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Authors: Shenglan Qi, Huida Guan, Gang Deng, Tao Yang, Xuemei Cheng, Wei Liu, Ping Liu, Changhong Wang

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Rapid, reliable, and sensitive detection of adenosine deaminase activity by UHPLC-Q-Orbitrap HRMS and its application to inhibitory activity evaluation of traditional Chinese medicines

Shenglan Qi [a, 1], Huida Guan [a, 1], Gang Deng [a], Tao Yang [d], Xuemei Cheng [a, b], Wei Liu [c]*, Ping Liu [c]*, Changhong Wang [a, b] *

[a]*Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine; The MOE Key Laboratory for Standardization of Chinese Medicines and The SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, 1200 Cailun Rood, Shanghai 201203, China*

[b]*Shanghai R&D Centre for Standardization of Chinese Medicines, 199 Guoshoujing Road, Shanghai 201203, China*

[c]*Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 528 Zhangheng Road, Shanghai 201203, China*

[d]*Institute of Cardiovascular Disease, Shuguang Hospital Affiliated with Shanghai University of Traditional Chinese Medicine, 528 Zhangheng Road, Shanghai 201203, China*

[1]Shenglan Qi and Huida Guan contributed equally to this work.

*Correspondence to: Professor Chang-hong Wang, Ping Liu and Wei Liu, The Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China. Tel: 086-021-51322511, Fax: 086-021-51322519, E-mail: wchcxm@hotmail.com (C.H. Wang); Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China. liuliver@vip.sina.com (P. Liu); Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China. lwhzayl@163.com (W. Liu).

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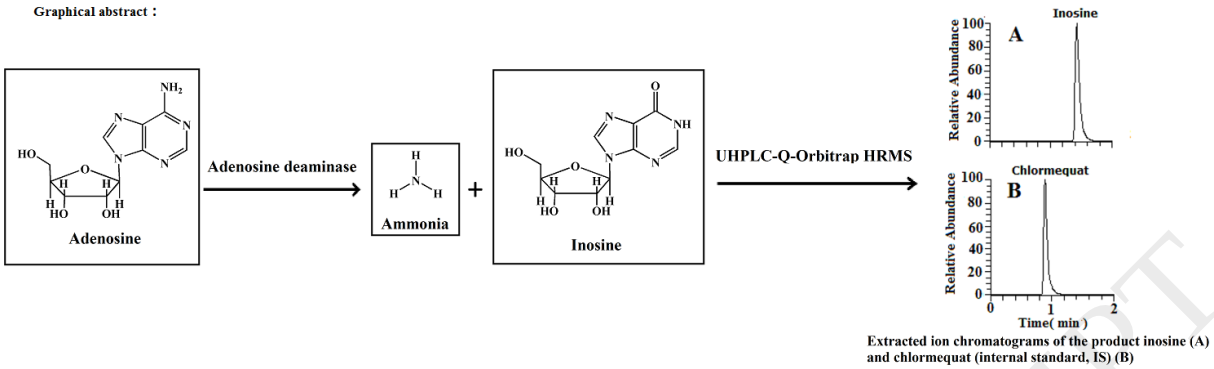
Graphical abstract



Extracted ion chromatograms of the product inosine (A) and chlormequat (internal standard, IS) (B)

Highlights

- ADA plays an important role in drug development and diseases detection
- An UHPLC-Q-Orbitrap HRMS method was developed to determine ADA activity.
- The assay is run within 2 min with acceptable sensitivity and repeatability.
- The method is economical for substrate and enzyme consumption.
- The method is successfully applied to detect inhibitive activity of herbal medicines.

Abstract

Adenosine deaminase (ADA), which is a key enzyme in the metabolism of purine nucleosides, plays important roles in diverse disorders, such as tuberculosis, diabetes, liver disorders, and cancer. Determination of the activities of ADA and its isoenzymes in body fluids has received considerable attention in the diagnosis and treatment of relative diseases. Ultraviolet spectroscopy with adenosine (AD) as a substrate is a classical approach for screening potential ADA inhibitors by measuring the decrease in substrate (AD) at 265 nm or increase in the product (inosine) at 248 nm. However, AD and inosine share a very close maximum absorption wavelength, and the reaction is uncertain and is frequently interfered by the background color of matrix compounds or plant extracts. Thus, the method usually yields false positive or negative results. In this study, a novel, rapid, sensitive,

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and accurate ultra-high-performance liquid chromatography-Q exactive hybrid quadrupole orbitrap high-resolution accurate mass spectrometric (UHPLC-Q-Orbitrap HRMS) method was developed for determining and screening ADA inhibitors by directly determining the deamination product of AD, inosine. A proper separation was achieved for inosine and chlormequat (internal standard) within 2 min via isocratic elution (0.1% formic acid:methanol = 85:15, v/v) at a flow rate of 0.3 $mL \cdot min^{-1}$ on a Waters ACQUITY HSS T3 column (2.1 mm × 100 mm, 1.8 μm) following a simple precipitation of proteins. The intra- and inter-day precisions of the developed method were below 7.17% and 8.99%, respectively. The method exhibited advantages of small total reaction volume (60 μL), short running time (2 min), high sensitivity (lowest limit of quantification of 0.02 μM for inosine), and low cost (small enzyme consumption of 0.007 $unit \cdot mL^{-1}$ for ADA and substrate of 3.74 μM for AD in individual inhibition), and no matrix effects (101.64%–107.12%). Stability results showed that all analytes were stable under the investigated conditions. The developed method was successfully applied to the detection of the inhibitory activity of ADA from traditional Chinese medicines.

