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Merits of HPLC-based method over spectrophotometric method for assessing the kinetics and inhibition of mammalian adenosine deaminase

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Abstract

Measurement of adenosine deaminase (ADA) activity using spectrophotometric method presents problem, regarding the quantitative estimation of the substrate degradation and product formation, due to the closely apposed λ_{max} of the substrates, product and the inhibitor. The feasibility of applying reverse-phase HPLC technique, for studying adenosine deaminase-catalyzed reaction product and inhibition study was examined. We have drawn a comparison between the HPLC-based method over the corresponding spectrophotometric method. A gradient elution pattern was used to separate substrate (adenosine and deoxyadenosine), product (inosine and deoxyinosine) and standard adenosine deaminase inhibitor (erythro-9-(3-nonyl-ρ-aminobenzyl)-adenine) in the HPLC method. The product formation was quantitated by monitoring the absorbance at 260 nm with the progress of time. The limit of detection as well as the limit of quantification of the respective enzymatic product were found to be in nano molar (nM) range in the HPLC method. This study was also extended to monitor adenosine deaminase activity in different cancer cells of hematological origin. The HPLC-based method is found to be suitable for the quantitative estimation of adenosine deaminase-catalyzed reaction product and for studying inhibition mechanism of different inhibitors. The HPLC-based method has specific advantages over the spectrophotometric method. Moreover, the concentration of different nucleotides in cell lysate and body fluid can be measured using this HPLC method.

Keywords: Reverse-phase HPLC; Spectrophotometric-enzyme kinetics; Adenosine deaminase; Adenosine; Inosine; EHNA

1. Introduction

Enzymes like adenosine deaminase (ADA) and hypoxanthine guanine phosphoribosyl transferase (HGPRT), responsible for the purine metabolism (catabolism or anabolism) are important targets for cancer chemotherapy [1]. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) is an important zinc containing metallo-enzyme of considerable physiological importance and is essential for the proliferation and differentiation of lymphoid cells [2]. ADA catalyzes the irreversible deamination of adenosine and deoxyadenosine to produce inosine and deoxyinosine, respectively (Scheme 1) [3]. The physiological function of ADA is critical in controlling the effect of adenosine and deoxyadenosine in a variety of tissues [4]. High level of ADA is present in different tissues such as thymus, lymphoid tissues and peripheral lymphocytes [5].

On the other hand, severe combined immunodeficiency disease (SCID) is characterized by chronic ADA deficiency, leading to the accumulation of ADA substrates that exert lymphotoxic effect either directly or after conversion to phosphorylated derivatives like dATP, dADP and dAMP [6]. Hence inhibition of ADA may be quite effective in inducing cell death in human lymphoid malignancies, as lymphocytes lack 5′-nucleotidase, which provide protection to other non-lymphocytic cells by metabolizing ADA substrates [7]. Standard ADA inhibitors like 2′deoxycoformycin (DCF) and erythro-9-(3-nonyl-ρ-aminobenzyl-adenine (EHNA) are already in the clinical trials against lymphoid malignancies [8,9]. ADA inhibitors have also been used as co-drugs in combination with adenine nucleoside [10].

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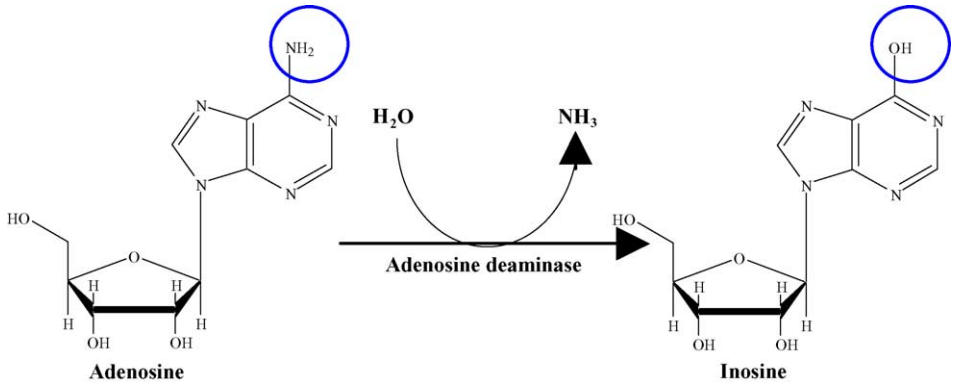

Scheme 1. Adenosine deaminase-catalyzed reaction scheme.

