Ion Chromatography Assay for Ammonia in Adenosine

Lipika Basumallick and Jeffrey Rohrer
Thermo Fisher Scientific, Sunnyvale, CA USA

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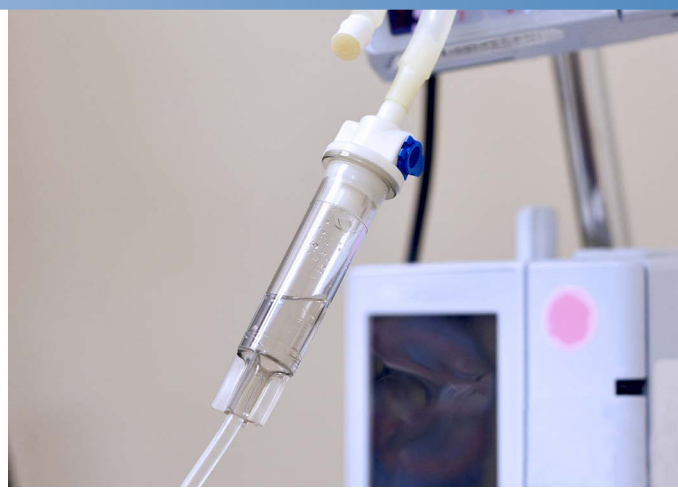
Suppressed Conductivity, Fast Separation, Pharmaceuticals, Drug Products, Dionex IonPac CS12A-5 µm Column



Introduction

Adenosine (Figure 1) is a naturally occurring purine nucleoside that forms from the breakdown of adenosine triphosphate, the primary energy source for living cells. This nucleoside is composed of adenine attached to ribose in the furanose conformation via a β-N9-glycosidic bond. The major therapeutic uses of adenosine are for treating surgical and nerve pain, pulmonary hypertension, and irregular heartbeat; for controlling blood pressure during anesthesia/surgery; and for cardiac stress tests. For these indications, adenosine is administered either as a bolus intravenous injection or as an intravenous infusion.

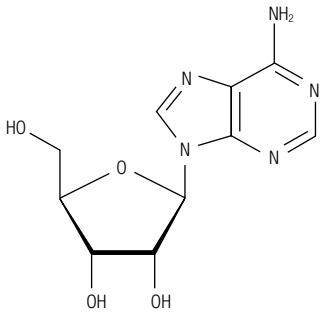


Figure 1. The structure of adenosine.

Adenosine is produced by the condensation of ribose and adenine biochemically or by fermentation. Ammonia is an impurity in adenosine preparations and the U.S. Pharmacopeia (USP) adenosine monograph describes an assay to determine the amount of ammonia in adenosine. That assay measures ammonia by comparing the color of an adenosine sample and a 0.4 mg/L ammonium chloride standard solution after the addition of an alkaline mercuric/potassium iodide solution to each. The acceptance criteria is defined as "The Sample solution does not exhibit a more intense yellow color than that of the Standard solution (NMT [not more than] 4 ppm of ammonia)."[1] However, that assay is subjective, potentially exposes the analyst to mercury, and generates hazardous waste.

Goal: To develop an ion chromatography (IC)-based method for the determination of ammonia in adenosine that can meet the assay requirements and replace the USP adenosine monograph's color-based method

Thermo SCIENTIFIC

Equipment and Software

- Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free™ HPIC™ system, capable of supporting high-pressure IC, including:
  - SP Single Pump or DP Dual Pump
  - EG Eluent Generator
  - DC Detector/Chromatography Compartment
  - CD Conductivity Detector
- Thermo Scientific Dionex AS-AP Autosampler with 10 µL PEEK Sample Loop (P/N 036104)
- Thermo Scientific Dionex EGC III Methanesulfonic Acid (MSA) Eluent Generator Cartridge (P/N 074535)
- Thermo Scientific Dionex CR-CTC Continuously Regenerated Cation Trap Column (P/N 066262)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software

Reagents and Standards

- Deionized (DI) water, 18 MΩ-cm resistance or better
- Combined Six-Cation Standard-I (Fisher Scientific P/N DX040187)
- Adenosine 99+% (Fisher Scientific P/N AC16404-0050)
- Ammonia Standard, 1000 mg/mL in water (Fisher Scientific P/N US-ICC-101))

