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Short Communication

HPLC determination of adenosine in human synovial fluid

Enzo Sottofattori [a,*], Maria Anzaldi [a], Luciano Ottonello [b]

[a] *Dipartimento di Scienze Farmaceutiche, Viale Benedetto XV, 3, 6-16132 Genova, Italy*
[b] *Dipartimento di Medicina Interna e Specialità Mediche, Viale Benedetto XV, 3, 6-16132 Genova, Italy*

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Abstract

A high-performance liquid chromatographic method has been developed for the quantitative determination of adenosine in human synovial fluid. The method is simple, rapid and, overall, selective. No interference with the components of the biological matrix was observed in these chromatographic conditions. An ODS (250 × 4.6 mm) 5 μm column was used with an isocratic elution of a phosphate buffer–acetonitrile mobile phase. Detection was carried out on a UV detector at 260 nm. Calibration curve was found to be linear in the 0.7–70 μg ml^{-1} range. Linear regression analysis of the data demonstrates the efficacy of the method in terms of precision and accuracy. The precision of this method, calculated as the relative standard deviation (RSD) of the recoveries (1.57–2.21%), was excellent. The limits of quantitation (LOQ) and detection (LOD) were respectively 0.7 and 0.2 μg ml^{-1}. The method was applied to some samples of synovial effusion from patients affected by rheumatoid arthritis. The concentrations of adenosine which were found were included in the range of the calibration curve.

Keywords: Adenosine; Synovial fluid; Rheumatoid arthritis; HPLC

1. Introduction

Adenosine (I) is an endogenous nucleoside produced by the degradation of intracellular adenosine triphosphates. After being released in the extracellular surroundings, adenosine interacts with specific cell surface receptors exerting multiple physiologic effects on a variety of cell types, including immune cells. In particular, adenosine is endowed with immunesuppressive properties towards lymphocytes, neutrophils, mast cells and monocyte/macrophages. Thus, its role in the pathogenesis of several inflammatory diseases, such as rheumathoid arthritis, has been investigated [1,2].

In particular some studies have been carried out in order to correlate the role of adenosine in the anti rheumatoid arthritis effect of methotrexate [3–5]. Nevertheless, a reliable methodology for

* Corresponding author. Tel.: +39-10-3538396; fax: +39-10-3538358.
E-mail address: enzo@unige.it (E. Sottofattori).

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detecting and dosing adenosine in the synovial effusions from patients affected by rheumatoid arthritis is not currently available.

In many papers, reverse-phase high performance liquid chromatography has been used to quantify adenosine in biological liquids [6–8]. As this nucleoside is, in general, easily determined using an octadecylsilane stationary phase, the analytical methods must be addressed more to the biological matrix in order to avoid interference of its component with the adenosine.

In the present work, we developed a selectively chromatographic method quantifying adenosine in the synovial fluid. This chromatographic method has been successfully utilized for studying the correlation between adenosine and rheumatoid arthritis.

2. Experimental

2.1. Reagents and standards

Adenosine was purchased from Calbiochem-Behering (San Diego, CA), hyaluronidase and adenosine deaminase from Sigma (Milan, Italy), monobasic sodium phosphate from Merck (Darmstadt, Germany). All chemicals used were of analytical grade. Acetonitrile and water (Merck) were HPLC grade. Solvents were filtered through a 0.45 μm membrane and degassed.

2.2. Chromatographic conditions and instrumentation

The chromatographic analyses were performed with a liquid chromatograph Perkin–Elmer Model Series 4 chromatograph (Norwalk, PA), a Rheodyne 7125 (Berkeley, CA) injector valve with a 20-μl loop, a model Perkin–Elmer LC235 diode-array UV detector. Retention times, peak areas and UV spectra were recorded on Perkin–Elmer LCI-100 integrator.