Moreover, ADA inhibition has been reported to prevent free radical generation and contractyle dysfunction in the postischemic heart [11]. Elevated ADA activity has also been reported in pleural effusion from patients with pleural tuberculosis and is considered to be an useful diagnostic marker in the evaluation of mycobacterial infection [12].

Unfortunately, the kinetic assays and inhibition characterization of ADA are difficult to evaluate using spectrophotometric method, as the substrates, products and even the inhibitors share almost identical λ_{max}. Therefore proper monitoring of substrate degradation with concomitant product formation are difficult to measure quantitatively. Generally, the adenosine deaminase reaction is assayed spectrophotometrically by measuring the decrease in absorbance at 265 nm (decrease in adenosine concentration) or increase in absorbance at 235 nm (increased formation of inosine) [13,14]. Moreover, in cell lysates or in body fluids the increase in inosine formation cannot be properly studied due to non-specific contributions. In our spectrophotometric experiments we have found that adenosine and inosine have very closely apposed λ_{max}. Hence spectrophotometrically, it is very difficult to quantitatively evaluate the product formation at their respective absorbance maxima or at the usually selected wavelengths of (265 or 235 nm) as both adenosine and inosine contribute at these respective wavelengths. ADA activity is also followed through the formation of ^{14}C inosine from ^{14}C adenosine [15]. Although the radioactive method is effective but it is inconvenient to use and are environmentally toxic. ADA activity is also followed spectrophotometrically by measuring the NH_3 liberated using the Berthelot method. This method suffers from non-specific contribution and lack of accuracy due to bleaching of the chromogen [16]. Recently, capillary zone electrophoresis has been used to study ADA activity, but capillary zone electrophoresis itself has many limitations [17]. Hence, ADA activity determination, for the purpose of studying kinetic, mechanism of action and inhibition studies possesses significant challenge, demanding an effective and improved assay method.

We hypothesized that the polarity difference of the substrate, product and inhibitors may be exploited in order to study adenosine deaminase kinetics and inhibition. Though HPLC method has been used to separate and quantify purine nucleotides in body fluids [18,19], but to the best of our knowledge, it has not been reported to study enzyme kinetics and inhibition studies of ADA earlier. Keeping in view the discrepancies of the spectrophotometric method the purpose of this work was to develop a simple, efficient, single step HPLC-based method to study the inhibition and activity of ADA with pure enzyme system, as well as in cell culture and in body fluids. In light of the fact, that adenosine deaminase is a target enzyme, for the different types of lymphocytic disorder, development of such kind of HPLC-based method was urgently required.

2. Experimental

2.1. Materials

Standard adenosine, inosine, and the standard ADA inhibitor, EHNA were purchased from Sigma (St. Louis, MO, USA). 2′3′ deoxyadenosine was purchased from Calbiochem (La Jolla, CA, USA). ADA (high purity grade) from calf intestine (approximately 200 U/mg protein) (in 50% glycerol and 5 mM potassium phosphate, pH 6.0) was purchased from Sigma chemicals. The purity of the enzyme was further studied by SDS-PAGE, which demonstrated a single band of $M_r \cong 50{,}000$. Mobile phase components, methanol, dipotassium hydrogen phosphate and potassium dihydrogen phosphate for HPLC were purchased from Merck Inc. Ltd (Germany).

Hut78, a T cell lymphoma cell line was purchased from National Centre for Cell Science (NCCS) (Pune, India) and was maintained in RPMI-1640 as per the suggested growth conditions.

2.2. Instrumentation

A Shimadzu Inc (Japan) HPLC system was used for our experiments, attached with UV detector. The chromatographic separations were carried out on a 10 μm Supelco C18 RP (250 mm × 4.6 mm i.d.) equipped with security guard col-

umn. The spectrophotometric assays were performed using a temperature controlled, double beam Perkin-Elmer Lambda 25 spectrophotometer.

