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Short communication

Simple and rapid determination of adenosine in human synovial fluid with high performance liquid chromatography–mass spectrometry

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Abstract

A simple, fast, sensitive and selective reversed-phase high performance liquid chromatography–mass spectrometry coupling with an electrospray ionization (ESI) interface method is described for the determination of adenosine in human synovial fluid. This method involved the use of the $[M + H]^+$ ions of adenosine and 2-chloroadenosine (internal standard for the assay) at *m*/*z* 268 and 302 in positive ion mode with selective ion monitoring (SIM). Separation was carried out on a 2.0 × 150 mm Shimadzu VP-ODS column by using an isocratic elution with a mobile phase consisting of water (94%),methanol (5%) and formic acid (1%). No interference with the components of the biological matrix was observed in the determination conditions. The calibration curve was linear in the range of 0.2–140 μg ml^{-1}. The limits of quantification (LOQ) and detection (LOD) were 0.2 and 0.03 μg ml^{-1}, respectively. The standard recoveries were between 93.3 and 104.0%. The method was successfully applied to determination of adenosine in some synovial fluids of patients affected by rheumatoid arthritis.

Keywords: High performance liquid chromatography; Mass spectrometry; Electrospray ionization (ESI) interface; Adenosine; Synovial fluid

1. Introduction

Adenosine, which is produced by the degradation of intracellular adenosine triphosphates, is an important component of nucleic acid. Adenosine is involved in the regulation and modulation of various physiological processes in the central nervous system (CNS) and is known to depress the excitability of CNS neurons and to inhibit release of various neurotransmitters presynaptically [1,2]. Adenosine also has immunosuppressive properties towards lymphocytes, neutrophils, mast cells and monocyte/macrophages. Therefore, some studies have been reported about its role in the pathogenesis of several inflammatory diseases, such as rheumathoid arthritis [3,4]. There are also some studies about the role of adenosine in the anti rheumatoid arthritis effect of methotrexate [5–7]. For these reasons, the quantification of adenosine in human synovial fluid has great clinical importance [8–10]. Many methods for the separation and determination of adenosine in biological samples have been developed. These methods include high performance liquid chromatography (HPLC) [8–17], co-electroosmotic capillary electrophores [18] and flow-injection method [19]. Of these methods, the most useful one for the determination of adenosine in biological samples is HPLC method. However, a reliable method for determining adenosine in the synovial effusions from patients affected by rheumatoid arthritis is not available. Because of complexity of biological liquids, the

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analytical methods must pay more attention to the biological matrix in order to avoid interference of its component with the adenosine. For a good separation of each component, complex gradient systems are usually required [11,12] or the sample preparation is tedious, for examples, solid phase extraction [13,14,17]. In addition, these methods need a relatively large amount of sample to reach a low quantification limit. Liquid chromatography–mass spectrometry (LC/MS) has been widely used for the determination of drugs and their metabolites in biological samples because of its high sensitivity and specificity [20–22]. As we know, there is no report about the detection of adenosine in the human synovial sample using LC/MS. The purpose of the present study is to develop a simple, fast and sensitive LC/MS method for determination of adenosine in the synovial fluid.

2. Experimental

2.1. Reagents and standards

Adenosine, the internal standard (IS) 2-chloroadenosine, hyaluronidase and adenosine deaminase (ADA) were purchased from Sigma (St. Louis, MO, USA). Molecular structures of adenosine and 2-chloroadenosine are shown in Fig. 2. A stock solution(0.2 mg ml^{-1}) of adenosine standard and IS was prepared in methanol and stored in a refrigerator. Dilutions of adenosine standard and IS (10 μg ml^{-1}) were prepared by diluting the stock solution with methanol. Formic acid used was analytical grade. HPLC-grade methanol was obtained from Hanbang Science and Technology Co. (Jiangsu, P.R. China) and Milli-Q quality water was used in the preparation of the mobile phase. Solvents were filtered through a 0.45 μm membrane and degassed.

2.2. Instrumentation

The Shimadzu (Kyoto, Japan) LC/MS equipment consisted of a LC-10ADvp solvent delivery pump, an FCV-10ALvp low pressure gradient unit, a DGU-14A degasser, a SPD-M10Avp diode array detector, a LCMS-2010 mass spectrometer, a CTO-10Avp column oven. The column utilized for separation was a 2.0 × 150 mm Shimadzu VP-ODS column with a particle size of 5 μm. The analytical column was protected by a C_{18} guard-pak cartridge (Waters, Milford, MA, USA).

