Adenosine Stability in Cardioplegic Solutions

Danny W. C. Lau, Scott E. Walker, Stephen E. Fremes and John Iazzetta

ABSTRACT

Adenosine is currently used for the treatment of supraventricular tachycardia and for pharmacologic dilation associated with thallium imaging. The cardioprotective role of adenosine has been thoroughly evaluated in experimental ischemia-reperfusion models. There exists a strong rationale to supplement cardioplegic solutions with adenosine during coronary bypass surgery, however, human studies have not been conducted and the stability of these solutions is unknown.

The objective of this study was to evaluate the stability of adenosine solutions containing 0.08 and 0.33 mg/mL of adenosine in cardioplegic solutions using a validated, stability-indicating, liquid chromatographic method during 14-days storage at 4°C and room temperature (23°C). Physical inspections and pH determinations were also completed on each of the nine study days during the 14-day storage period.

During the 14-day study period all solutions stored at room temperature (23°C) and in the refrigerator (4°C) retained more than 90% of the initial adenosine concentration. The pH in solutions stored at both 23°C and 4°C did not change by more than 0.5 of a pH unit in any solution over the 14-day study period.

We conclude that adenosine solutions of 0.08 and 0.33 mg/mL stored at 4°C or 23°C for 14 days are stable and retain more than 90% of the initial adenosine concentration during the storage period.

Key Words: *adenosine, adenine, stability, cardioplegic solutions*

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RÉSUMÉ

L'adénosine est actuellement utilisée pour le traitement de la tachycardie supraventriculaire et pour ses propriétés dilatatrices dans l'imagerie au thallium. Le rôle cardioprotecteur de l'adénosine a fait l'objet d'évaluations rigoureuses dans des modèles expérimentaux d'ischémie-reperfusion. Il existe une forte logique qui veut que l'on ajoute de l'adénosine aux solutions cardioplégiques au cours d'un pontage coronarien. Cependant, aucune étude chez l'humain n'a été menée et la stabilité de ces solutions demeure inconnue.

L'objectif de cette étude était d'évaluer la stabilité de solutions cardioplégiques contenant 0,08 et 0,33 mg/mL d'adénosine, à l'aide d'une technique de chromatographie liquide validée mesurant la stabilité des solutions entreposées durant 14 jours à une température d'une part de 4°C et d'autre part à la température ambiante de 23°C. Les inspections physiques et la mesure du pH ont également été effectuées pour chacune des neuf journées d'étude de la période d'entreposage de 14 jours.

Toutes les solutions gardées à la température ambiante (23°C) et au réfrigérateur (4°C) ont conservé plus de 90% des concentrations initiales d'adénosine. Le pH des solutions conservées à 23°C et à 4°C n'a pas varié de plus de 0,5 d'une unité pH au cours de la période d'entreposage de 14 jours.

On a conclu que les solutions à 0,08 et 0,33 mg/mL d'adénosine conservées à 4°C ou à 23°C durant 14 jours sont stables et retiennent plus de 90% des concentrations originales d'adénosine au cours de la période d'entreposage.

Mots clés : *adénine, adénosine, solutions cardioplégiques stabilité,*

INTRODUCTION

Adenosine (9-beta-D ribofuranosyladenine) is an endogenous nucleic acid formed by glycosidic linkage between adenine and ribose. It is a naturally occurring substance widely distributed in nature and currently indicated for conversion of supraventricular tachycardia and for pharmacologic dilation associated with thallium imaging. The cardioprotective role of adenosine has been thoroughly evaluated in experimental laboratory models of ischemia-reperfusion.[1-3] The cardioprotective effect of adenosine has resulted in the initiation of a number of studies which utilize adenosine supplemented cardioplegic solutions during coronary bypass surgery.

As part of this investigational protocol, knowledge of the stability of adenosine in cardioplegic solutions was required. The manufacturer recommends that adenosine be given as a rapid intravenous injection and any unused portion of any vial be discarded immediately.[4] However, no information is provided concerning compatibility or stability in any solutions.[4]

Danny W. C. Lau, Dip Pharm Tech, is a Senior Research Assistant, Department of Pharmacy, Sunnybrook Health Science Centre, North York, Ontario.

Scott E. Walker, M.Sc.Phm. is Coordinator, Research and Quality Control, Department of Pharmacy and Division of Pharmacology, Sunnybrook Health Science Centre, and Associate Professor, Faculty of Pharmacy, University of Toronto.

Stephen E. Fremes, MD. is a Staff Surgeon, Division of Cardiovascular Surgery Department of Surgery, Sunnybrook Health Science Centre, and Assistant Professor, Faculty of Medicine, University of Toronto.