2.3. Sample preparation

Purified enzyme purchased from Sigma was diluted accordingly for the in vitro assays. For the studies regarding cell lines, approximately 10^8 Hut 78 cells were lysed by homogenization (20 cycles × 2) in a Polytron homogenizer at 2000 rpm in homogenization buffer (250 mM sucrose, 20 mM K + HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM EGTA). The cell lysates were then subjected to centrifugation at 2500 × *g* for 5 min to pellet down the cell debris and the supernatant was used for both the spectrophotometric as well as the HPLC assays.

2.4. Spectrophotometric analysis of adenosine deaminase activity

Adenosine deaminase activity was assayed spectrophotometrically using saturating substrate conditions. The assay mixture contained different concentrations of adenosine, and 0.0055 units of adenosine deaminase in 20 mM potassium phosphate buffer (pH 7.4) at 25 °C. The decrease in the absorbance at 265 nm and simultaneous increase in absorbance at 235 nm resulting from the deamination of adenosine to inosine were continuously monitored in a temperature regulated (Heto circulating water bath, HMT 200, CBN 8-30 model) UV–vis Perkin-Elmer Lambda 25 spectrophotometer. Different concentrations of standard inhibitor, EHNA were used to study the effect of the inhibitor on the enzymatic reaction. All the kinetic and inhibition experiments represent the results obtained from three individual experiments.

2.5. HPLC-based method for the analysis of adenosine deaminase activity

A gradient elution profile was obtained by employing a combination of 20 mM potassium phosphate buffer (pH 7.4) and methanol, this gradient solution was used to separate the substrate and product of the enzyme reaction. The gradient was as follows: isocratic phase at 7% methanol for 3 min, then up to 30% methanol holding for 3 min and then 7% methanol for 6 min to restore initial conditions. The injection volume was 20 μl, total run time was 12 min and the flow rate was adjusted to 1 ml/min. The HPLC procedure was performed at room temperature and the absorbance of both the substrate and the product were monitored at 260 nm (i.e. approximate λ_{max} of adenosine).

Kinetic measurements for ADA were carried out using different concentrations of adenosine upto 500 μM in 20 mM of potassium phosphate buffer (pH 7.4) in a final volume of 1 ml assay mixture. The reaction was started with the addition of 0.00275 units of adenosine deaminase and was terminated by adding perchlorate ($HClO_4$) to a final concentration of 0.3 M. Denatured proteins were precipitated by centrifugation at 3000 × *g* for 5 min and the final pH of the supernatant was adjusted to pH 7.4 with the addition of K_2CO_3 (final concentration 2.5 mM). Clear supernatants were processed for HPLC experiments.

The mobile phase solvents (methanol and potassium phosphate) were filtered through 0.22 μm nylon membrane and further degassed before use. The injection volume was 20 μl and the mobile phase flow rate was kept constant at 1 ml min^{-1}.

2.6. Method validation for HPLC-based method

2.6.1. Linearity and range

Stock solution of inosine (3 mM) was prepared. The stock solution was diluted accordingly, to prepare solutions of concentrations ranging from 5 to 500 μM and the solutions were injected in triplicate for obtaining a representative result.

2.6.2. Precision

Injections of three different concentrations (25, 50 and 100 μM), each in triplicate were injected on the same day and the relative standard deviation was calculated to determine the precision of intra-day and inter-day variation of our experimental result.

2.6.3. Accuracy

Percentage recovery was calculated from the difference between the calculated and expected concentration (calculated from the corresponding peak area). The recovery studies were carried on with four concentrations of the product (25, 50, 100 and 200 μM inosine) to find out the accuracy of the method.

3. Results

Spectrophotometry-based kinetic study of few key target enzymes like adenosine deaminase and hypoxanthine-guanine phosphoribosyltransferase, raise the problem of overlapping spectra of the substrates, products and the inhibitors. Moreover, other substrates of adenosine deaminase like deoxyadenosine and oxipurine also have almost identical λ_{max} as that of adenosine. Even the corresponding products also absorb in the same wavelength region and overlap with that of the substrate. Due to this problem it was necessary to develop an alternative assay system for adenosine deaminase. Hence we have applied the HPLC technique in which the molecules are separated based on the inherent polarity difference as reflected in their retention time (R_t) values.