Conditions	
Columns:	Thermo Scientific™ Dionex™ IonPac™ CG12A-5 µm Guard, 3 × 30 mm (P/N 057184) Dionex IonPac CS12A-5 µm Analytical, 3 × 150 mm (P/N 057185)
Eluent:	33 mM MSA
Flow Rate:	0.7 mL/min
Inj. Volume:	10 µL
Column Temp:	30 ºC
Detector Temp:	30 ºC
Detection:	Suppressed conductivity, Thermo Scientific™ Dionex™ CSRS™ 300 Cation Self-Regenerating Suppressor, 2 mm (P/N 064557) or Dionex SC-CSRS 300 Salt-Converter Cation Self-Regenerating Suppressor (P/N 067529), autosuppression recycle mode, power setting 68 mA
Back Pressure:	2600 psi
Background Conductance:	<0.250 µS
Noise:	~0.1–0.2 nS

Preparation of Solutions and Reagents

Ammonium Standards

Prepare standards gravimetrically by making appropriate dilutions of a commercial 1000 mg/L standard with DI water. Store standard solutions at 4 °C when not in use.

Sample Preparation

Adenosine sample solution (use sample within 24 h)

In the current USP monograph for ammonia assay in adenosine, sample preparation steps are: (i) suspend 0.5 g in 10 mL of water, (ii) stir for 30 s, (iii) pass through a coarse filter, (iv) dilute the filtrate to 15 mL and use the filtrate for the assay.[1]

For this IC-based method, prepare adenosine samples according to the USP monograph steps described above, except do not dilute the filtrate to 15 mL; instead, directly inject the filtrate into the IC system.

General Design of Robustness Study

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in the procedural parameters. The robustness of each method was studied by determining the response of a 1 mg/L ammonia standard solution under typical variations of analytical conditions. Variations in the following were tested:

- Mobile phase concentration (±2 mM MSA*)
- Flow rate (±10%)
- Temperature of column (±2 °C)
- Column-to-column (column sets from two different production batches, Column Sets 1 and 2)

** Not typical for eluent generation but rather for manually prepared eluents*

Results

Separation

For determining ammonia in adenosine, a medium-capacity cation-exchange column designed for fast isocratic separation of common cations was selected. Figure 2 shows separation of ammonium in Combined Six-Cation Standard-I and adenosine (50 mg/mL) using a Dionex IonPac CS12A-5 µm column set. Figure 2, Chromatograms A and B show that ammonia elutes at 2.1 min with a peak resolution value of 2 (relative to sodium) and a peak asymmetry value of 1.2. The total run time is 5 min, which enables high sample throughput.

Figure 2, Chromatogram B shows the determination of ammonia in an adenosine sample, prepared as described in the current USP National Formulary (USP-NF), except for the final dilution step. The filtrate was directly injected into the IC system. The amount of ammonia in the adenosine sample is 0.04 mg/L, well under the limit of 4 mg/L.

Columns:	Dionex IonPac CG12A-5 µm Guard Dionex IonPac CS12A-5 µm Analytical
Eluent:	33 mM MSA
Flow Rate:	0.7 mL/min
Inj. Volume:	10 µL
Temp:	30 °C

Peaks:	A	B	
1. Lithium	—	—	
2. Sodium	—	—	
3. Ammonium	2.5	0.04	mg/L
4. Potassium	—	—	
5. Magnesium	—	—	
6. Calcium	—	—	

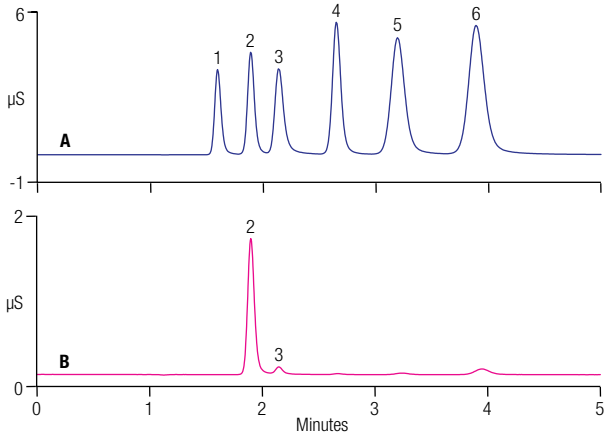


Figure 2. (A) Combined Six-Cation Standard-I and (B) adenosine (50 mg/mL) on the Dionex IonPac CS12A-5 µm column.