John Iazzetta, Pharm.D. is Coordinator of Drug Information, Department of Pharmacy and Division of Pharmacology, Sunnybrook Health Science Centre, and Assistant Professor, Faculty of Pharmacy, University of Toronto.

Address correspondence to: Scott Walker, Department of Pharmacy, Sunnybrook Health Science Centre, 2075 Bayview Avenue, North York, Ontario. M4N 3M5.

Therefore, the purpose of this investigation was to evaluate the chemical stability of adenosine in cardioplegic solutions containing high and low concentrations of potassium in order to determine reasonable expiry dates for adenosine in these solutions. In the study of adenosine as a cardioplegic additive during coronary bypass surgery, adenosine concentrations in blood cardioplegia were expected to range up to 0.066 mg/mL. Since blood cardioplegia is prepared by mixing four parts of oxygenated blood with a solution containing potassium, tris [hydroxymethyl] amino methane (THAM), magnesium, dextrose, adenine and adenosine,[5] concentrations of adenosine in the additive solution would have to range up to 0.33 mg/mL to achieve the required concentrations in blood. Therefore, in this study an upper concentration of 0.33 mg/mL was studied as well as a lower concentration of 0.08 mg/mL.

METHODS
Assay Validation
Following the development of the chromatographic system for adenosine, the suitability of this method for use as a stability-indicating assay was tested by accelerating the degradation of adenosine. 39.7 mg of adenosine, as the free base, (cat. # A9251; Lot # 129F0625; Sigma Chemical Company, St. Louis, MO) was dissolved in 50 mL of distilled water. The pH of 15 mL of this solution was adjusted to 0.8 with 5 N HCl and was placed in a glass vial and incubated in a water bath at 79°C protected from light for 76.5 hours. The pH of a second solution was not adjusted (pH = 6.2). Samples were drawn from both solutions just prior to incubation at 79°C and at 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 10, 24, 27, 29, 32, 34, 49.5, 53, 56, 73.5, and 76.5 hours. Chromatograms were inspected for the appearance of additional peaks and the adenosine peak was compared between samples

for changes in concentration, retention time, and peak shape.

Following this first phase of evaluation and validation, the accuracy and reproducibility of standard curves was tested over five days, and system suitability criteria (theoretical plates, tailing and retention time) were developed to ensure consistent chromatographic performance. On each day, 40 mg of adenosine powder, as the free base, (cat # A9251; lot #129F0625: Sigma Chemical Company, St Louis, MO) was dissolved in 100 mL of distilled water resulting in a final concentration of 0.40 mg/mL. Samples of this stock solution were diluted with water to obtain standards with final concentrations of 0.04, 0.08, 0.10, and 0.20 mg/mL. These standards served to construct a standard curve. Each sample was chromatographed in duplicate. As well, a 0.15 mg/mL sample of adenosine, as the free base, was prepared on each day, chromatographed and its concentration determined and compared to its known concentration. Intra-day error was assessed by the coefficient of variation of the peak area for six replicates each chromatographed in duplicate.

Stability Study
On study day zero, a total of 24 - 30 mL solutions containing adenosine were prepared. Adenocard® (adenosine, 3 mg/mL; lot #330548; exp. Jan 95: Fujisawa, Markham, Ontario) was diluted in cardioplegic solutions containing either 100 mmol/L of potassium [high] or 30 mmol/L of potassium [low], to prepare 12 - 30 mL solutions with final concentrations of adenosine of approximately 0.33 mg/mL and an additional 12 - 30 mL solutions containing approximately 0.08 mg/mL of adenosine. Three solutions of each adenosine concentration, potassium concentration combination were stored at room temperature (23°C ± 2°C) and the other three were refrigerated (4°C ± 2°C). An additional six high potassium cardioplegic

solutions were prepared, storing three at 4°C and three at 23°C and a further six low potassium cardioplegic solutions were prepared and served as blank controls for the study. Cardioplegic solutions, both high and low potassium also contain 12 mmol/L of THAM, 9 mmol/L of magnesium sulphate, 250 mmol/L of dextrose and 20 mL/L of citrate-phosphate-dextrose-adenine (CPD-adenine) solution.

Adenosine Analysis
Two solutions of each adenosine (0.08 and 0.33 mg/mL), potassium (100 mmol/L and 30 mmol/L) and temperature (4°C or 23°C) combination, as well as two blank solutions of each temperature and potassium combination were designated for LC analysis (16 different adenosine solutions and eight blank control solutions). From these solutions, samples were drawn on each of nine study days (0, 1, 4, 5, 6, 7, 8, 11, and 14). One microlitre of each sample was directly chromatographed in duplicate.