3.1. Spectrophotometric analysis of adenosine deaminase activity

Representative scans of the substrate (adenosine λ_{max} = 259 nm), product (inosine λ_{max} = 249 nm) and the

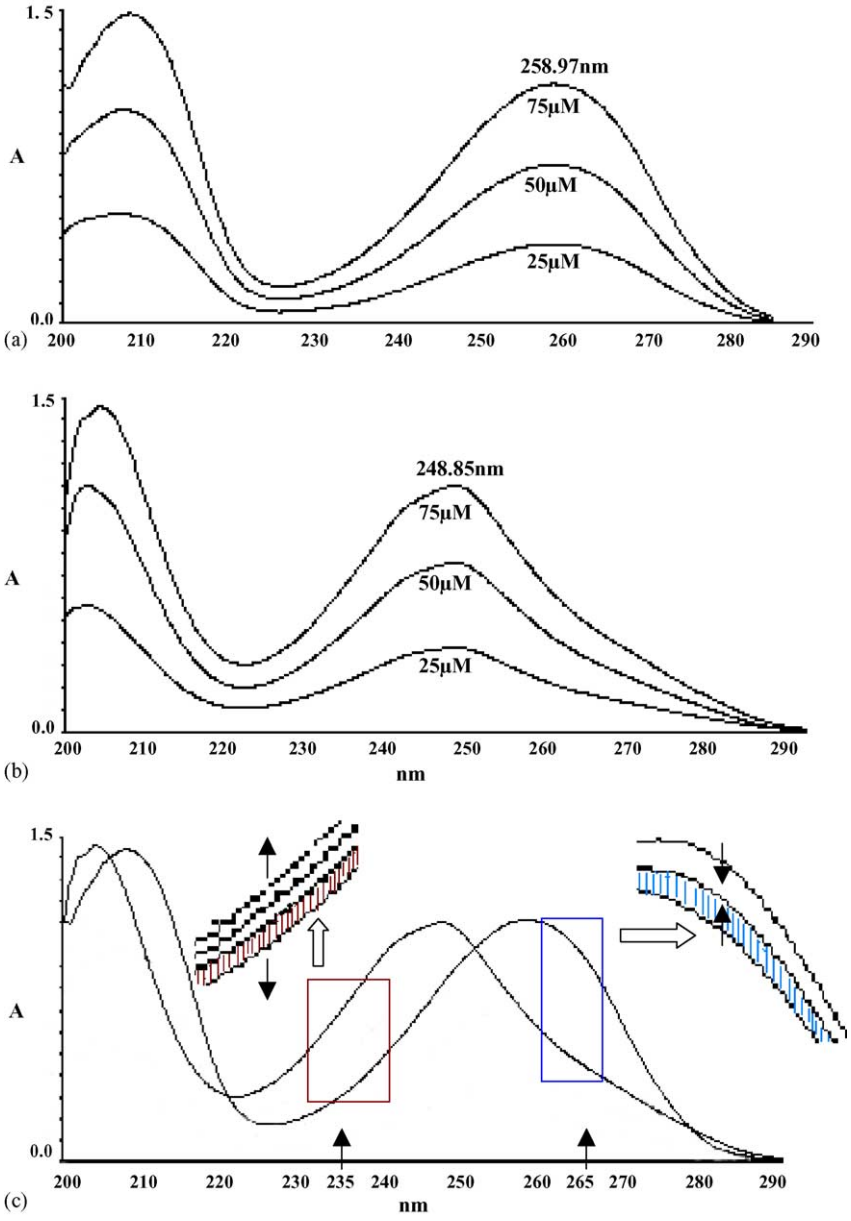

Fig. 1. (a) Overlapping spectra of 25, 50 and 75 μM adenosine (300–200 nm) in potassium phosphate buffer, pH 7.4 showing the λ_{max} at 258.97 nm. (b) Overlapping spectra of 25, 50 and 75 μM inosine (300–200 nm) in potassium phosphate buffer, pH 7.4 showing the λ_{max} at 248.85 nm. (c) Mixed spectra of adenosine and inosine showing overlapping contribution at 265 and 235 nm. At 265 nm the inset shows the concomitant decrease in absorbance of adenosine (substrate utilization) and increase in absorbance of inosine (product formation) as shown by arrows. The blue shaded area at 265 nm describes the compromised zone that cannot be detected. Similarly the inset at 235 nm shows the concomitant substrate utilization and product formation as indicated by arrows. The brown shaded area at 235 nm represents the compromised zone that is masked by the spectral overlapping. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