A 250 × 4.6 mm i.d. stainless-steel Hibar column prepacked with 5 μm RP-18 phase was used (Hypesil, Runcorn, UK); the column was protected with a LiChroCart 4-4 guard cartridge system (Merck). The mobile phase consisted of acetonitrile – 0.01 M monobasic sodium phosphate (5:95, v/v), and it was delivered by a high-pressure solvent-mixing valve. The flow rate was 1.0 ml min^{-1}. Detection was performed at 260 nm. All separations were carried out at room temperature.

2.3. Calibration curve

To prepare standards for the calibration curve and assessment of validation, a stock solution of adenosine was prepared with a concentration of 0.07 mg ml^{-1}. This solution was diluted with distilled water to yield 0.7, 0.35, 7.0 and 70 μg ml^{-1} working standard solutions for preparation of calibration curve. A 20-μl aliquot was injected (5 replicated) and the calibration curves were constructed by plotting the peak area of adenosine (y) against concentration of adenosine (x), using linear regression analysis. Unknown concentrations of adenosine were quantified by relating the respective peak area to the regression line. In Table 1 are reported the validation data.

In order to determine the matrix effect, a 0.7 μg $ml^{-1}$of adenosine was spiked to a free-adenosine synovial fluid sample. This solution (5 replicated) was injected into the chromatograph.

2.4. Sample preparation

The synovial samples were filtered through a 0.8 μm membrane. Fifty microlitres of hyaluronidase (initial concentration of 3000 U/ml) were added to 1 ml of sample until a final concentration of 150 U/ml was reached. The mixture was incubated at 37° for 20 min.

Table 1
Validation data ($n = 5$)

Calibration range (μg/ml)	0.7–70
Slope	0.37
Intercept	0.011
Regression coefficient	>0.999
Precision (%RSD)	0.95–1.66
Recovery (%)	98.65–100.85
LOQ (μg/ml)	0.7
LOD (μg/ml)	0.2
Recovery average % at 0.7 μg/ml in water	99.88
Recovery average % at 0.7 μg/ml in synovial fluid	100.21

The synovial liquid blank was prepared from human synovial fluid treated with adenosine deaminase. The procedure was similar to that of the synovial analytical sample, but the enzyme was added after incubation. Twenty microlitre of this mixture was injected into the chromatography immediately after incubation.

3. Results and discussion

3.1. Validation of the method

The aim of the present chromatographic method was to quantify the adenosine in human synovial fluid. The most important target was, therefore, checked so that there is no interference between the adenosine and the components of the biological matrix. This is quite difficult in the case of synovial fluid because of the presence of components with different polarities and consequently, different retention times.

We studied the influence of phosphate buffer and acetonitrile on the retention time of adenosine in the presence of biological matrix. Although the phosphate buffer molarity did not have a great influence on the retention time, the found value (Section 2) gave the best results in terms of shape of peak and selectivity. An acetonitrile percentage $>5\%$, accelerates the adenosine peak e does not allow its determination among the biological matrix peaks.

Fig. 1 shows typical chromatograms of synovial fluid without adenosine (a) and with adenosine (b). According to the conditions described, the adenosine retention time was about 10 min. No interference with the components of the biological matrix was observed in these chromatographic conditions. To confirm the nature of the peak of adenosine, we treated the sample with adenosine deaminase: in this case, the adenosine signal disappeared.

A crucial problem was regarding the sensibility of the method. The lower limit of the range of the calibration curve can be considered the limits of quantitation (LOQ) of the method. Althought at a concentration of 0.5 μg ml^{-1} the statistical data worsened ($r=9989$, relative standard deviation

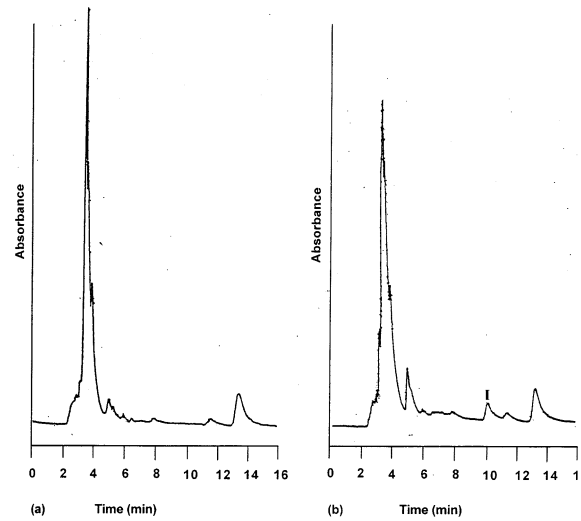

Fig. 1. Chromatograms of synovial fluid without (a) and with adenosine **I** (b).

