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Development and validation of a high-performance liquid chromatography assay and a capillary electrophoresis assay for the analysis of adenosine and the degradation product adenine in infusions

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Abstract

A high-performance liquid chromatography (HPLC) method and a capillary electrophoresis (CE) method for the analysis of adenosine and the degradation product adenine in infusion solutions have been developed and validated. The HPLC separation of the analytes was achieved on a RP-18 column, using a mobile phase, consisting of 20 mM ammonium acetate, pH 6.0, containing 5% of acetonitrile at a flow rate of 1 ml/min. Thymidine was used as internal standard. The CE separation was performed in a fused-silica capillary with a 100 mM sodium phosphate buffer, pH 2.7, at an applied voltage of 25 kV, using cytidine as internal standard. The assays were validated with regard to linearity, range, limit of detection (LOD), limit of quantitation (LOQ), specificity, and precision. Both methods were specific allowing reliable quantification of the analytes. Compared to the CE method, HPLC analysis yielded a two- to five-fold lower LOD. With respect to analysis time, CE was faster than HPLC. The applicability of both methods for the determination of the purity and stability of adenosine in the infusion solutions is demonstrated.

Keywords: Adenosine; Validation; Liquid chromatography; Capillary electrophoresis; Infusion

1. Introduction

Adenosine (Fig. 1) is a potent agent for the treatment of paroxsymal supraventricular tachycardia [1]. In Germany, the compound also used as a diagnostic agent in cardiology [2]. During cardiac catheterization adenosine is infused to produce a maximal hyperemia. At steady state, the coronary blood flow reserve is measured as an indicator of stenosis. The standard intravenous dose of adenosine for diagnostic use is 140 μg/kg/min. Due to the high price of commercial intravenous adenosine solutions, an infusion solution of the drug is produced at the hospital pharmacy of the University of Jena at a concentration of 2.8 mg/ml adenosine in 0.9% sodium chloride solution. This preparation requires analytical methods to test the purity and stability of the drug in saline.

The stability of adenosine in aqueous solutions has been investigated by HPLC. Naud et al. separated adenosine and the hydrolysis product adenine (Fig. 1) on a RP-18 column using a phosphate buffer, pH 3.7, containing 10% (v/v) methanol as the mobile phase [3]. Proot et al. analyzed the compounds also on a RP-18 column with a mobile phase consisting of a potassium phosphate buffer, pH 6.0, containing 7% (v/v) methanol as modifier [4]. However, when applied to the analysis of infusion solutions in our laboratory both methods were unsuitable due to shifting retention times of the analytes under the experimental conditions applied by us.

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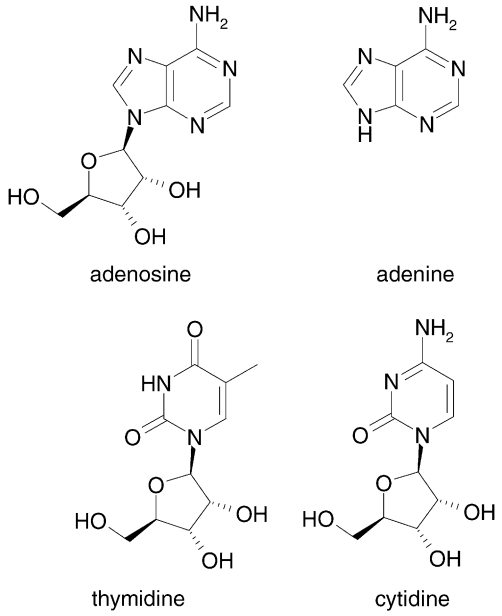

Fig. 1. Structures of adenosine, the degradation product adenine, and the internal standards thymidine and cytidine.

In recent years, capillary electrophoresis (CE) has emerged as a powerful separation technique that requires only small amounts of samples and chemicals. CE has also been applied to study the stability of drugs. Several reports describe the separation of adenine and adenosine along with other purine and pyrimidine bases as well as nucleosides and nucleotides by CE [5–8] including their analysis in biological samples, such as urine [9] or human cord plasma [10], in *Xenopus* oocytes [11], or in beer samples [12]. However, the analysis of adenosine and adenine in pharmaceuticals, has not been investigated by CE, to date. Accordingly, the aim of this study was to develop, validate, and compare HPLC and CE assays for the analysis of the purity and stability of adenosine infusions produced by hospital pharmacy of the University of Jena.