2.3. Chromatographic conditions

Mobile phase consisting of water (94%), methanol (5%) and formic acid (1%) was degassed ultrasonically before use. Each component of the mobile phase was filtered through a 0.22 μm membrane. All separations were at room temperature and a flow-rate of 0.2 ml min^{-1}. The wavelength of photo-diode array detector was 200–300 nm.The volume of injection was 5 μl.

2.4. Mass spectrometric detection conditions

MS coupling with an electrospray ionization (ESI) interface was used in positive ion mode by selective ion monitoring at *m/z* 268 and 302. $[M + H]^+$ was selected as the SIM ion in quantification. Sensitivity optimization was performed by injection of an adenosine standard(20 μg ml^{-1}). ESI temperature was 400 °C. Curved desolvation line (CDL) and block temperature was 250 and 200 °C, respectively. Probe voltage was +4.5 kV.Detector voltage was1.5 kV. CDL voltage was −20 V. Q-array Bios was 50 V. Nebulizing gas flow was 4.5 l min^{-1}.

2.5. Sample preparation

About 1 ml human synovial samples were filtered through a 0.45 μm membrane. An amount of 60 μl hyaluronidase (initial concentration of 3000 U ml^{-1}) were added to 1 ml of sample until a final concentration of 180U ml^{-1} was reached. The mixture was incubated at 37 °C for 30 min. Samples were stored at −20 °C until analysis. The synovial liquid blank was prepared from human synovial fluid treated with ADA. The procedure was similar to that of the synovial analytical sample, but adenosine deaminase was added after incubation.

2.6. Validation

A set of seven non-zero calibration standards, ranging from 0.2 to 140 μg ml^{-1} was prepared in order to calculate the standard curve for the determination of adenosine, which was calculated by plotting peak area ratio (*Y*) of adenosine and IS in total ion chromatogram (TIC) of LC/ESI-MS versus concentration (*X*, μg ml^{-1}) with least squares linear regression. The human synovial fluid samples spiked with adenosine at concentrations of 0.2, 2.0, 50 and 100 μg ml^{-1} were used to determine intra-day and inter-day variance. To determine intra-day variance, the replicate assays were carried out on the same samples at different times during the day. Inter-day variance was determined by analyzing samples over six consecutive days. Coefficients of variation were calculated from these values. For the determination of recovery, 3.0,5.0 and 10.0 μg ml^{-1} adenosine at sample level were spiked to 1 ml of human synovial fluid. The resulting peak area ratios of adenosine and IS were used to calculate the recovery. As part of the method validation, stability was evaluated. The processed sample stability was evaluated by comparing the processed samples that were injected immediately (time 0) with the samples that were re-injected after placing a period at room temperature for 24 h. The stability of spiked samples stored at −20 °C (long-term stability) was evaluated by analyzing 1.0, 10.0 and 50.0 μg ml^{-1} processed samples for 10 weeks.