overlapping spectra of adenosine and inosine are shown in Fig. 1a–c. The shaded area in the left inset (Fig. 1c) represents the additional absorbance contribution due to the overlapping spectra, while the right inset represents the decrease in the contribution due to the overlapping nature of the spectra for a time-based enzymatic reaction. Furthermore EHNA, the standard adenosine deaminase inhibitor (EHNA λ_{max} = 260 nm) absorbs in the same wavelength region (inset of Fig. 3.) and also contributes to the spectral overlapping at higher concentration. The problem, due to the overlapping spectral characteristics of the substrates (adenosine and deoxyadenosine) and their respective enzymatic products, formed with the progress of time is shown in Fig. 2a and b (indicated by arrows), respectively. It is quite interesting to note that at the wavelength maxima of inosine and adenosine (249 and 259 nm), the decrease in the absorbance of the substrate and the corresponding increase in the absorbance of the product with the course of time are not quantitatively

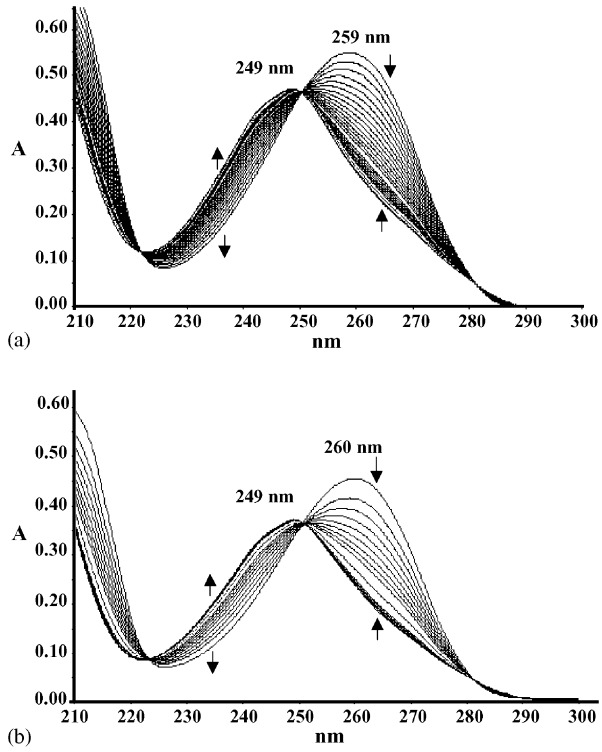

Fig. 2. (a) Adenosine (25 μM) reaction with 0.0055 units of adenosine deaminase in potassium phosphate buffer, pH 7.4, temperature −25 °C absorbs maximally at 258.97 nm and product maximally absorbs at 248.85 nm, with the formation of an isobastic point at 254 nm. (b) .2′3′dideoxyadenosine (25 μM) reaction with 0.0055 units of adenosine deaminase in potassium phosphate buffer, pH 7.4, temperature−25 °C absorbs maximally at 260 nm and product maximally absorbs at 249 nm almost equal to that of adenosine and inosine, respectively (Fig. 3).

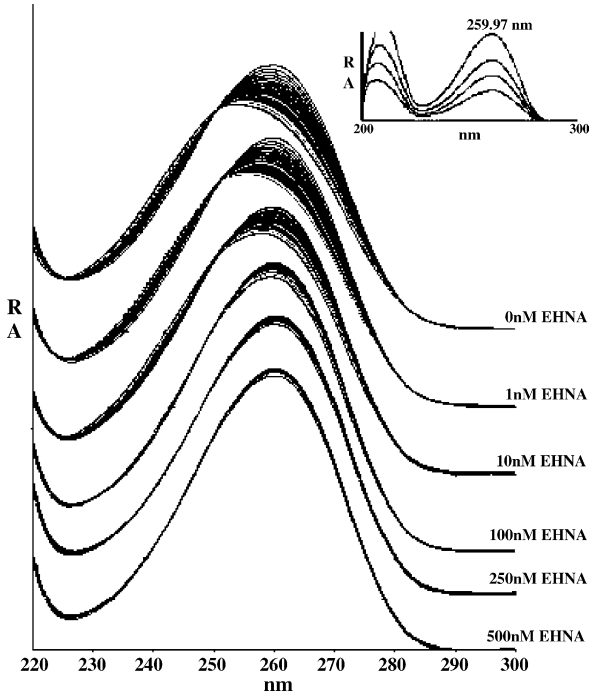

Fig. 3. Adenosine deaminase-catalyzed adenosine (50 μM) to inosine conversion was inhibited with different concentrations of EHNA (1, 10, 100, 250 and 500 nM) in the presence of 0.0055 units of adenosine deaminase in potassium phosphate buffer, pH 7.4, temperature-25 °C. The spectra of different concentration of EHNA are shown in the inset and the λ_{max} was found to be 260.00 nm.