Accuracy

Method accuracy was verified by determining recoveries of ammonium in spiked adenosine samples over three consecutive days (Table 1). The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)—the combined pharmaceutical regulatory authority for Europe, Japan, and U.S.—and the USP General Chapter <1225> guidelines recommend that a minimum of nine measurements (i.e., three concentration levels and three replicates of each concentration) be used to determine accuracy.[2,3]

Table 1. Accuracy.

Day	Spike Level (mg/L)	Replicate #	Amt. Measured (mg/L)	Recovery (%)
1	0.05	1	0.093	110
		2	0.093	110
		3	0.093	109
	1	1	1.025	99
		2	1.026	99
		3	1.027	99
	2.5	1	2.250	88
		2	2.259	89
		3	2.258	89
2	0.05	1	0.088	100
		2	0.088	100
		3	0.090	104
	1	1	1.075	104
		2	1.070	103
		3	1.073	104
	2.5	1	2.347	92
		2	2.347	92
		3	2.345	92
3	0.05	1	0.090	103
		2	0.091	106
		3	0.096	116
	1	1	1.066	103
		2	1.068	103
		3	1.090	105
	2.5	1	2.334	92
		2	2.333	92
		3	2.332	92

The amount of ammonium in the adenosine sample was 0.034 mg/L. The samples were spiked with 0.05, 1, and 2.5 mg/L ammonium. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The average recovery of ammonium ranged from 88 to 116%.

Precision

The precision of an analytical procedure is typically expressed as the RSD of a series of measurements. It is determined by assaying a sufficient number of aliquots of a sample that have undergone the complete analytical procedure from sample preparation to final test.

Table 2. Precision for ammonia.

Day	Sample	Retention Time (min)	Retention Time RSD	Peak Area (µS * min)	Peak Area RSD	Asymmetry	Resolution
1	1 mg/L Standard	2.144	0.09	0.165	0.19	1.3	—
	4 mg/L Standard	2.148	0.09	0.4385	0.08	1.4	—
	10 mg/L Standard	2.150	<0.01	0.9416	0.22	1.6	—
	Adenosine Solution	2.147	<0.01	0.0055	1.83	1.2	2.2
2	1 mg/L Standard	2.144	0.09	0.1651	0.88	1.4	—
	4 mg/L Standard	2.148	0.09	0.4403	0.01	1.4	—
	10 mg/L Standard	2.152	0.09	0.9433	0.24	1.6	—
	Adenosine Solution	2.144	<0.01	0.0051	2.27	1.2	2.2
3	1 mg/L Standard	2.149	0.09	0.1673	0.34	1.3	—
	4 mg/L Standard	2.152	0.09	0.4451	0.06	1.4	—
	10 mg/L Standard	2.158	0.09	0.9561	0.38	1.6	—
	Adenosine Solution	2.151	0.09	0.0052	1.79	1.2	2.2

The ICH guidelines recommend that repeatability be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration).[3] The retention time RSDs were <0.1 and the peak area RSDs ranged from 0.01 to 2.3 (Table 2).

Detection Limit

The limit for ammonia in adenosine in the current monograph is NMT 4 ppm (i.e., mg/L) of ammonia.

USP/ICH defines limit of detection (LOD) in terms of the signal-to-noise ratio (S/N) as 2:1 or 3:1 and limit of quantitation (LOQ) as 1:10. Currently, USP defines S/N as 2H/h, where H is the peak height from the middle of the noise band to the top of the peak. The value of h is the "difference between the largest and smallest noise values observed equal to at least five times the width at the half height of the peak and, if possible, situated equally around the peak of interest" or the "range of the noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to at least five times the width at half height of the peak in the chromatogram obtained with the prescribed reference solution, and if possible, situated equally around the place where this peak is found."[2,3]