Keywords: adenosine deaminase; inhibitor; UHPLC-Q-Orbitrap HRMS; traditional Chinese herbal medicine

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Introduction

Adenosine deaminase (adenosine aminohydrolase, EC3.5.4.4, ADA) plays a relevant role in intracellular and extracellular nucleotide metabolism and specifically participates in the irreversible hydrolytic deamination of adenosine (AD) and deoxyadenosine to produce inosine and 2'-deoxyinosine, respectively, and ammonia (Fig. 1) [1, 2]. Accumulating evidence indicates that dysfunctional ADA in human body is closely related to many important diseases, such as tuberculosis [3], diabetes [4, 5], liver disorders [6, 7], and cancer [8-11]. Given its significance in pathology, ADA is an important target for drug development and disease detection. Elevated ADA levels in the sera or tissue of cancer patients have important implications. For instance, cancer cells require high ADA activity to metabolize AD for maintaining their division, whereas high AD concentrations can inhibit cell division [12]. Therefore, an effective treatment strategy is to restrain the deamination function and to elevate the AD level by inhibiting ADA. 2'-deoxycoformycin (dCF), a potent inhibitor of ADA, has been officially approved as a drug by the FDA for the treatment of acute lymphoblastic leukemia [13]. However, the proposed inhibitors exhibit some disadvantages, such as irreversibility, side effects, high inhibition constant (*Ki*), and toxicity on different cells [14]. In recent years, natural sources have received growing attention, and most ADA inhibitors have been isolated from natural plants or structurally modified from natural compounds. Therefore, identification of ADA inhibitors as potential drug candidates has been a major topic in the field of drug discovery.

Ultraviolet (UV) spectroscopy is commonly used for screening potential ADA inhibitors [15]. In the UV method, the ADA reaction is calculated by measuring the decrease in absorbance of the substrate at 265 nm or increase in absorbance of the product at 248 nm. However, AD and inosine share a very close maximum absorption wavelength. Thus, exact evaluation of the product formation at the usually selected wavelength is difficult. In addition, the reaction is uncertain and is frequently interfered by the background color of compounds

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or plant extracts. The use of the UV method to screen potential ADA inhibitors usually yields false positive or negatives results. To overcome these drawbacks, many methods, such as radiative method [16], Berthelot method [17], capillary electrophoresis (CE) [18], high-performance liquid chromatography (HPLC) [19], and aptasensor method [20], have been developed for screening potential ADA inhibitors. However, these methods still exhibit some shortcomings. The radioactive method is effective but is inconvenient to use and is environmentally toxic [16]. The Berthelot method measures the amount of liberated NH_3 but suffers from lack of accuracy due to the interference of exogenous NH_3 [17]. Given its high efficiency separations and short analysis times, CE has been widely used to evaluate ADA activity. However, the measuring sensitivity of the method is very low [18]. HPLC is also applied in ADA analysis to separate the substrate and the product. Despite its superior repeatability, it requires intensive sample pretreatment and long running time [19]. The aptasensor approach allows for exclusive analysis of the substrate AD and indirect detection of ADA activity. Despite its low detection limit, this method requires many labeled molecules and relevant testing instruments, which result in high costs and limitation for the general laboratory without these conditions [20]. Therefore, convenient and sensitive methods for the assay of ADA activity should be developed. UPLC-MS with high sensitivity and selectivity is especially applicable for detecting enzyme activities [21]. Orbitrap is a new developed mass analyzer in high-resolution mass spectrometry (HRMS). It promises high mass resolution and mass accuracy, wide dynamic range, and good duty cycle and sensitivity [22]. Therefore, this work developed a selective, convenient, quick, and sensitive ultra-high-performance liquid chromatography-Q exactive hybrid quadrupole orbitrap high-resolution accurate mass spectrometric (UHPLC-Q-Orbitrap HRMS) method for detecting and screening ADA inhibitors. The direct product (inosine) from the selective substrate (AD) catalyzed by ADA in the incubation was quantitated. The method not only involves the classic principle of ADA activity assay *in vitro* but also performs rapid and sensitive screening of inhibitors and avoids the interferences of the matrix effects as much as possible.

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2. Experimental

2.1. Chemicals and reagents

ADA, AD, inosine, chlormequat, and erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (EHNA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2'-dCF was purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, Liaoning China). The purities of all reference standards were over 98%. HPLC-grade methanol was obtained from Fisher Co. (Santa Clara, CA, USA). Formic acid was obtained from Tedia Inc. (Fairfield, OH, USA). Ultra-pure water (18.2 MΩ) was purified using a Milli-Q Academic System (Millipore, Billerica, USA). All other reagents were of analytical reagent grade.