complementary (Fig. 1c, inset). At a certain wavelength the substrate degradation (decrease in absorbance) is partially nullified or amplified by the contribution of the product formation (increase in absorbance). Therefore a compromised net result is obtained.

While monitoring inhibition of ADA reaction with adenosine as substrate and using different concentrations of EHNA as inhibitor, we confronted the same spectral overlapping problem, where the real inhibition is partially masked (Fig. 3). Studies of the inhibition kinetics of ADA, using deoxyadenosine as the substrate and EHNA as inhibitor, suffer from similar drawback as that of adenosine (data not shown).

3.2. Development of HPLC based method for the kinetic analysis of adenosine deaminase activity

In order to eliminate the spectroscopy related difficulty we have used the HPLC based method as a simple and efficient alternative method for the purpose of assaying the same reactions catalyzed by adenosine deaminase. This reverse-phase HPLC-based assay system depends on the polarity of the molecules yielding a difference in the migration time. A clear separation of peaks of the substrate, product and inhibitor was observed and was reflected in their well-separated R_t values. The R_t values of the substrates (adenosine, R_t = 11.8 min), (deoxyadenosine, R_t = 8.00 min), products (inosine, R_t = 7.8 min), (deoxyinosine, R_t = 4.517 min.), (Fig. 4), and standard inhibitor (EHNA, R_t = 11.3 min) (Fig. 7) were found to be reasonably different and the corresponding peaks were well separated in the chromatogram. Fig. 5 shows the time dependent enzymatic reaction, where we observed a decrease in the area under the peak of adenosine and a gradual increase in the area under the peak of inosine. Using this HPLC-based method we further calculated the initial rates of the adenosine deaminase reaction.

The effect of the standard inhibitor, EHNA and certain anticancer drugs (data not shown) were also studied and the adenosine deaminase reaction kinetics was followed using the HPLC-based method. EHNA exhibited a clear dose dependent inhibition of the adenosine to inosine conversion (Fig. 6). We found that as compared to the HPLC-based method, the results obtained from the spectrophotometric method did not

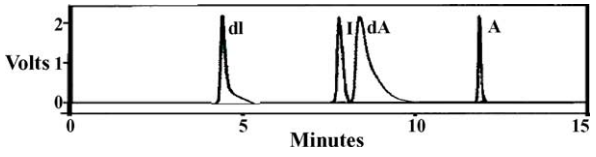

Fig. 4. HPLC chromatogram showing the retention time of: adenosine (R_t = 11.8 min) (A), inosine (R_t = 7.8 min) (I), deoxyadenosine (R_t = 8.0 min) (dA) and deoxyinosine (R_t = 4.5 min) (dI).