2. Experimental

2.1. General

Adenosine was purchased from BUFA B.V., Pharmaceutical Products (Uitgest, Netherlands). Adenine, cytidine, thymidine, and HPLC grade acetonitrile were obtained from Sigma-Aldrich (Deisenhofen, Germany). Monobasic sodium phosphate, ammonia, phosphoric acid, and acetic acid were from E. Merck (Darmstadt, Germany). All chemicals were purchased at the highest purity available and used without further purification. Sodium chloride solution (0.9%, w/v) was obtained from Braun–Melsungen (Melsungen, Germany). All buffers were prepared in double-distilled water and filtered (0.45 μm). Stock solutions of the compounds were prepared and diluted in physiological saline solution.

2.2. Instrumentation

2.2.1. HPLC

HPLC was performed on a Dionex instrument (Sunnyvale, CA) equipped an ASI-100 autosampler and an 170S UV–vis detector set at 260 nm. Chromeleon^{TM}, version 6.20 (Dionex) was used for data aquisition. Separation of analytes was performed on a C-18 reverse-phase column Acclaim^{TM}, 250 × 4.6 mm, 5 μm (Dionex, Idstein, Germany). The mobile phase consisted of 20 mM ammonium acetate buffer, pH 6.0, containing 5.0% (v/v) acetonitrile. The flow rate was 1.0 ml/min. UV detection was performed at 260 nm. Quantitation of the analytes was based on the peak area ratio method. All analyses were performed at room temperature.

2.2.2. Capillary electrophoresis

CE experiments were performed on a Beckmann P/ACE 5500 instrument (Beckmann Coulter GmbH, Unterschleißheim, Germany) equipped with a diode array detector, using 50 μm i.d. and 365 μm o.d. fused-silica capillaries (Polymicro Technologies, Phoenix, Arizona, USA) at 20 °C. The effective length of the capillary was 40 cm, the total length was 47 cm. UV detection at 260 nm was performed at the cathodic end of the capillary. Samples were injected hydrodynamically at a pressure of 0.5 p.s.i. for 3 s. The applied voltage was 25 kV. The run buffer consisted of 100 mM monobasic sodium phosphate adjusted to pH 2.7 by the addition of 100 mM phosphoric acid. The buffer solution was filtered (0.45 μm) and degassed by sonication. New capillaries were conditioned by rinsing for 20 min with 100 mM phosphoric acid, 20 min with water, and 20 min with the run buffer. Between analyses, the capillary was rinsed with 100 mM NaOH for 1 min followed by the run buffer for 2 min. Quantitation was performed, using the corrected peak area ratio method.

2.3. Incubations

Solutions of adenosine at a concentration of 2.8 mg/ml were prepared in 0.9% aqueous sodium chloride and incubated at 120 °C. At selected intervals, 5 ml aliquots were withdrawn and diluted 10-fold with distilled water containing thymidine or cytidine as internal standards for HPLC and CE, respectively. The final concentration of thymidine was 242 μg/ml, the final concentration of cytidine was 200 μg/ml. The samples were thoroughly vortexed and stored at −20 °C until analyzed.

3. Results and discussion

3.1. Method development

3.1.1. HPLC

Initial experiments on the reversed-phase separation of adenosine and adenine were performed using a mobile phase