2.2. Materials

The collected traditional Chinese herbal medicines were purchased from local medicinal markets in Shanghai in 2017 (Supplementary materials Table S1). The authentication of herbal medicines was confirmed by Professor Lihong Wu, and the voucher specimens were deposited at the Herbarium of the Shanghai R&D Center for Standardization of Traditional Chinese Medicine (Shanghai, China).

2.3. Preparation of extracts

The extracts of 26 traditional Chinese herbal medicines were extracted by methanol for inhibitor screening of ADA. In brief, air-dried Chinese herbal medicine was ground into fine powder in a grinder. Then, 2 g of Chinese herbal medicine powder was poured into a 100 mL stoppered Erlenmeyer flask, which was then added with 50 mL of methanol. The flask sealed with Parafilm (Parafilm, Chicago, IL, USA) was placed into an ultrasonic cleaner for ultrasonic extraction of powder for 1 h. The supernatant was acquired by centrifugation at 2300 × *g* for 10 min and then evaporated to dryness on vacuum. The dried extract was stored in a closed desiccator for inhibitor screening.

2.4. Standard stock solutions and quality control samples

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The primary stock solutions of the product (inosine) and internal standard (IS, chlormequat) were prepared by dissolving the appropriate amount of each standard substance in 10 mL of methanol to obtain 3.73 and 6.33 mM, respectively. The stock solutions were prepared separately for calibration curves and quality control (QC) samples by serially diluting with the initial mobile phase (0.1% formic acid:methanol = 85:15, v/v) to provide working solutions of desired concentrations. The IS working solution (1.26 μM) was prepared by diluting the stock solutions with methanol. All solutions were stored at 4 °C and brought to room temperature before use.

2.5. Sample preparation procedure

ADA at 2.3 unit·mL^{-1} was prepared by dissolving in 20 mM sodium phosphate buffer (pH 7.6) to make stock solution, and the solution was stored at −80 °C before use. The substrate AD was dissolved in 20 mM sodium phosphate buffer (pH 7.6) to achieve a concentration of 3.75 mM.

The stock solutions of the Chinese medicine extract were prepared by dissolving an adequate quantity of each extract (obtained in Section 2.3) in 10% DMSO to obtain 100 mg·mL^{-1} solutions (calculated by the amount of crude herb). The stock solutions of the tested positive compounds (EHNA and 2'-dCF) were prepared by dissolving an adequate quantity of each compound in DMSO to obtain 10 mg·mL^{-1} solutions. All the stock solutions were diluted to a series of concentrations with a 20 mM sodium phosphate buffer solution (pH 7.6) before each experiment. All solutions were filtrated through a 0.22 μm membrane filter before use.

2.6. Chromatography conditions for UHPLC-Q-Orbitrap HRMS

Chromatographic separation was performed on a UHPLC-Q-Orbitrap system (Thermo Fisher Scientific Inc., Grand Island, NY, USA). The UHPLC system consisted of a Thermo Scientific Dionex Ultimate 3000 Series RS pump coupled with a Thermo Scientific Dionex Ultimate 3000 Series TCC-3000RS column compartments and WPS-3000 autosampler controlled by Chromeleon 7.2 Software. The cooling autosampler was set at 4 °C and protected from light, and the column heater was set at 40 °C. A Waters ACQUITY UPLC HSS T3 column (2.1 ×

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100 mm, 1.8 μm) was employed with the temperature set at 40 °C. The mobile phase consisted of A (methanol) and B (0.1% formic acid) at a flow rate of 0.3 $mL \cdot min^{-1}$ with isocratic elution of 15% A and 85% B. The injection volume was 5 μL.

The mass spectrometer Q-Orbitrap system was connected to the UHPLC system via heated electrospray ionization and controlled by Xcalibur 4.1 software that was used for data capture and analysis. The electrospray ionization source was operated and optimized in positive ionization mode. The optimized parameters of mass spectrometry were as follows: capillary temperature, 320 °C; sheath gas (N_2) flow rate, 40 arbitrary units; auxiliary gas (N_2) flow rate, 10 arbitrary units; sweep gas flow rate, 0 arbitrary units; spray voltage, 3.50 kV; S-lens RF level, 50 V; auxiliary gas heater temperature, 300 °C; scan mode, full MS; scan range, 50–500 m/z; maximum injection time, 200 ms; scan resolution, 70,000 FWHM (m/z/s); and automatic gain control target, $1.0e^6$. The ions of the product inosine (m/z 269.0880) and the IS (m/z 122.0733) were extracted for quantitative and qualitative analyses.

2.7. Method validation

The developed UHPLC-Q-Orbitrap HRMS method was validated for selectivity, calibration curve, lowest limit of quantification (LLOQ), accuracy, precision (inter- and intra-day precisions), matrix effects, and stability in accordance with the European Medicines Agency's guideline on bioanalytical method validation and the literature [23].