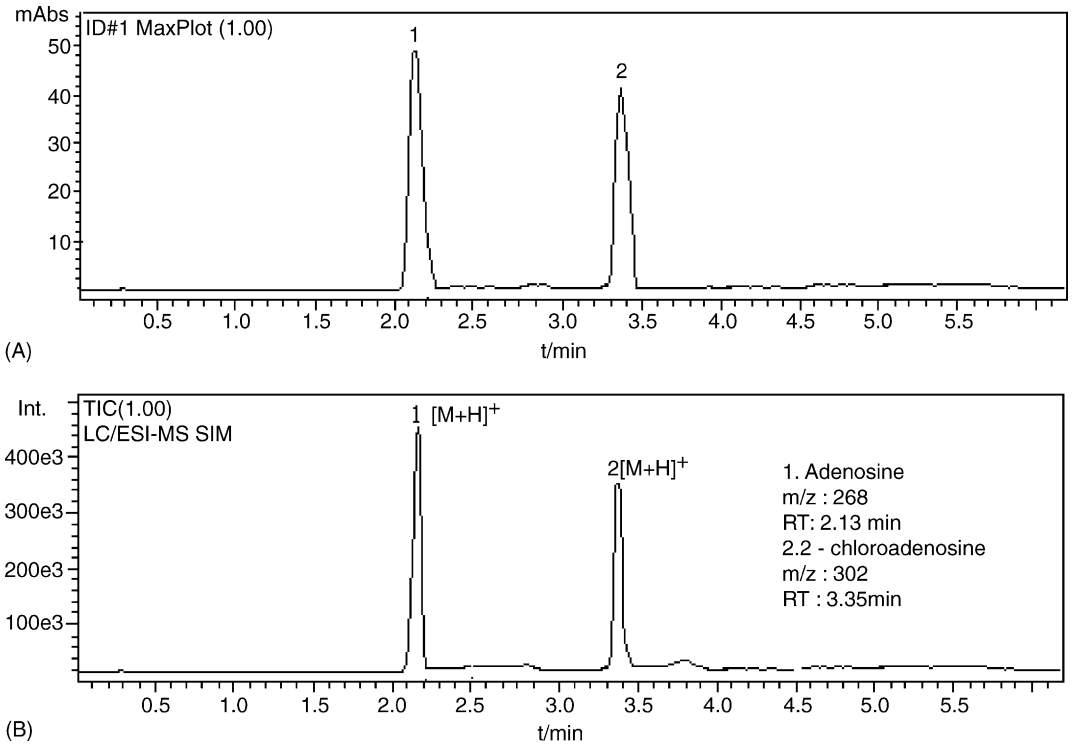

Fig. 1. Simultaneous HPLC–UV chromatogram detected with a photo-diode array detector set at 260 nm (A) and LC/ESI-MS TIC (B) of adenosine standard (20 μg ml^{-1}) and IS (20 μg ml^{-1}). Chromatographic conditions are listed in Section 2.3 and SIM mode parameters are listed in Section 2.4. Peaks are detected in SIM mode. 1: Adenosine, 2: IS.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The purpose of the present LC/MS method was to quantify the adenosine in human synovial fluid within a short analysis time. In a preliminary study, it was found that separation of adenosine and 2-chloroadenosine was achieved on a 2.0 × 150 mm Shimadzu VP-ODS column by using the mobile phase consisting of water, methanol and formic acid. Separation conditions such as percentage of water, methanol and formic acid were optimized. Of the optimizable parameters, the pH value of mobile phase and organic modifier percentage had the greatest effect on the separation, peak shape and detection sensitivity of adenosine. The percentage of formic acid was the most important factor. When the percentage of

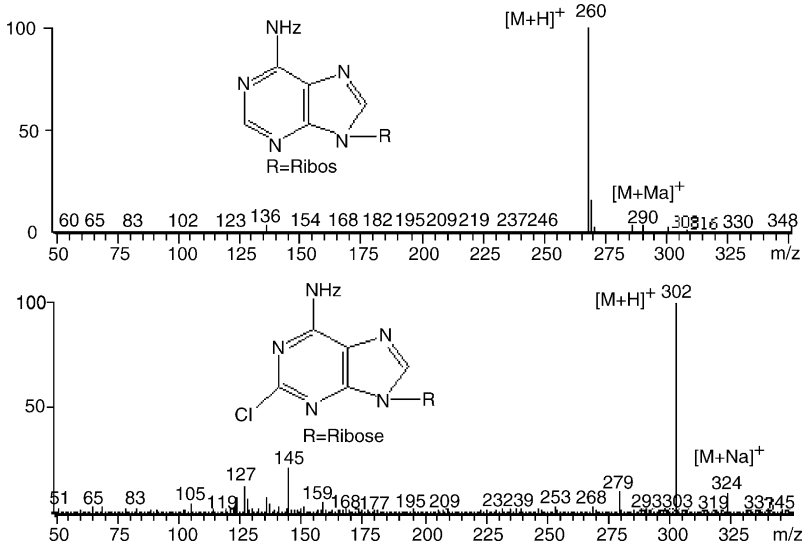

Fig. 2. LC/ESI-MS mass spectra of adenosine standard (top) and IS (bottom) in the positive ion mode by scanning between *m*/*z* 50–350 per s. Interpretation of major peaks is shown in this figure.

