESI source, resulting in weak ionization. Finally, 5 mM of ammonium acetate was chosen in our study.

The pH would also influence the retention time and peak shape [19, 20]. The chemical structures of ATP and ADP were the most stable under neutral conditions; for this reason, the pH of the aqueous phase was adjusted to 7.4 by aqueous ammonia.

Optimization of MS Conditions

Flow injection analysis (FIA) was employed to determine the best MS source conditions, including capillary voltage, drying gas temperature, and drying gas flow. The result showed that the capillary voltage greatly influences the MS signal response. When it was decreased from 5,500 to 3,000 V, the signal response of ATP and ATP also demonstrated a significant increase, but when decreased to 2,500 V, the response dropped. Considering a polar compound, a faster drying gas speed, higher drying gas temperature, and stronger capillary voltage would be good for solvent vaporization as well as for ionization of molecules, so as to increase the response signal [21]. However, it has

been reported that the phosphate group of ATP and ADP would be easily broken under fierce physical effects [10], and their response signals are unstable. Thus, a mild MS source condition was applied for better stability and reproducibility.

Inhibition Kinetics and Study of the Anti-Cancer Mechanism of *Momordica cochinchinensis*

In our laboratory, the quality of the inhibition data was assessed by the Z' factor calculated by the following formula:

$$z' = 1 - \frac{3\sigma_s + 3\sigma_c}{|\mu_s - \mu_c|},$$

where μ_s and μ_c represent the mean of the signal of the standard (in the absence of the inhibitor) and on 100% inhibition, respectively, and σ_s and σ_c represent the standard deviations of the standard and on 100% inhibition, respectively [22]. A Z' factor value between 0.5 and 1 implies that the data quality is excellent for an assay method. In the case of MBZA, the Z' factor value was calculated as 0.72 (for 9 assays).

With these data, we constructed the IC_{50} curve using an extrapolation method, which is shown in Fig. 7. Further, the IC_{50} value was estimated to be 85 μg mL^{-1}, showing moderate intensity inhibitor kinetics. The MBZA came from the ethanol-soluble part of the *Momordica cochinchinensis*, which is a high polarity part; this indicated that some polar ingredients of *Momordica cochinchinensis* possessed certain hexokinase inhibitor activity, which might explain one possibility of anticancer mechanism of the *Momordica cochinchinensis*.

Conclusion

An LC–MS method was established for a hexokinase inhibitor study based on ATP determination. Our new method shows excellent specificity and sensitivity when compared to the commonly used analytical methods such as LC-UV and spectrophotometric methods in vivo. We used this method to determine the activity of 3-BrPA (a known inhibitor of hexokinase) and an extract of *Momordica cochinchinensis*. The calculation results for 3-BrPA matched with those in the relevant reports [14], and the *Momordica cochinchinensis* extract also showed inhibitory activity, which might indicate its anticancer mechanism.