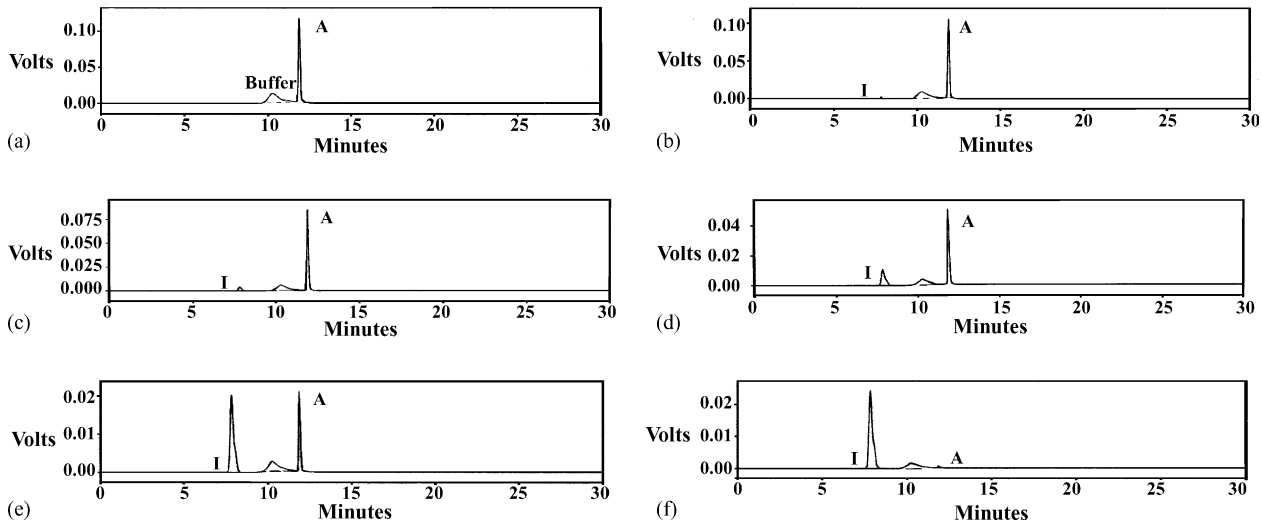

Fig. 5. HPLC chromatogram showing the adenosine deaminase reaction at different time point, reaction stopped at: (a) 0 min; (b) 15 s; (c) 30 s is (d) 1 min; (e) 2 min; (f) 5 min by incubating at 30 °C. Peak 1–3 corresponds to inosine (I), buffer and adenosine (A), respectively.

provide us the quantitative interpretation (Fig. 3) of the kinetics and inhibition because of the appearance of the time dependent overlapping of the spectra. Hence data obtained from HPLC-based method gives a true representation of the enzymatic rate and inhibition mechanism and can be easily extended to calculate the IC_{50} and the inhibition constant of an inhibitor or drug.

Though the inhibitor EHNA has a very close R_t value (11.3 min.) as that of adenosine (11.8 min.) but its peak is quite separated from that of adenosine (R_t = 11.8 min.) and moreover, its molar absorptivity is so less that it could not be detected in the chromatogram under the experimental condition. Fig. 7 depicts the adenosine peak and the scaled up version of the EHNA peak, which is otherwise not detectable under the experimental conditions.

3.3. HPLC method validation

3.3.1. Linearity and range

With the methanol:potassium phosphate mobile phase, the response of the product (inosine) was linear in the concentration range between 5 and 500 μM. The mean correlation coefficient was calculated to be 0.9997 (±.03).

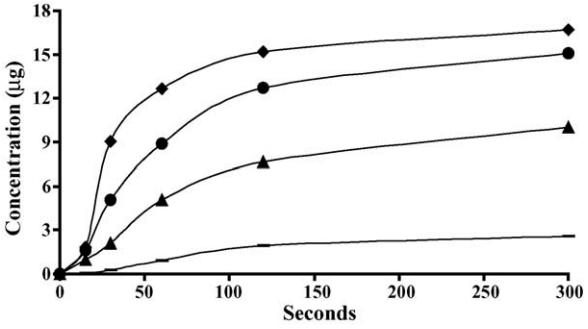

Fig. 6. Graphical representation of the inhibition studies by HPLC method using 0.0025 U ADA, 25 μM adenosine and different concentrations: control (♦); 1 nM (●); 10 nM (▲) and 100 nM (▬) of EHNA.

3.3.2. Accuracy

The recovery percentage was calculated for triplicate sets of 25, 50, 100 and 200 μM concentrations of the product (inosine) and were found to be 100.7, 107.1, 101.7 and 100.3%, respectively.

3.3.3. Precision

The reproducibility and precision of the data were evaluated through intra-day and inter-day studies, for the prod-

