Short Communication

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A rapid and simple chemiluminescence method for screening levels of inosine and hypoxanthine in non-traumatic chest pain patients

Don E. Farthing,[a,b]* Domenic Sica,[a] Michael Hindle,[b] Les Edinboro,[c] Lei Xi,[d] Todd W.B. Gehr,[a] Lynne Gehr,[e] Christine A. Farthing,[a] Terri L. Larus,[a] Itaf Fakhry[a] and H. Thomas Karnes[b]

ABSTRACT: A rapid and simple chemiluminescence method was developed for detection of inosine and hypoxanthine in human plasma. The method utilized a microplate luminometer with direct injectors to automatically dispense reagents during sample analysis. Enzymatic conversions of inosine to hypoxanthine, followed by hypoxanthine to xanthine to uric acid, generated superoxide anion radicals as a useful metabolic by-product. The free radicals react with Pholasin®, a sensitive photoprotein used for chemiluminescence detection, to produce measurable blue-green light. The use of Pholasin® and a chemiluminescence signal enhancer, Adjuvant-K™, eliminated the need for plasma clean-up steps prior to analysis. The method used 20 μL of heparinized plasma, with complete analysis of total hypoxanthine levels (inosine is metabolized to hypoxanthine using purine nucleoside phosphorylase) in approximately 3.7 min. The rapid chemiluminescence method demonstrated the capability of differentiating total hypoxanthine levels between healthy individuals, and patients presenting with non-traumatic chest pain and potential acute cardiac ischemia. The results support the potential use of chemiluminescence methodology as a diagnostic tool to rapidly screen for elevated levels of inosine and hypoxanthine in human plasma, potential biomarkers of acute cardiac ischemia.Copyright ©2009 John Wiley & Sons, Ltd.

Keywords: inosine; hypoxanthine; biomarker; chemiluminescence; Pholasin®; acute cardiac ischemia

Introduction

Cardiovascular disease is one of the leading causes of mortality in the world and includes acute myocardial infarction (MI), which can be preceded by acute cardiac ischemia (1–4). The standard diagnostic procedure for patients suspected of having acute cardiac ischemia or acute MI includes: patient history and exam, electrocardiogram (ECG) and sequential assessment of biomarkers in the blood to detect myocardial damage [e.g. creatine kinase muscle brain isoenzyme (CK-MB), cardiac troponin] (5–7). Current test methods for cardiac biomarkers (e.g. myoglobin, CK-MB and cardiac troponin) include liquid chromatography mass spectrometry (LC-MS) analysis (8,9) and fluorescence immunoassay (6,10–12); however, elevation of these protein biomarkers reflects some level of myocardial tissue necrosis, and they are typically elevated in the diagnostic range several hours after acute MI. Non-traumatic chest pain is a leading cause of individuals rushing to the hospital emergency department (ED), and may be caused by more than 10 medical conditions (e.g. anxiety, angina, acid reflux, heartburn) (13); however, one serious medical condition is acute cardiac ischemia, which can precipitate into acute MI and cause eventual death. Our previous research has focused on evaluation of potential biomarkers, inosine and hypoxanthine, finding elevated during conditions of non-traumatic acute cardiac ischemia (14).

During periods of acute cardiac ischemia, affected heart tissues undergo constant oxidative stress. The affected cardiac tissue accumulates significant amounts of ATP metabolic by-products (i.e. ADP, AMP), which activates normally dormant cardiac cellular enzymes, to generate ATP catabolic by-products (i.e. inosine and hypoxanthine), which become systemically available for elimination by the kidney. Inosine (9-β-D-ribofuranosylhypoxanthine;

* Correspondence to: D. E. Farthing, Department of Internal Medicine, Virginia Commonwealth University, MCV Station Box 980160, Richmond, VA 23298-0160 USA. defarthi@vcu.edu

[a] Department of Internal Medicine, Divisions of Nephrology, Clinical Pharmacology and Hypertension, Virginia Commonwealth University, Richmond, VA, USA

[d] Department of Internal Medicine, Division of Cardiology, Virginia Commonwealth University, Richmond, VA, USA

[b] Department of Pharmaceutics, Virginia Commonwealth University, Richmond, VA, USA

[e] Department of Anesthesiology, Virginia Commonwealth University, Richmond, VA, USA

[c] Department of Forensic Science, State of Virginia, Richmond, VA, USA

purine nucleoside) and hypoxanthine (1,7-dihydro-6H-purin-6-one; metabolite of inosine) are endogenous plasma constituents normally found at low levels (e.g. ~200–400 ng/mL), resulting from normal dietary and purine metabolism (15).