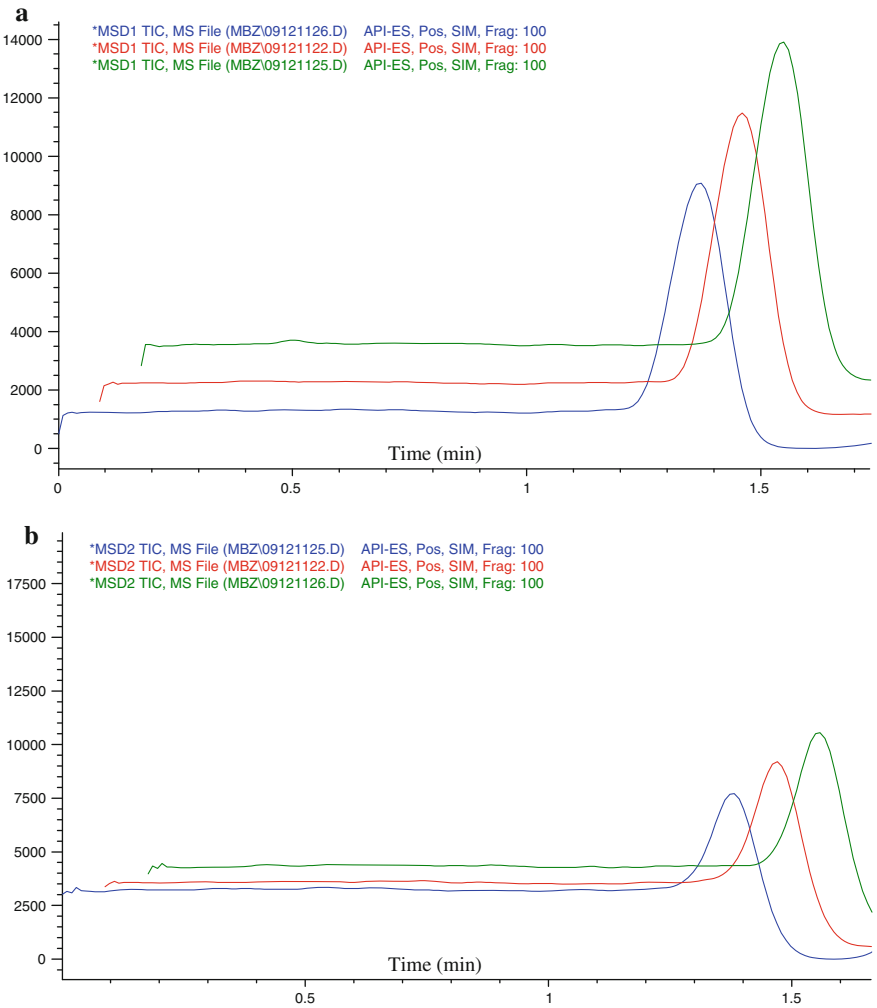

Fig. 3. Typical chromatogram for inhibitor assay of hexokinase. **a** for ATP, the upper (green) peak was for the blank sample, the middle (red) one was for the inhibitor, and the lower (blue) one was for the standard control sample (without any inhibitor). **b** For ADP, the upper (green) peak was for the standard control sample; the middle (red) one was for the inhibitor; and the lower (blue) one was for the blank sample. Colored peaks are shown in the on-line version

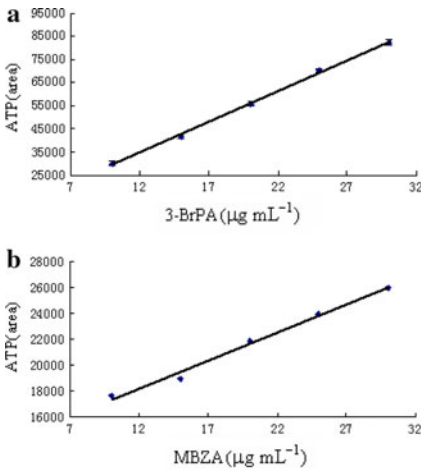

Fig. 4. Inhibition behavior of 3-BrPA (**a**) and MBZA (**b**)

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30873405). The authors wish to thank Mr. Hu Yingkang (Shanghai Baike Pharmaceuticals, Ltd) for technical contributions.

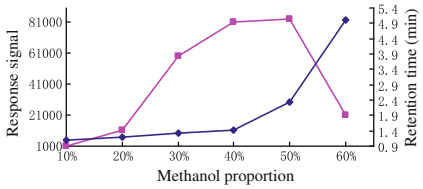

Fig. 5. The effect of methanol on retention time and response signal: the upper (pink) curve refers to response signal and the lower (blue) curve refers to retention time

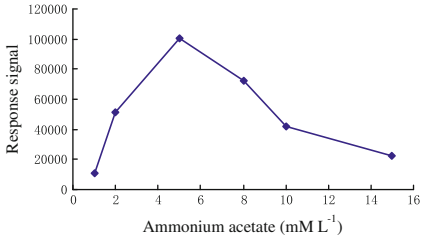

Fig. 6. The effect of ammonium acetate on response signal

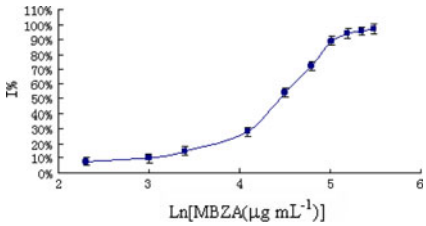

Fig. 7. The IC_{50} curve of MBZA

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