According to the new recommendation outlined in Hinshaw and Dolan's article, S/N must be measured as S/Np-p, with S defined as measurement of peak height from the middle of a noise band to the highest point of the peak (this is synonymous with H); Np-p is defined as peak-to-peak baseline noise, or noise measured over ≥5× peak width measured at half of the peak height.[4]

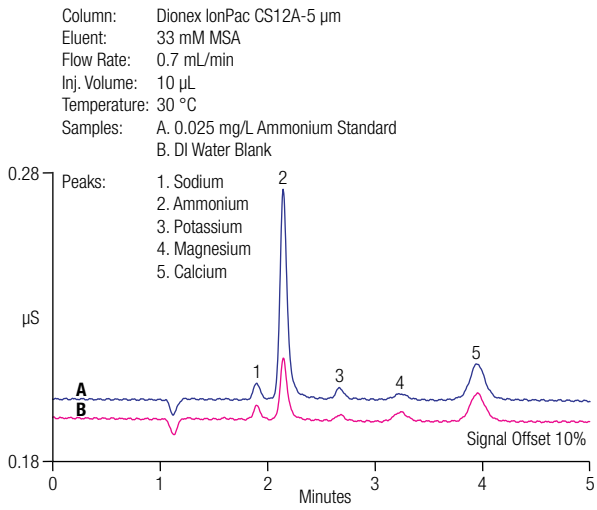


Figure 3. Chromatograms of (A) a 0.025 mg/L ammonium standard and (B) a DI water blank.

For this IC method, the LOD and LOQ for ammonia in adenosine—i.e., concentrations that resulted in peaks that were 3× and 10× of the noise (both as h and Np-p)—are 0.001 and 0.004 mg/L, respectively.

Figure 3 shows ammonium at 0.025 mg/L (Chromatogram A) and a DI water blank with a background level of ~0.008 mg/L ammonia (Chromatogram B).

Linearity

The USP/ICH recommendations for establishing linearity of an impurity in a drug substance or a finished product are a minimum of five concentrations ranging from 50 to 120% of the acceptance criteria.[2,3]

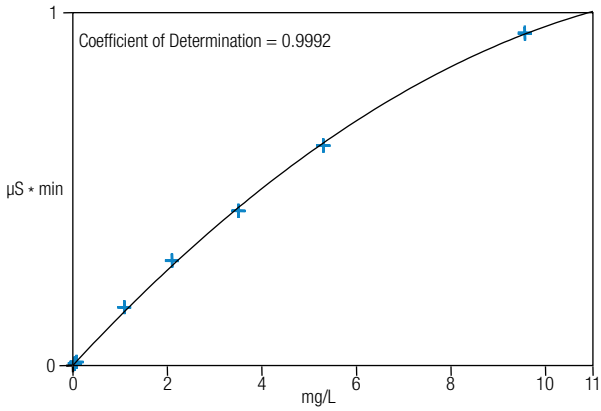


Figure 4. Calibration curve for ammonium using a Dionex CSRS 300 cation self-regenerating suppressor.

For ammonium, linearity was investigated in the range of 0.025 to10 mg/L (0.025, 0.05, 1.0, 2.0, 4.0, 6.0, and 10.0 mg/L). The highest concentration investigated was 2.5× the acceptance criteria (NMT 4 mg/L) for ammonia in adenosine. Weak bases—like ammonium—are partially dissociated and thus give a nonlinear response as the concentration increases. The coefficient of determination was 0.9992 using a quadratic curve-fitting function for ammonium (Figure 4) in the range of 0.025 to 10 mg/L.

For a narrow range (0.025–2 mg/L), response can be linear and the coefficient of determination for a linear fit is 0.9984. Linear response can be extended by converting

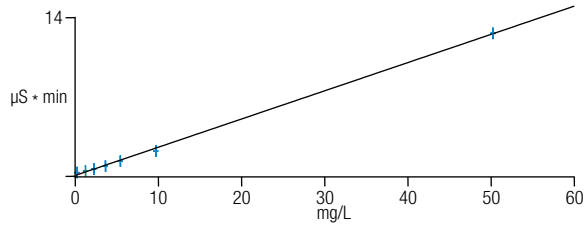


Figure 5. Calibration plot for ammonium using a Dionex SC-CSRS 300 suppressor.

the weak base to the fully ionized salt form using a Dionex SC-CSRS 300 suppressor. Figure 5 shows the linear calibration plot for ammonium with a Dionex SC-CSRS 300 suppressor over the 0.025–50 mg/L range (coefficient of determination 0.9989).