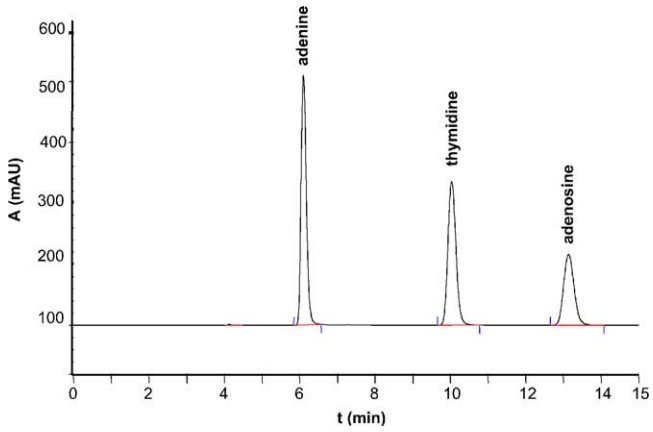

Fig. 2. Typical chromatogram of adenosine, adenine, and the internal standard thymidine. Analytical conditions: Acclaim^{TM}, 250 mm × 4.6 mm, 5 μm; mobile phase: 20 mM ammonium acetate buffer, pH 6.0, containing 5% acetonitrile; flow rate: 1.0 ml/min; UV detection: 260 nm.

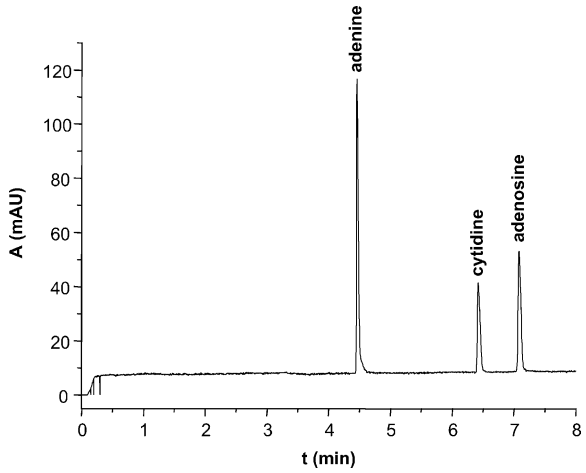

Fig. 3. Typical electropherogram of adenosine, adenine, and the internal standard cytidine. Analytical conditions: 47/40 cm capillary, 50 μm i.d.; 100 mM sodium phosphate buffer pH 2.7; applied voltage: 25 kV; UV detection: 260 nm.

consisting of 10 mM monobasic potassium phosphate buffer, pH 3.7 or 6.0, which contained 10% (v/v) methanol as described in refs. [3,4]. However, retention times of adenosine and adenine drifted considerably when analyzed on the Acclaim^{TM} RP-column. Changing the buffer to 20 mM ammonium acetate, pH 6.0, containing 10% (v/v) methanol improved the reproducibility of the retention times, but the analysis time exceeded 18 min. Analysis time was subsequently shortened to 13 min by replacing the content of the organic modifier methanol of 10% (v/v) with 5% (v/v) acetonitrile. Thymidine was selected as internal standard as its structure is similar to adenine and because it eluted between adenosine and adenine. A representative chromatogram is shown in Fig. 2. These experimental conditions were used for method validation.

3.1.2. Capillary electrophoresis

For the development of the CE assay, phosphate buffers in the pH range 2.5–4.0 were investigated. Under these conditions the compounds are protonated and migrate to the detector as cations. As thymidine is uncharged under these conditions, cytidine was selected as internal standard for CE analysis to compensate for injection errors and fluctuations of migration times. Optimized conditions employed a background electrolyte consisting of a 100 mM sodium phosphate buffer, pH 2.7, and an applied voltage of 25 kV. Separation was completed within 8 min (Fig. 3). These experimental conditions were used for method validation.

3.2. Method validation

Both methods were validated with respect to linearity, range, limit of quantitation (LOQ) and limit of detection (LOD) as well as precision. The terms are used according to the definition of the ICH guideline Q2B [13].

Adenosine was calibrated in the concentration range 5–400 μg/ml in both assays, while adenine was determined in the range 1–200 μg/ml for HPLC and 5–200 μg/ml for CE. Calibration curves were constructed from six or seven different concentrations. Each concentration was prepared in triplicate and each individual solution was injected twice. The data are summarized in Table 1. Linear relationships with a regression coefficient R of at least 0.9990 were obtained. At the lowest concentrations assayed, the signal/noise ratio was at least 10:1, and this concentration was subsequently regarded as LOQ for the present assays. The LOD defined as a signal/noise ratio of 3:1 was 1.2 and 0.3 μg/ml for adenosine and adenine, respectively, in the HPLC assay. In CE, the LOD values of 2.5 μg/ml (adenosine) and 1.5 μg/ml (adenine) were two- to five-fold higher compared to HPLC. On a molar basis, these values correspond to 4.5 and 9.4 nM for adenosine in HPLC and CE, respectively, and to 2.2 nM (HPLC) and 11.0 nM (CE) for adenine. The differences in LOD between adenosine and adenine in the respective assays are caused by the different molar absorptivities of the compounds. Generally, the LOD may be improved in CE by using a capillary with a larger inner diameter or by applying