Selectivity was confirmed by comparing chromatograms of the blank matrix (0.00736 $unit \cdot mL^{-1}$ of ADA was prepared by dissolving in 20 mM of sodium phosphate buffer) pretreated with methanol (containing IS) against the matrix added to inosine and the IS. The standard curve was obtained by plotting the peak area ratio of the product to the IS (Y) and applying a weighted ($1/y^2$) least-squares linear regression analysis. The standard solution of inosine was diluted to seven concentrations with the buffer solution ranging from 0.02 μM to 11.93

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μM to evaluate the linear relationship of the method. The lowest concentration of inosine threefold the signal-to-noise ratio was used as the limit of detection (LOD). The LLOQ was defined as the lowest concentration giving a signal-to-noise ratio at least 10-fold on the calibration curve for which acceptable accuracy (within 80%–120%) and precision (< 20%) were confirmed.

The precisions of the assay were appraised by performing replicate analysis of the QC samples (n = 5) at five levels (0.02, 0.05, 0.76, 1.91, and 11.93 μM). Intra-day precision was assessed by repeating the analysis of the QC samples three times a day, whereas inter-day precision was evaluated by repeating the analysis on three subsequent days. Relative standard deviation (RSD) was used to determine the precision value calculated from the observed concentrations (C_{obs}) in accordance with the following equation: %RSD = [standard deviation (SD)/C_{obs}] × 100. Accuracy was expressed by the recovery of the QC samples at five levels (0.02, 0.05, 0.76, 1.91, and 11.93 μM, n=5). The apparent concentrations of these samples were calculated using the calibration curves. The recovery rates (%) were calculated from the mean value of the observed concentrations (C_{obs}) and the theoretical concentrations (C_{the}) by using the following equation: % = [C_{obs}/C_{the}] × 100. The matrix effects were determined by comparing the peak responses of the standard QC samples at three levels (0.02, 0.76, and 11.93 μM, n = 5) dissolved in the blank matrix pretreated with methanol (A) against those dissolved in the initial mobile phase (B, n = 5). The mean value (A)/(B) × 100 was considered the matrix effect, which is acceptable when the ratio is more than 85% and less than 115%.

The stability of inosine was conducted on QC samples at three concentration levels (0.02, 0.76, and 11.93 μM) with five replicates after keeping in the autosampler (4 °C) or refrigerator (4 °C) for 3 days and at ambient temperature (25 ± 2 °C) for 1 day. The measured concentrations were then compared with those of freshly prepared QC samples. RSD was used to determine the stability value calculated from the observed concentrations (C_{obs}) in accordance with the following equation: %RSD = [standard deviation (SD)/C_{obs}] × 100.

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2.8. In vitro anti-adenosine deaminase assay

2.8.1 UHPLC-Q-Orbitrap HRMS

ADA activity assay was carried out to rapidly screen by UHPLC-Q-Orbitrap HRMS using AD as the substrate under the optimal incubation conditions. The extract of individual traditional Chinese medicines was tested in concentration ranges between 0.01 and 10 mg·mL^{-1}. Positive compounds (EHNA and 2'-dCF) were tested in concentration ranges between 0.01 and 10000.0 ng·mL^{-1}. A 60 μL incubation system composed of a 6 μL solution of the tested traditional Chinese medicine extract and 34 μL of the enzyme solution (final concentration of 0.00736 unit·mL^{-1} for ADA) was mixed and pre-incubated for 10 min in a 1.5 mL centrifuge tube. Thereafter, 20 μL of the substrate solution (final concentration of 5.50 μM for AD) was added and incubated for 20 min at 30 °C. The reaction was terminated by immediately adding 180 μL of ice methanol solution (0 °C) containing 3.29 μM IS. The reaction system was diluted by adding 960 μL of ultra-pure water. The tube was tightly capped and vigorously mixed for 0.5 min using a vortex (Scientific Industries, New York, USA) mixer at maximum speed. The solution was then centrifuged (15,000 × *g*, 10 min), and the supernatant was used for the analysis. The inhibitory activity of the analyte was calculated in accordance with the production of inosine. The formula of inhibition rate was as follows: Inhibition % = $(C_{control}-C_{sample})/C_{control}\times100$. $C_{control}$ and C_{sample} represent the production of inosine without and with the test sample, respectively. The assay was carried out in triplicate. The inhibitory IC_{50} values of the traditional Chinese medicines on ADA were calculated using the Prism software (GraphPad Software Inc., San Diego, CA).