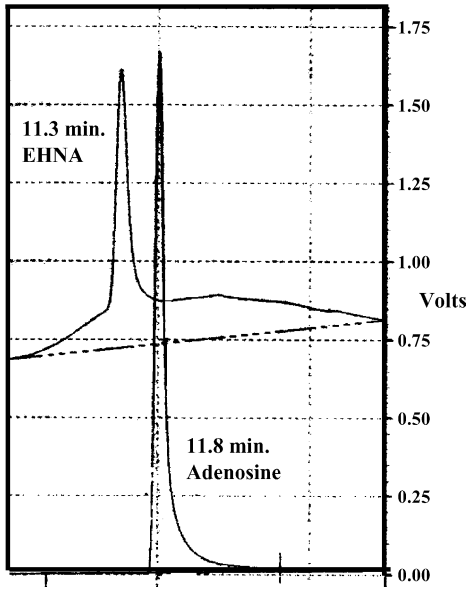

Fig. 7. HPLC chromatogram showing retention time of adenosine (R_t = 11.8 min.) and that of EHNA (R_t = 11.3 min.). The figure shows no contribution from EHNA to the adenosine peak at the inhibitory concentration.

uct concentrations of 25, 50 and 100 μM. Concentrations measured for the intra-day concentrations (±S.D.) were found to be 26.783 ± 0.852 μM, 49.286 ± 0.195 μM and 99.007 ± 0.348 whereas the inter-day concentrations were calculated as 27.563 ± 1.12 μM, 51.693 ± 2.105 μM and 105.236 ± 5.053, respectively. As is evident from the resolution of the peaks, it is well evident that this process is sufficiently specific for the separation of adenosine and inosine. The reproducibility and precision of the spectrophotometric method are also comparable under our experimental condition.

3.3.4. Limit of detection and quantification

The HPLC-based method is much more sensitive (limit of detection is in nano molar range, limit of quantitation is also in nano molar range) as compared to the spectrophotometric method.

3.4. Extension of the HPLC-based to cell line lysate

Assays for the presence of the enzyme, substrate and the product using spectrophotometric method with cell line or tissue lysate present more problems of non-specific contribution. Therefore we have analyzed the time dependent product formation profile in the Hut-78 (T cell lymphoma) cell line lysates using HPLC-based method and have been able to monitor the substrate utilization kinetics (Fig. 8).

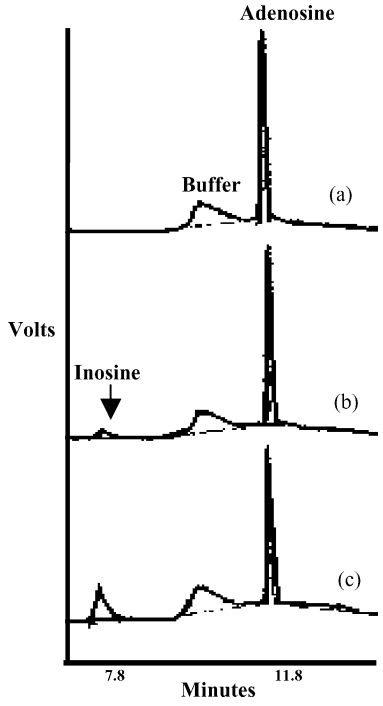

Fig. 8. HPLC chromatogram showing the adenosine deaminase reaction in Hut-78 cell extract at different time point, reaction stopped at: (a) 0 min (b) 30 min and (c) 60 min.

4. Discussion

Spectrophotometric method is popularly used for the estimation of adenosine deaminase activity but it suffers from the spectral overlapping problem of the substrate, product and inhibitor. Moreover, higher concentration of enzyme is also required in the spectrophotometric method to give a noticeable change for quantitative estimation of the product formation or substrate degradation and to check the effect of the inhibitor. The main drawback with the HPLC-based method lies in the time required for the preparation of samples, hence high throughput studies cannot be effectively made possible. ADA inhibitors are used as drugs and hence an exact dose is needed to be prescribed, therefore any technical problem in calculating the true dose is not at all desirable. Thus the alternative HPLC-based method is suitable for the quantitative measurement of the kinetics and inhibition of mammalian adenosine deaminase. The method can also be used for the screening of a large number of inhibitors of adenosine deaminase. Keeping in mind the role of adenosine deaminase inhibitors in cancer, SCID, ROS mediated cardiovascular ischemia and other diseases, the proposed HPLC-based method for the assay of adenosine deaminase enzyme activity or inhibition was proved to be quantitative, reliable, accurate and cheap.

5. Conclusion

We have developed a new HPLC method for quantitatively studying the kinetics and inhibition of mammalian adenosine deaminase using pure enzyme system or using cell extracts and body fluids. The same method can also be extended for screening a large number of compounds for the discovery of adenosine deaminase inhibitors.

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