Table 1
Calibration data of adenosine and its degradation product adenine for LC and CE

	Adenosine		Adenine	
	LC	CE	LC	CE
Range (μg/ml)	5–400	5–400	1–200	5–200
R	0.9999	0.9993	0.9998	0.9990
LOD				
(μg/ml)	1.2	2.5	0.3	1.5
(nM)	4.5	9.4	2.2	11.0
LOQ				
(μg/ml)	5	5	1	5
(nM)	18.7	18.7	7.4	37.0

Table 2
Intra-day precision and inter-day precision of the concentrations adenosine and adenine vs. the internal standard thymidine of the LC assay and vs. the internal standard cytidine of the CE assay

	Intra-day precision[a]		Inter-day precision[a]	
	c (μg/ml)	R.S.D. (%)	*c* (μg/ml)	R.S.D. (%)
Adenosine				
LC	50.5	1.19	50.4	0.29
	200.7	0.26	200.48	0.12
CE	51.1	1.82	51.9	1.46
	203.0	0.93	206.8	1.92
Adenine				
LC	50.2	0.42	50.3	018
	200.9	0.44	200.5	0.17
CE	50.5	1.73	48.8	3.58
	190.1	2.90	190.6	0.87

[a] Six samples were analyzed within 24 h (intra-day precision) or on three consecutive days (inter-day precision). Each sample was injected twice.

stacking techniques for on-line analyte concentration [14]. However, this was not evaluated in the present assay.

Repeatability and intermediate precision of the methods were evaluated by analyzing two concentrations of approximately 50 and 200 μg/ml in six independent series on the same day (repeatability of intra-day precision) and on three consecutive days (intermediate precision of inter-day precision). Within these series, each sample was analyzed twice. The data are summarized in Table 2. Generally, higher precision was found for the HPLC assay with relative standard deviations (R.S.D.) varying between 0.12 and 1.19%. Despite the use of an internal standard to compensate for injection errors and fluctuations of migration times, high R.S.D. values between 0.87 and 3.58% were observed in CE. However, these values are still acceptable for the quantitative analysis of the compounds [13]. Both assay methods are selective and sensitive and, therefore, suitable for the analysis of adenosine and its degradation product adenine. The HPLC method is somewhat more sensitive than the CE, but this difference can readily be compensated for by increasing concentrations of the sample solutions by reducing the dilution of the test infusion solutions prior to analysis (see below), by increasing the inner diameter of the capillary or by stacking procedures. HPLC also exhibited low R.S.D. values in the tests of repeatability and intermediate precision. Advantages of CE are a shorter time of analysis, avoiding organic solvents, and more importantly, less consumption of chemicals, resulting in lower overall costs as well as a greater environmental safety.

3.3. Application of the methods

The assays were applied to the analysis of adenosine infusion solutions prepared at the hospital pharmacy of the