The standard curves were prepared daily by dissolving 40 mg of adenosine, as the free base, (cat # A9251; Lot # 129F0625: Sigma Chemical Company, St. Louis, MO) in 100 mL of distilled water. This stock solution was then diluted to prepare four additional concentrations ranging from 0.04 mg/mL to 0.2 mg/mL. One additional accurate weight of 15 mg of adenosine, as the free base, (cat # A9251; Lot # 129F0625: Sigma Chemical Company, St. Louis, MO) was also dissolved in 100 mL of distilled water and used to calculate accuracy. One microlitre of each of these standards and a blank were directly chromatographed in duplicate.

The liquid chromatographic (LC) system consisted of an isocratic solvent delivery pump (Model P100; Spectra Physics, San José, CA) which pumped a mixture of acetonitrile (cat.#A998; Fisher Scientific,

Toronto, Ontario), and 0.01 molar potassium phosphate monobasic (cat. #P286; Fisher Scientific, Toronto, Ontario) through a 25 cm x 4.2 mm reversed-phase C-18, 5 mm column (Ultrasphere ODS, #235329; Beckman, Mississauga, Ontario) at 1.0 mL/min. The ratio of acetonitrile to phosphate buffer was 5:95 and was held constant during a chromatographic run. On each day, the strength of the mobile phase was prepared to achieve a retention time for adenosine between 270 and 360 seconds. Samples were introduced into the LC system using an autoinjector (WISP 715; Waters Scientific, Toronto, Ontario).

The column effluent was monitored with a variable wavelength ultra-violet detector (Model 1050; Hewlett Packard; Waldbronn, FRG) at 220 nm. A signal from the detector was integrated and recorded with a chromatographic integrator (Model 4240; Spectra Physics, San José, CA). The area under the adenosine peak at 220 nm was subjected to least squares linear regression and the actual adenosine concentration in each sample determined by interpolation from the standard curve. Adenosine concentrations were recorded to the nearest 0.01 mg/mL.

Cardioplegic solutions contain 0.005 mg/mL of adenine. The concentration of adenine, also the major degradation product of adenosine, was not measured quantitatively against a standard curve during the study. Instead, the peak area of the adenine peak was recorded daily and compared between days for changes. Although actual concentrations cannot be reported because a standard curve for adenine was not prepared, changes in degradation product concentration between days can be monitored and are a more sensitive indicator of degradation.[6,7]

Physical Evaluation

One solution of each adenosine concentration (0.08 and 0.33 mg/mL),

potassium concentration (100 mmol/L and 30 mmol/L) and temperature (4°C or 23°C) combination, was designated for pH and physical evaluation (eight different solutions) as were four blank samples. Samples were drawn from each of these solutions on each of nine study days (0, 1, 4, 5, 6, 7, 8, 11, and 14). On each of the study days, a 1 mL sample was drawn, placed in a 10 x 75 mm glass test tube and inspected visually for colour and clarity. Visual particulate matter inspection was performed against a black and white background. The pH of each solution was then measured and recorded to the nearest 0.001 of a pH unit. The pH meter (Accumet-model 925; Fisher Scientific, Toronto, Ontario) was equipped with a microprobe glass body electrode (cat# 13-639-280; Fisher Scientific, Toronto, Ontario) and was standardized each day with two commercially available buffer solutions.

Data Reduction and Statistical Analysis

Means were calculated for analyses completed in duplicate. Error was assessed by the coefficient of variation (CV: standard deviation divided by the mean). Mean results from different days of an identical test were compared statistically by least squares linear regression to determine if an association existed between the observed result and time. Log-linear and linear-linear fits for the data from the accelerated degradation study (79°C) were compared for goodness of fit by the Maximum Likelihood Method of Box and Cox.[8,9] Analysis of variance and the least significant difference multiple range test was used to compare differences between temperature, and/or solutions for similar analytical tests. The 5% level was used as the a priori cut-off for significance and all reference to significance refers to this level.

Adenosine concentrations were considered "acceptable" or "within

acceptable limits" if the concentration on any day of analysis was not less than 90% of the initial (day-zero) concentration.