Table 1
Summary of adenosine calibration standards

Analyte	Concentration added ($\mu g\,ml^{-1}$)	Concentration found ($\mu g\,ml^{-1}$)	RE (%)	RSD (%, $n = 6$)
Adenosine	0.2	0.19	−5.0	3.2
	2.0	2.03	1.5	3.6
	20	20.46	2.3	2.1
	50	50.32	1.0	1.6
	75	74.39	−0.8	1.1
	100	98.93	−1.1	2.3
	140	136.47	−3.5	1.9

formic acid was below 0.6%, the resolution of adenosine and IS was poor. When the percentage of formic acid was between 0.6–1.4%, the resolution of adenosine and IS could be well separated. The adenosine and 2-chloroadenosine were best separated using a mobile phase of water (94%), methanol (5%) and formic acid (1%). Fig. 1A was HPLC–UV chromatogram detected with a photo-diode array detector set at 260 nm. Based on this, a very simple, fast, sensitive and selective LC/MS method to quantify the adenosine was developed.

3.2. Optimization of the ESI-MS conditions

Although the analyte can be monitored at 265 nm with a photo-diode array detector (see Fig. 1A), this is quite difficult to quantify the adenosine in the case of synovial fluid because of the presence of components with different polarities and consequently, different retention times. However in LC/MS method, using positive ion detection with SIM mode, adenosine and IS were detected without interferences from human synovial fluid matrix. Both positive and negative ion mode were tested. It was found that almost the same ion intensities were achieved in the chromatographic conditions. But negative ion detection mode has poorer selectivity and higher background than positive ion detection method. Consequently, positive ion detection mode was chosen because quantification was easy for the low concentration in real samples. Also the concentration of formic acid affected heavily on ionization. When the percentage of TFA was below 0.8%, the ionization efficiencies of adenosine and IS were low. When the percentage of formic acid was above 0.8%, the ionization was good. LC/ESI-MS mass spectra of adenosine standard and IS in positive ion mode were obtained by scanning between *m*/*z* 50–350 per s (see Fig. 2). The mass spectra of the adenosine and IS obtained from scan mode were characterized by a protonated molecular ion $[M + H]^+$(at *m*/*z* 268 and 302,respectively) as base peak. SIM mode involved the use of the $[M + H]^+$ions at *m*/*z* 268 and 302 (IS ion) was chosen for the quantitative analysis of adenosine. ESI-MS parameters were optimized by injection of an adenosine standard (20 $\mu g\,ml^{-1}$). The best ESI-MS conditions were listed in Section 2.4. Fig. 1B showed LC/ESI-MS TIC of adenosine standard and IS, in which peaks were detected in SIM mode. The retention time of adenosine and IS in TIC was 2.13 and 3.35 min, respectively.

3.3. Analytical properties

The selectivity that differentiates and quantifies the analyte in the presence of other components in the sample is very important to an analytical method. Thus, the most important aim was checked so that there is no interference between the adenosine and the components of the biological matrix. Due to using SIM mode in this method, no interference at the retention times of adenosine was observed. Under these conditions, adenosine and IS exhibited favorable selectivity. The results of the calibration samples were presented in Table 1. The calibration curve, which was linear in the range of 0.2 to 140 $\mu g\,ml^{-1}$, was $Y = 0.1206X + 0.01075$ with correlation coefficients being 0.9992. A crucial problem was regarding the sensitivity of the method due to the very low content of adenosine in synovial fluid. The method reported here was very sensitive due to using optimal ESI-MS conditions and the advantages of LC/ESI-MS in the SIM mode. Because human synovial fluid contains analyte, no real blank was available for preparation of standards or controls. A solvent blank was analyzed for determining limit of detection (LOD) and no peaks at *m*/*z* 268 and 302 were observed in the blank.

Table 2
Intra-assay and inter-assay precision and accuracy for the determination of adenosine in human synovial fluid

Concentration added ($\mu g\,ml^{-1}$)	Intra-assay			Inter-assay		
	Concentration found ($\mu g\,ml^{-1}$)	RE (%)	RSD (%)	Concentration found ($\mu g\,ml^{-1}$)	RE (%)	RSD (%)
2.0	2.07	3.5	6.2	2.10	5.0	6.8
50	51.13	2.3	2.7	52.04	4.1	3.4
100	98.62	−1.4	1.8	97.41	−2.6	2.1