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in the procedural parameters.

An ammonium standard (1 mg/L) was used as the check standard. The data are summarized in Table 3. The peak asymmetry for ammonia ranged from 1.3 to 1.4 (USP) in the 1 mg/L standard and 1.2 to 1.25 in the adenosine solution. The resolution of ammonia from sodium in the adenosine solution ranged from 1.96 to 2.32 under the different conditions listed in Table 3. The retention time (RT) RSD was <0.2 and the peak area RSD ranged from 0.17 to 1.8.

Table 3. Robustness.

Condition	Sample	RT (min)	RT RSD	Peak Area (µS * min)	Peak Area RSD	Asymmetry (USP)	Resolution (USP)
Column Set 1							
30 °C, 0.7 mL/min, 33 mM MSA	Combined Six-Cation Standard-I	2.14	0.19	0.3226	0.17	1.36	1.96
30 °C, 0.7 mL/min, 33 mM MSA	1 mg/L Standard	2.144	0.09	0.165	0.19	1.33	—
30 °C, 0.7 mL/min, 33 mM MSA	Adenosine Solution	2.147	<0.01	0.0055	1.83	1.2	2.24
30 °C, 0.7 mL/min, 35 mM MSA	1 mg/L Standard	2.082	0.11	0.1675	0.53	1.4	—
30 °C, 0.7 mL/min, 35 mM MSA	Adenosine Solution	2.083	<0.01	0.0058	0.87	1.22	2.14
30 °C, 0.7 mL/min, 31 mM MSA	1 mg/L Standard	2.223	<0.01	0.1669	1.12	1.34	—
30 °C, 0.7 mL/min, 31 mM MSA	Adenosine Solution	2.225	0.11	0.006	0.75	1.2	2.32
32 °C, 0.7 mL/min, 33 mM MSA	1 mg/L Standard	2.115	0.11	0.1673	0.14	1.37	—
32 °C, 0.7 mL/min, 33 mM MSA	Adenosine Solution	2.117	<0.01	0.0061	0.39	1.22	2.19
28 °C, 0.7 mL/min, 33 mM MSA	1 mg/L Standard	2.17	0.22	0.1663	0.79	1.36	—
28 °C, 0.7 mL/min, 33 mM MSA	Adenosine Solution	2.17	<0.01	0.0061	0.73	1.22	2.23
30 °C, 0.65 mL/min, 33 mM MSA	1 mg/L Standard	2.287	<0.01	0.1763	0.26	1.35	—
30 °C, 0.65 mL/min, 33 mM MSA	Adenosine Solution	2.283	<0.01	0.0067	1.72	1.23	2.22
30 °C, 0.75 mL/min, 33 mM MSA	1 mg/L Standard	2.017	<0.01	0.1533	0.20	1.36	—
30 °C, 0.75 mL/min, 33 mM MSA	Adenosine Solution	2.02	<0.01	0.0057	1.23	1.25	2.18
Column Set 2							
30 °C, 0.7 mL/min, 33 mM MSA	Combined Six-Cation Standard-I	2.16	<0.01	0.3111	<0.01	1.23	1.98
30 °C, 0.7 mL/min, 33 mM MSA	1 mg/L Standard	2.162	0.09	0.1667	0.87	1.29	—
30 °C, 0.7 mL/min, 33 mM MSA	Adenosine Solution	2.168	0.09	0.0052	1.49	1.2	2.17

Conclusion

The Dionex IonPac CS12A-5 µm column with its small-diameter resin enables a fast and accurate isocratic method for the determination of ammonia in adenosine. A run time of 5 min yields high sample throughput. The electrolytically generated eluent and the self-regenerating suppressor contribute to the robustness and sensitivity of the method. In addition, only water is used for eluent generation; no hazardous chemicals are required.

The precision (RT RSD <0.1, peak area RSD <2), accuracy (average recovery 88–116%), linearity, detection and quantitation limits, and robustness for the assays presented in this study show that the proposed IC method for ammonia determination in adenosine meets the analytical performance characteristics outlined in USP General Chapter <1225>.

References

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