2.8.2 Ultraviolet spectroscopy method

The ADA activity assay by the UV method was performed as previously described [24] with slight modifications. The extract of individual traditional Chinese medicines was tested in concentration ranges between 0.01 and 10 mg·mL^{-1}. Positive compounds (EHNA and 2'-dCF) were tested in concentration ranges

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between 0.01 and 10000.0 ng·mL^{-1}. A 150 μL incubation system consisting of 50 μL of the tested traditional Chinese medicine extract solution and 50 μL of the enzyme solutions (final concentration of 0.38 unit·mL^{-1} for ADA) was mixed and then pre-incubated for 10 min in a 96-well UV plate (UV transparent flat bottom, Corning 3635). Then, 50 μL of the substrate solution (final concentration of 0.50 mM for AD) was added. The 96-well UV plate was immediately placed in a Powerwave XS microplate spectrophotometer reader (Bio Tek Instruments, Winooski, VT, USA). The absorbance was measured at 265 nm every 10 s for 60 s. The straight line was drawn with time as the abscissa and light absorption value as the ordinate. The inhibitory activity of the compound was calculated in accordance with the change in slope of the linear equation. The formula of inhibition rate was as follows: Inhibition % = $(K_{control}-K_{sample})/K_{control} \times 100$. $K_{control}$ and K_{sample} represent the slope of the linear equation without and with the test sample, respectively. The assay was carried out in six replicates. The inhibitory IC_{50} values of the traditional Chinese medicines on ADA were calculated using the Prism software (GraphPad Software Inc., San Diego, CA).

2.9. Data analysis

All tabulated results were shown as mean ± SD. The IC_{50} values and the enzyme kinetic parameters (K_m and V_{max}) were all calculated from the concentration–response curves via nonlinear regression analysis using the Prism software.

3. Results and discussion

3.1. UHPLC-Q-Orbitrap HRMS conditions

To achieve suitable retention times, good peak shapes, and high MS sensitivity of inosine and the IS, various mobile phase conditions were studied. Methanol or acetonitrile as organic phase and water as polar phase with different concentrations of ammonium formate, ammonium acetate (from 1 mM to 10 mM), and acetic and formic acid (from 0.05 to 0.5%) were examined. Finally, methanol–water containing 0.1% formic acid was

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employed as the mobile phase to obtain good peak shapes for inosine and the IS. Moreover, an optimal isocratic elution and 0.3 $mL \cdot min^{-1}$ flow rate provided the improved peak shape of inosine and the IS with the column temperature set at 40 °C. At the aforementioned UHPLC conditions, the baseline separation of inosine and the IS was achieved on an HSS T3 column, and both were eluted at 1.53 and 0.95 min, respectively (Fig. 2B). The total analysis time for a single run was completed within 2 min.

The molecular weight of inosine ($C_{10}H_{12}N_4O_5$, M.W. 268.23) was approximately 1.0 Da greater than that of AD ($C_{10}H_{13}N_5O_4$, M.W. 267.24). When inosine and AD did not reach baseline separation, the interference peak of AD isotope was found in the extracted ion chromatogram of inosine by the low-resolution model (Supplementary materials Fig. S1A). The challenge, however, could be overcome by high-resolution MS perfectly. It can provide ion spectra with accurate mass measurement. The interference peak of AD isotope was undetected in the extracted ion chromatogram of inosine (Supplementary materials Fig. S1B). Therefore, a proper separation was achieved for inosine and the IS within 2 min via simple isocratic elution, which ignored the interference peak of AD isotope by UHPLC-Q-Orbitrap HRMS. However, the complex gradient elution conditions needed at least 6 min to achieve the proper separation of AD, inosine, and IS by UHPLC coupled with low-resolution MS (data not shown).

3.2. Method validation

The chromatographic separation is shown in Fig. 2. No significant interfering peaks were observed at the retention times of either analytes or internal standard in the blank matrix. The calibration curve showed a good linear behavior over the inosine concentration range of 0.02–11.93 μM ($y = 0.312x + 0.00603$, $R^2 = 0.9951$, weighed: $1/y^2$). The LOD (S/N = 3) of inosine was 0.013 μM, and the LLOQ (S/N = 10) was 0.02 μM for the product. The LLOQ is appropriate for the quantitative detection of inosine in the enzymatic studies.

The results of intra- and inter-day precisions, accuracy, matrix effect, and stabilities of the method are

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summarized in Table 1. The RSD values of intra- and inter-day precisions ranged from 2.14% to 7.17% and from 2.55% to 8.99%, respectively. The average recoveries of inosine at five concentrations ranged from 85.69% ± 1.13% to 111.3% ± 0.85%. These results suggested that the method exhibited satisfactory precision and accuracy. The matrix effects for inosine ranged from 101.64% ± 3.24% to 107.12% ± 7.05%. The data suggested that no ionization enhancement or suppression was observed for inosine under the present conditions. Inosine was stable after being placed at room temperature for 24 h and kept in the autosampler or refrigerator (4 °C) for 3 days.

3.3. Influence of temperature on the enzymatic reaction

The effect of temperature on the enzymatic reaction was studied by varying the temperature in the range of 20 °C–45 °C (Fig. 3A). No obvious influence of temperature from 30 °C to 45 °C was observed on ADA reaction. Therefore, the temperature for enzymatic reaction was selected at 30 °C.