RESULTS
Accelerated Degradation and Assay Validation

The degradation of adenosine was observed to be pH dependant. No noticeable degradation of adenosine occurred at a pH of 6.2 solution over a 72.5 hour period at 79.1+0.2°C. However, at a pH of 0.8, 38.4% of the adenosine was lost within two hours and it had completely degraded after 10 hours. These data were not fit significantly better by either a first order or zero order rate. Three degradation products can be observed in chromatograms, although only one is visible at the scale in which Figure 1 is drawn. The major degradation product can be identified as adenine (inset - Figure 1). None of the degradation products interferes with adenosine quantification. The predictable degradation and the chromatographic separation of degradation products and adenosine, indicated that this analytical method was stability-indicating.[10-12]

Analysis of accuracy and reproducibility evaluations indicated that the adenosine concentration was measured accurately. Recovery, based on the mean of duplicate determinations of the 0.15 mg/mL quality control sample was within 95-103% of the theoretical concentrations. Analytical reproducibility, within a day (as measured by CV determined on six replicates) averaged 1%, for concentrations between 0.04 and 0.40 mg/mL. Inter-day reproducibility was assessed by analysis of the 0.15 mg/mL quality control sample determined on five consecutive days following storage at 4°C and averaged 4.8%. This indicates that differences of 10% or more can be confidently detected with acceptable error rates.[13] System suitability criteria were developed based on daily calculations
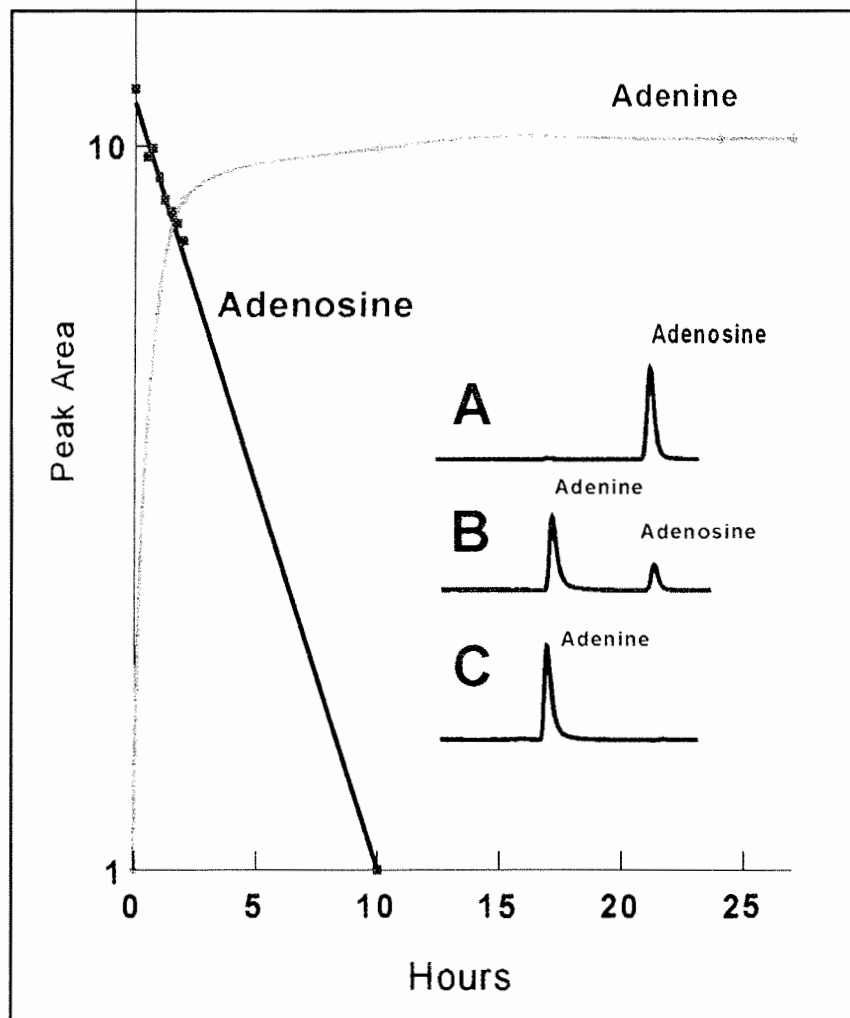
Figure 1: Rate of loss of adenosine during the accelerated study at a pH of 0.8 and 80°C. Log-linear coefficient of determination: $r^2 = 0.9892$ versus linear-linear: $r^2 = 0.9720$. As the adenosine concentration declined three products were observed; the major degradation product, adenine, and two minor unidentified products [which are not apparent in chromatograms drawn at this scale [see inset A, B and C above]. Inset panels A, B and C represent chromatograms of samples drawn at time zero, two hours (38.4% remaining) and 10 hours (no adenosine remaining), respectively.

of theoretical plates, tailing, retention time and accuracy observed during the validation period and were used to ensure continued chromatographic performance during the study period.