The LOD of adenosine was 0.03 μg ml^{-1} with LC/ESI-MS, which determined from signal-to-noise ratio of 3:1. Nevertheless, after applying the method to clinical samples, the limit of quantification (LOQ) was more important. The lower limit of the range of the calibration curve can be considered the limit of quantification of this method. Reproducibility of retention times of adenosine and IS, which was calculated from six replicate analysis, was 0.19 and 0.22. Because adenosine is endogenous substances, there is no "real" blank available. Therefore, spiked samples were used for the determination of precision, accuracy and recovery. Before spiking, blank of human synovial fluid was screened to ensure it was free of endogenous interference at the areas of retention time of adenosine and IS. The accuracy of the method was calculated using the equation: accuracy = [(concentration found − concentration added)]/concentration added] × 100. Concentration found was equivalent to (measured concentration-sample concentration). The results of inter- and intra-assay precision and accuracy were summarized in Table 2. The inter-assay precision and accuracy were in the range of 2.1–6.8 and −2.6–5.0%, respectively. The intra-assay precision and accuracy were in the order of 1.8–6.2 and −1.4–3.5%, respectively. The intra-assay precision and accuracy for samples at LOQ level were 6.8 and −5.0%. The recoveries of 3.0, 5.0 and 10.0 μg ml^{-1} adenosine at sample level were 93.3, 104.0 and 97.0%, respectively. The results showed that precision, accuracy and recovery were very satisfactory. Stability experiment indicated that the difference of measured concentration from time 0 to 4 h was 4.2%, which allowed us to conclude that processed samples were stable for at least 4 h at room temperature. In long-term stability experiments, the difference of measured concentration from time 0 to 42 days for 1.0 μg ml^{-1}, from time 0 to 57 days for 10.0 μg ml^{-1} and from time 0 to 61 days for 50.0 μg ml^{-1} was 4.6, 4.3 and 4.8%, respectively. The results showed that the spiked samples were stable at least 42, 57, 61day for 1.0, 10.0 and 50.0 μg ml^{-1},respectively.

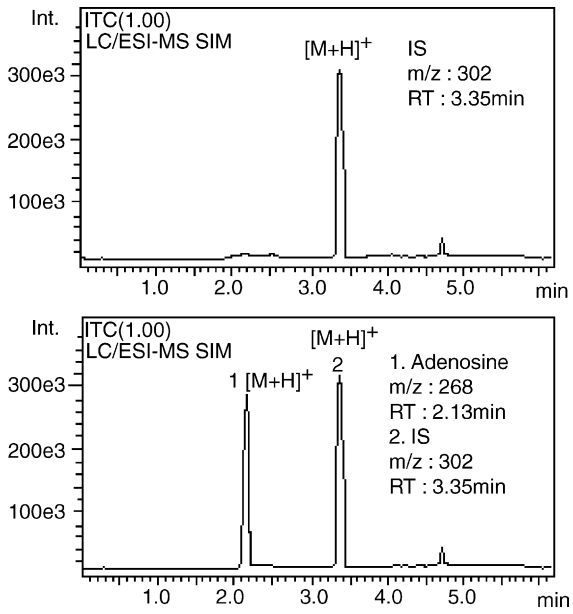

Fig. 3. LC/ESI-MS TIC of a representative human synovial fluid without adenosine (top) and with adenosine (bottom). Chromatographic conditions and SIM mode parameters are described in Fig. 2. Peaks are detected in SIM mode and $[M + H]^+$ ions (at *m/z* 268 and 302) are selected. Peak identification is like in Fig. 2.

3.4. Application to clinical samples

Fig. 3 was a typical LC/ESI-MS TIC of a representative human synovial fluid affected by rheumatoid arthritis without adenosine and with adenosine according to the conditions described (see Fig. 3). It was suggested that an adenosine deaminase inhibitor should be added in a routine analysis of adenosine. The treatment of clinical samples with hyaluronidase was very effective in order to make the sample more fluent and allow for direct injection. Using this method, the concentration of adenosine in synovial fluids of patients affected by rheumatoid arthritis was determined. The average concentration (mean ± S.D.) of adenosine in human synovial fluid from 20 human subjects affected by rheumatoid arthritis was found to be 8.72 ± 3.26 μg ml^{-1}. The results were in agreement with earlier report [10].

4. Conclusions

Using mobile phase consisting of water (94%), methanol (5%) and formic acid (1%), a very simple, fast isocratic LC/ESI-MS method is presented. The described LC/ESI-MS method in SIM mode involving the use of the $[M + H]^+$ allows us to determine adenosine in human synovial fluid with good selectively and sensitivity, which is completely suitable to quantify the real concentration of adenosine in synovial fluid. These advantages have made the application of this method possible to study new correlations between adenosine and rheumatoid arthritis and thus to better understand the mechanism of this illness.

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