3.4. Optimization of reaction time, substrate concentration, and ADA concentration

The enzyme concentration, substrate concentration, and reaction time of the assay should be appropriate prior to its development. To ensure the utilization of the initial rate velocities for the calculation of enzyme constants in accordance with steady-state kinetics, the optimum reaction time, substrate concentration, and ADA concentration should be within the linear region of the formation of the product. Three reaction functions were generated by monitoring the product as functions of reaction time, substrate concentration, and enzyme concentration. The reaction time was set as 20 min for the ADA assay (Fig. 3B). The formation of inosine showed good linear equation of $y = 0.186x + 0.779$ ($R^2 = 0.987$) for ADA assay (Fig. 3C) when the substrate concentration of AD ranged from 1.87 μM to 37.42 μM. Thus, the substrate concentration of AD was selected at 3.74 μM. The formation of inosine showed a good linear equation of $y = 1325.272x + 1.321$ ($R^2 = 0.946$) for ADA assay (Fig. 3D) when the enzyme concentration of ADA ranged from 0.0005 unit·mL^{-1} to 0.018 unit·mL^{-1}. Accordingly, the enzyme concentration was selected at 0.007 unit·mL^{-1}. The product inosine could be quantified

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sensitively, and even 99% of ADA enzyme activity was inhibited.

3.5. Michaelis constant (K_m) of ADA

From Supplementary materials Fig. S2, the linear Eadie–Hofstee plots (II) were found to exhibit Michaelis–Menten kinetics for ADA-catalyzed enzyme reaction. The values of K_m and V_{max} for ADA were 81.70 ± 13.82 μM and 779.2 ± 43.99 μM·min^{-1}·unit^{-1}, respectively. The values of K_m ranged from 25 μM to 550 μM in previous reports because K_m varies with the reaction conditions, analytical methods, and enzyme sources used [25-28]. The value of K_m in this assay is highly consistent with that obtained from the UV method (89 μM), which was used under similar reaction conditions (such as substrate, reaction temperature, and pH of buffer) [28]. This result indicated no significant change in the enzymatic property of the optimized incubation system.

3.6. Detection of inhibitory activity of traditional Chinese medicines against enzymes

The ADA inhibitory activities of traditional Chinese medicines and model compounds were evaluated *in vitro* by using the UHPLC-Q-Orbitrap HRMS and UV methods. As shown in Table 2, EHNA and 2'-dCF exhibited exceedingly strong inhibition on ADA, which was in good agreement with previous reports [29, 30]. Among the tested traditional Chinese medicines, 13 exhibited different potential inhibition activities on ADA. *Ligustrum lucidum* and *Rheum palmatum* showed strong inhibitory effects with IC_{50} values below 1 mg·mL^{-1} on ADA, and *Salvia chinensis* showed exceedingly strong inhibitory effects with IC_{50} values below 0.1 mg·mL^{-1} on ADA.

To interpret the correlation of the two methods, a bivariate correlation analysis for the IC_{50} values obtained from the UHPLC-Q-Orbitrap HRMS and UV methods was conducted (Supplementary materials Fig. S3). The IC_{50} values of the model compounds (EHNA and 2'-dCF) and 13 traditional Chinese medicines obtained from the two methods (Table 2) were used in the correlation analysis. A significant linear correlation was found between the UHPLC-Q-Orbitrap HRMS and UV methods with correlation factors (r) of 0.9654 (p< 0.001) and the linear equation of y = 1.163x + 0.0963 (R^2 = 0.9321). This result indicated the reliability of the newly

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established UHPLC-Q-Orbitrap HRMS method. Enzymatic reaction was a dynamic process, and the UV method did not end the reaction immediately. Thus, the standard deviations (SD) determined by the UV method were generally higher than that by the UHPLC-Q-Orbitrap HRMS method (Table 2). Therefore, the repeatability of the UV method was lower than that of the UHPLC-Q-Orbitrap HRMS method. In addition, the enzyme and substrate concentrations used in the UV method (ADA: 0.38 unit·mL^{-1}, AD: 0.50 mM) were several folds higher than those in the UHPLC-Q-Orbitrap HRMS method (ADA: 0.00736 unit·mL^{-1}, AD: 5.50 μM). In UHPLC-Q-Orbitrap HRMS, the potential instability of the dynamic enzymatic reaction was avoided (the enzymatic reaction was terminated by methanol) along with the interference of the background color of compounds or plant extracts. Therefore, the newly developed UHPLC-Q-Orbitrap HRMS method is sensitive and reliable and can be used to avoid false negative results.

4. Conclusion

A UHPLC-Q-Orbitrap HRMS method was developed using AD as the substrate for determining the activity of ADA inhibitors. The method achieved a proper separation for the product inosine and the IS within 2 min by isocratic elution on an efficient HSS T3 column without any matrix effect. The method is fast, sensitive, accurate, and repeatable. It also exhibits smaller total reaction volume, shorter running time, higher sensitivity, and lower cost than the conventional methods. Thus, this method is suitable for the high-throughput screening of potential ADA inhibitors from natural medicinal plants.