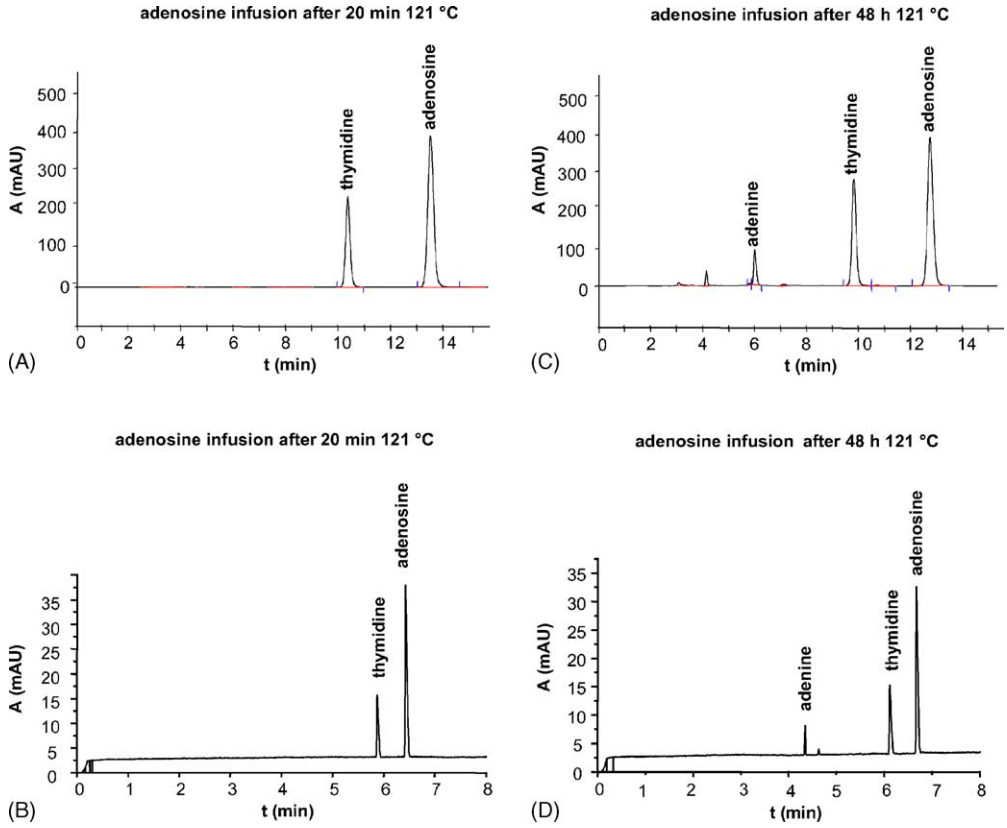

Fig. 4. Typical chromatogram (A, C) and electropherogram (B, D) of adenosine infusion solutions after sterilization and after 48-h incubation at 120 °C. For experimental conditions of LC see Fig. 2 for experimental conditions of CE, see Fig. 3.

University of Jena. The solutions contained adenosine at a concentration of 2.8 mg/ml in 0.9% aqueous sodium chloride solution. The rate of degradation of adenosine in aqueous solutions is known to be pH-dependent [15,16]. The pH of freshly prepared solutions was 7.0 and it remained stable even upon prolonged heating at 121 °C. Adenine was identified by co-injection of a standard solution.

Fig. 4 shows representative chromatograms and electropherograms of adenosine infusion solutions following sterilization of the product at 121 °C for 20 min and after further heating at this temperature for 48 h. No degradation was observed by either method following the 20-min sterilization procedure (Fig. 4 A and B). The concentration of adenosine in the infusion solution as determined by HPLC and CE was 2.83 and 2.89 mg/ml, respectively. In contrast, following prolonged incubation at 121 °C for 48 h adenine could be identified as a degradation product (Fig. 4C and D). Concentrations of 0.13 and 0.16 mg/ml of adenine were determined by HPLC and CE, respectively. These findings indicate that both new assays can be applied to study degradation kinetics of adenosine to adenine in aqueous solutions, although this was not the primary aim of the present work.

4. Conclusions

Sensitive, specific and reliable new HPLC, and CE methods have been developed and validated for the analysis of adenosine and adenine in infusion solutions of adenosine. The HPLC method displayed low R.S.D. value for repeatability and intermediate precision. Advantages of CE, where there is shorter analysis time and lower consumption of chemicals. Both assays were applied to the analysis of adenosine and adenine in aqueous solutions for clinical use as intravenous infusions following sterilization at 121 °C for 20 min and prolonged heating at 121 °C for 48 h. The degradation product adenine could not be detected after 20 min, but degradation of adenosine occurred with prolonged heating. In conclusion, both new assay methods can be applied for quality control of the purity and stability of adenosine infusion solutions. They are also suitable for studies of the degradation kinetics of adenosine in aqueous solution.

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