Adenosine Stability Study

Over the 14-day study period there was no significant change in adenosine concentration in any solution at either storage temperature (Table I). Due to the lack of degradation at both 4°C and 23°C, potassium concentration, adenosine concentration and storage temperature did not appear to affect

the degradation rate and confident estimates of the degradation rate could not be determined for either temperature or concentration. Cardioplegic solutions also contain approximately 0.005 mg/mL of adenine, one of the degradation products of adenosine. As a further indication of the lack of degradation, the adenine concentration did not appear to change in any sample during the stability study.

All solutions remained clear and colourless and the pH, which was initially between 8.15 and 8.25, did not change by more than 0.5 (average

0.15) of a pH unit in any of the solutions on any day.

During a preliminary investigation, some solutions stored at room temperature became contaminated. In these solutions the pH dropped by as much as 3 pH units beginning on day seven. This drop in pH was associated with the development of visible growth and a rapid decline in the adenosine concentration, often to zero. These contaminated solutions were not cultured and the organisms were not identified.

DISCUSSION

Statistical analysis of the adenosine concentration time data in this study was limited to least squares log-linear regression because demonstration of a trend for the concentration to decrease was considered more important than demonstrating a statistical difference in concentration between any two days. In fact, the random fluctuations in concentration around the initial concentration are not of practical importance and should be considered 'noise' or experimental error. Least squares log-linear regression indicated that much less than a 10% loss in the initial adenosine concentration occurred during 14-days storage at both 23°C and 4°C.

Since no change in adenosine concentration could be detected in any solution, assurance of the specificity of the analytical method is very important. The specificity of the analytical method was demonstrated during the accelerated degradation studies (Figure 1). In these studies reduced adenosine concentrations were measured as the concentration of apparent degradation products, including adenine, increased. The separation and detection of intact drug in the presence of degradation compounds must be assured before the method can be considered stability indicating.[10-12]

A reduction in the concentration of adenosine was only observed in a preliminary investigation in which the

Table I: Mean[a] Adenosine Concentrations

STUDY DAY	Refrigerated (4°C)		Room Temperature (23°C)	
	0.33 mg/mL	0.08 mg/mL	0.33 mg/mL	0.08 mg/mL
0	0.29 ± 0.06	0.06 ± 0.03	0.27 ± 0.06	0.06 ± 0.04
1	0.31 ± 0.13	0.07 ± 0.04	0.31 ± 0.04	0.07 ± 0.01
4	0.28 ± 0.06	0.07 ± 0.02	0.30 ± 0.08	0.07 ± 0.02
5	0.28 ± 0.04	0.07 ± 0.02	0.29 ± 0.11	0.06 ± 0.15
6	0.29 ± 0.07	0.07 ± 0.0	0.27 ± 0.03	0.06 ± 0.03
7	0.29 ± 0.06	0.07 ± 0.02	0.30 ± 0.08	0.07 ± 0.01
8	0.30 ± 0.07	0.07 ± 0.02	0.29 ± 0.04	0.06 ± 0.01
11	0.32 ± 0.05	0.08 ± 0.01	0.31 ± 0.05	0.07 ± 0.01
14	0.30 ± 0.07	0.07 ± 0.01	0.26 ± 0.03	0.06 ± 0.01
% remaining (Day 14/Day 0)*100	103.11	107.81	97.77	98.41

[a] Concentrations are the mean of two solutions of each of two different potassium concentrations, each determined in duplicate [a total of 8 concentration estimates made from four solutions] and reported in mg/mL + standard deviation for each study day.

reduction in adenosine concentration was associated with both a drop in pH and the development of visible growth. This growth could have been fungal, a common laboratory contaminant, or bacterial, but culture testing was never completed. Nonetheless, microbial contamination could have produced a decline in pH as the result of metabolism of dextrose in the cardioplegic solutions. This reduction in pH could have contributed to the degradation of adenosine which was observed in the accelerated portion of this study to be pH dependant. This demonstrates that, while expiry dates in any IV additive system must consider the possibility of contamination, checking every solution for particulate contamination and/or visible signs of growth represents an important quality control function in an IV additive program.

A recommended expiry date must consider that a prepared product may be stored for a period of time at both 4°C and room temperature. However, confident first order degradation rates could not be calculated from the data at 4° and 23°C because of the lack of degradation. Since it is known that greater than 90% of the initial adenosine concentration would remain after 13-days storage at 4°C, storage for this duration can be followed by an additional 24 hours storage at room temperature. This expiry date must only be used after due consideration of sterility and the contamination rate in an IV additive program. ⬛

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