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of this study.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. The irreversible hydrolytic deamination of adenosine (AD) and deoxyadenosine to produce inosine and 2'-deoxyinosine by adenosine deaminase.

Fig. 2. Extracted ion chromatograms of the product inosine and chlormequat (internal standard, IS), A: a blank matrix spiked with IS; B: a blank matrix spiked with the analyte and IS.

Fig. 3. Influence of temperature (A), incubation time (B), substrate concentration (C) and ADA concentration (D) on the production of inosine converted from adenosine.

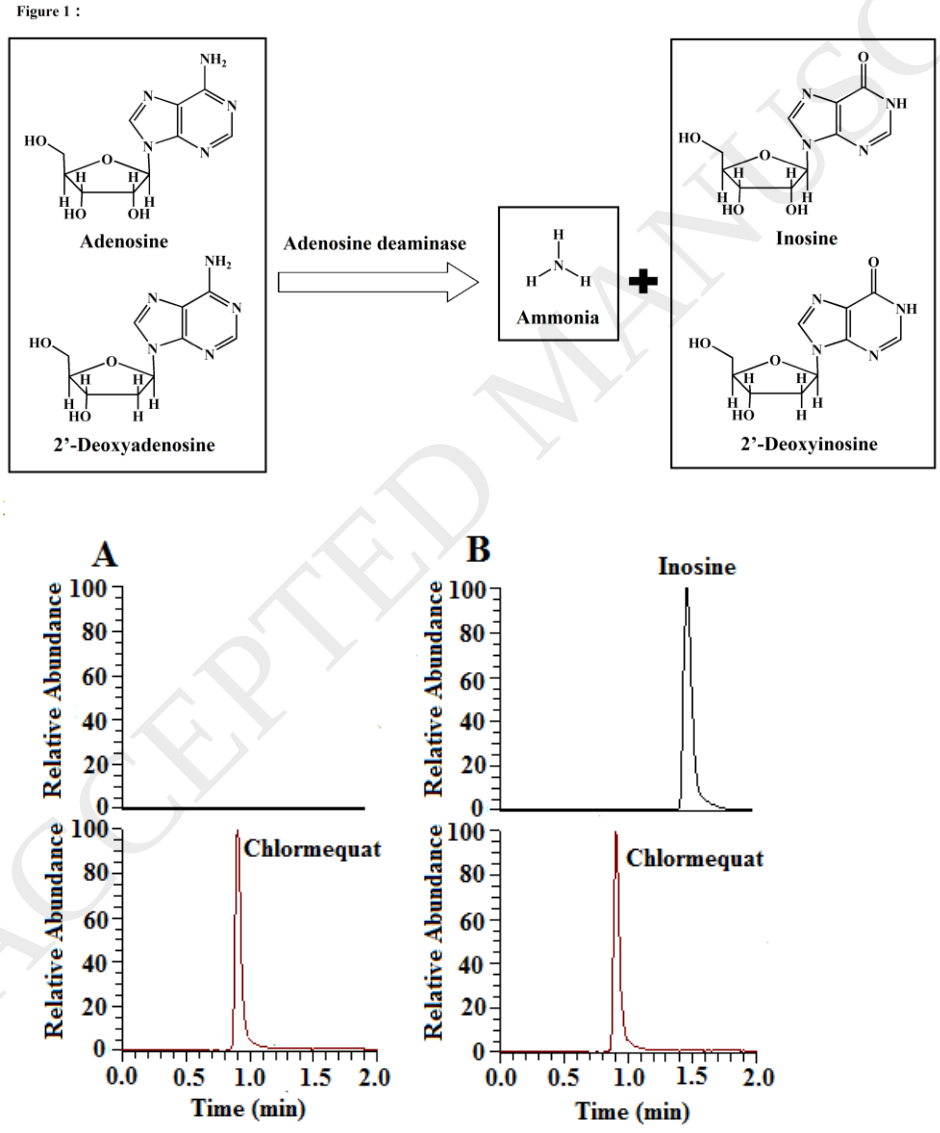

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Figure 3:

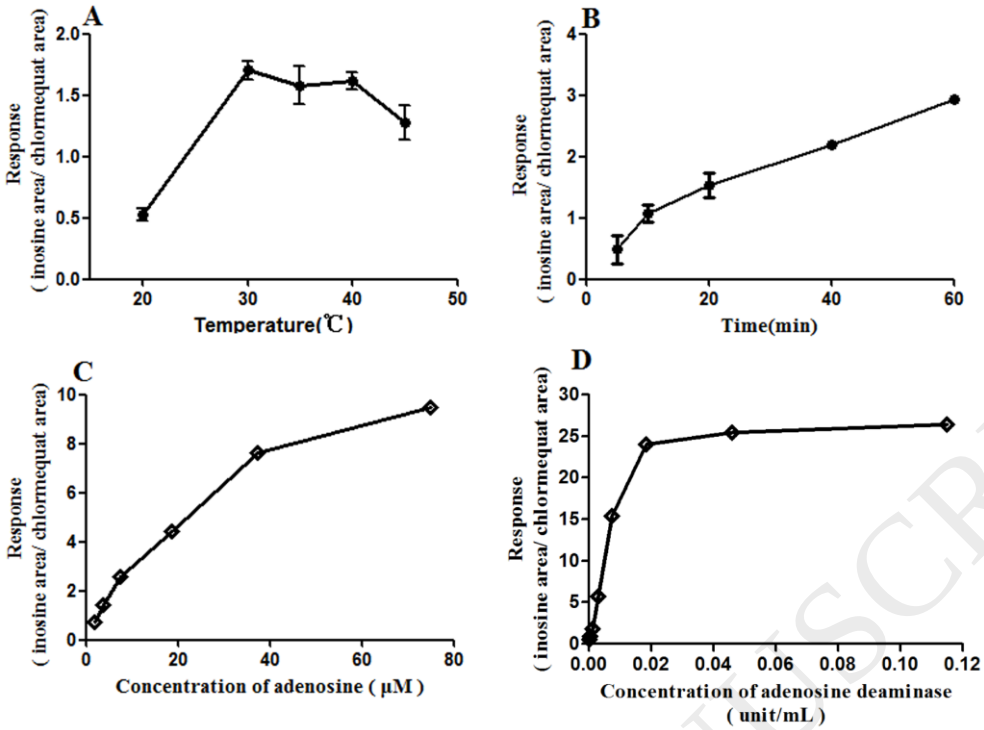

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Table 1. Results of method validation for the UHPLC-Q-Orbitrap HRMS analysis of inosine.

Nominal level (μM)	Measured concentration	Accuracy (%)	Precision (RSD%)		Matrix effect (%)	Stability (RSD%)	
	(μM)		Intra-day	Inter-day		(4℃)	(RT)
0.02 (LLOQ)	0.018±0.0004	89.17±2.04	7.17	8.99	107.12±7.05	7.64	8.36
0.05 (QCL)	0.048±0.0034	97.27±6.86	5.23	6.54	/	/	/
0.76 (QCM)	0.85±0.0065	111.30±0.85	2.23	3.29	101.64±3.24	2.31	3.02
1.91 (QCH)	2.09±0.04	109.33±1.99	3.61	5.36	/	/	/
11.93 (ULOQ)	10.23±0.13	85.69±1.13	2.14	2.55	103.00±2.68	2.27	3.13

RT: Room Temperature

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Table 2. Inhibitory activities (IC_{50}) of the tested traditional Chinese medicines and positive compounds investigated against ADA by UHPLC-Q-Orbitrap HRMS and UV methods (mean ±SD).

Natural extract/Compounds	IC_{50} for ADA ($mg \cdot mL^{-1}$)	
	UHPLC-Q-Orbitrap HRMS (n=3)	UV (n=6)
Bupleurum chinense DC.	2.27±0.04	2.56±0.04
Ligusticum chuanxiong Hort.	1.76±0.03	2.65±0.13
Angelica sinensis (Oliv.) Diels.	1.85±0.01	1.20±0.14
Sophora flavescens Ait.	4.38±0.07	5.33±0.07
Artemisia capillaris Thun.	2.96±0.02	3.55±0.11
Artemisia argyi LevL. et Vant.	1.65±0.03	2.48±0.12
Atractylodes macrocephala Koidz.	2.32±0.03	3.02±0.12
Isatis indigotica Fort.	1.41±0.004	1.50±0.04
Ligustrum lucidum Ait .	0.72±0.007	1.37±0.04
Schisandra chinensis (Turcz.) Baill.	0.05±0.003	0.13±0.003
Gardenia jasminoides Ellis	2.02±0.02	2.66±0.09
Rheum palmatum L.	0.57±0.006	0.78±0.01
Cordyceps sinensis (Berk.) Sacc.	2.67±0.01	2.86±0.05
Sophora tonkinensis Gagnep.	＞10	＞10
Polyporus umbellatus (Pers.)Frie.	＞10	＞10
Isatis indigotica Fort.	＞10	＞10
Prunus persica(L.) Batsch	＞10	＞10
Gynostemma pentaphyllum (Thunb.) Makino.	＞10	＞10
Paeonia lactiflora Pall.	＞10	＞10
Salvia miltiorrhiza Bge.	＞10	＞10
Stephania tetrandra S. Moore	＞10	＞10
Coptis chinensis Franch.	＞10	＞10
Astragalus membranaceus (Fisch.) Bge.	＞10	＞10
Curcuma Longa L	＞10	＞10
Eupolyphaga sinensis Walker	＞10	＞10
Glycyrrhiza uralensis Fisch.	＞10	＞10
EHNA	$6.47E^{-05}$±$5.77E^{-07}$	$7.12E^{-05}$±$3.07E^{-06}$
2’-dCF	$1.14E^{-07}$±$7.28E^{-09}$	$2.05E^{-07}$±$1.41E^{-08}$