(RSD) ranged from 0.95 to 3.40%) it was however acceptable. Nevertheless, after applying the method to clinical samples, we considered the limit of detection (LOD) more important. In fact, in some of them we have found no adenosine at all. In these cases the level of adenosine was, therefore, less then the LOD (0.2 μg ml^{-1}). In the other cases, the smaller adenosine concentration was ten times greater then the LOQ.

Another goal was to correlate the calibration data, made in water, with the real quantitation of adenosine in synovial fluid. In other words, to understand the matrix effect. For this reason, the matrix effect with concentration of 0.7 μg ml^{-1} has been studied. This concentration has been spiked in a free-adenosine sample of synovial fluid. In Table 1, the data in water and in synovial fluid are reported. Though the peak of adenosine in water is sharper, significant differences of peak areas were not noted.

Furthermore, the method was validated for linearity, accuracy and precision. Linearity was observed when a graph of the peak area against adenosine concentration over a range of 0.7–70 μg ml^{-1} was plotted. The calibration range has been selected on the base of mean data of previous analysis in biological fluids (2–30 μg ml^{-1}). In fact, the calibration range includes the concentrations of adenosine in our clinical samples (Table 2).

Table 2
Concentration of adenosine in synovial fluid of patients affected by rheumatoid arthritis

Sample	1	2	3	4	5	6	7	8	9	10	11
Concentration (mg/ml)	–	–	–	5×10^{-3}	6×10^{-3}	8×10^{-3}	8×10^{-3}	8×10^{-3}	8×10^{-3}	14×10^{-3}	14×10^{-3}

The correlation coefficient was $r > 0.999$ ($n = 5$). The precision of the method was calculated as the RSD of assays containing adenosine in the same range of concentrations. The RSD ranged from 0.95 to 1.66%. The accuracy was assessed by calculating relative recoveries of adenosine. Recoveries were calculated by relating the respective peak area to the regression line of the standard calibration curve. The analytical recoveries were 98.65–100.57%.

3.2. Application to clinical samples

Table 2 summarized the concentration of adenosine found in synovial fluids of patients affected by rheumatoid arthritis. The data ranged from 0 (samples without adenosine, or under the LOD) to 14×10^{-3} mg ml^{-1}. A direct correlation was found between the quantity of adenosine and the adenosine deaminase previously found in the same samples [9]. This explains the rapid decrease in the amount of adenosine when the samples were not kept frozen. For this reason, all the samples were rigorously treated in the same manner and in the same time. In a routine analysis of adenosine, an adenosine deaminase inhibitor could be added. In the samples tested we have not found important differences in the qualitative and quantitative composition of synovial fluid, therefore the chromatographic method was always proved suitable to determine the amount of adenosine. The treatment of clinical samples with ialuronidase was very effective in order to make the sample more fluent and allow for direct injection. After fifty injections, the back-pressure of the system continued to be quite low and no obstraction effects were noted. At the same time the column did not loss efficiency.

4. Conclusions

The chromatographic method herewith described allows us to quantify adenosine in synovial fluid. In particular, it selectively allows for the determination of adenosine in spite of the fact that the peaks of the matrix components take up the chromatogram until 25 min.

Another considerable advantage was the good sensitivity, which was completely suitable to quantify the real concentration of adenosine in synovial fluid. Both these peculiarities have made the application of this method possible to study new correlations between adenosine and rheumatoid arthritis [9] and therefore to better understand the mechanism of this illness.

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