Current test methods for measurement of selected ATP catabolic by-products inosine, hypoxanthine, xanthine and uric acid utilize high-performance liquid chromatography ultraviolet (HPLC-UV) detection with sample preparation techniques including solid-phase extraction (15), centrifugal filtration (16), protein precipitations (e.g. ethanol or TCA), as well as other methods requiring use of internal standards (17,18). Several HPLC methods utilized ion pairing reagents (19–21), while one method utilized a protein precipitation technique and enzyme-catalyzed chemiluminescence detection (22). One recent publication utilized a complex LCMS analysis and metabolomic evaluation of plasma samples from cardiac patients undergoing planned myocardial infarction as part of the medical treatment, and reported elevated levels of hypoxanthine (23).

There are no published articles describing a rapid test method (<5 min analysis) for the determination of inosine and hypoxanthine in plasma to meet the stringent sample turnaround time requirements of the emergency medical services (EMS) and ED environment. The rationales for using a chemiluminescence technology over commonly used LC and immunoassay technologies are as follows: LC and immunoassay methods can be very sensitive and specific techniques (e.g. monoclonal antibodies for immunoassay and MS detection for LC); however, an LC-MS system can be expensive to purchase and operate, both techniques require technical expertise to perform, and both lack a rapid turnaround time needed by an ED facility analyzing priority 'stat' patient samples. In contrast, a microplate luminometer can measure chemiluminescent light with high sensitivity, is relatively inexpensive to purchase and operate, and is currently used in many laboratories.

To address the biomarker specificity requirement, the developed chemiluminescence test method utilized biological enzymes purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO), which are specific for enzymatic conversions of inosine and hypoxanthine, respectively. The enzyme PNP converts inosine to hypoxanthine and XO converts hypoxanthine to xanthine, followed by XO conversion of xanthine to the final product uric acid (in human species). Each time XO reacts with 1 mole of hypoxanthine, and subsequently with 1 mole of xanthine, the metabolic by-products of each XO enzymatic turnover is production of 1 mole of hydrogen peroxide (H_2O_2) and 2 moles of superoxide anion radical ($O_2^{-}\cdot$). Both by-products can become substrates for use in chemiluminescence reactions.

Several commonly used luminescent materials [e.g. luminol (oxidation), lucigenin (reduction), and Pholasin® (oxidation)] were considered for this research. If using luminol or lucigenin as the luminescent material, the hydrogen peroxide (which has both oxidizing and reducing capabilities) can react with the horseradish peroxidase (HRP) enzyme, luminescent material, and signal enhancer to generate a measurable blue or green light (~425–500 nm), thus an amplification of signal effect (1 mole of hypoxanthine and xanthine can generate 2 moles of hydrogen peroxide) is observed. However, to achieve even greater sensitivity as low concentrations (1–3 μM) of inosine and hypoxanthine are typically found in human plasma, another chemiluminescence approach was investigated utilizing a highly sensitive photoprotein.

Pholasin®, a photoprotein isolated from the bivalve mollusc *Pholas dactylus*, was reported to possess a very sensitive chemiluminescent material (called lucidalin®), which can react with superoxide anion radical (SAR) and other reactive oxygen species (ROS) such as the hydroxyl free radical (24). Pholasin® is an approximately 34–36 kDa glycoprotein (25), and has been reported not to possess fluorescent properties. It has been extensively studied for more than 30 years with commercial use patented by Knight Scientific Ltd (Plymouth, UK).

The SAR generated by XO enzymatic turnover reacts with Pholasin® to generate measurable blue-green light (with a maximum ~490 nm) (Fig. 1), thus an amplification of signal is observed (1 mole of hypoxanthine can generate 4 moles of SAR). Theoretically, and assuming equivalent quantum yields, the use of Pholasin® should increase sensitivity and provide lower component detection limits representing an improvement of approximately two-fold, over using a hydrogen peroxide/horseradish peroxidase/luminol approach. Knight *et al*. cited Pholasin® improving chemiluminescence sensitivity by more than 100-fold, in comparison to using lucigenin for the same studies (26). The reaction of Pholasin® with SAR is rapid (flash type technique, typically seconds), and method sensitivity can be further increased with use of a signal enhancer [e.g. Adjuvant-K™ (proprietary) from Knight Scientific Ltd].

The primary goal of this research was the development of a rapid chemiluminescence method for determination of inosine and hypoxanthine levels in human plasma. The purpose was to provide the capability of rapidly screening patients (as a diagnostic tool for acute cardiac ischemia) for potential use in a hospital ED environment. To be effective, the method needed to be specific and sensitive for inosine and hypoxanthine, thus reducing potential errors in interpreting test results (i.e. minimize false positive and false negative results). The chemiluminescence method was successfully utilized on samples representing healthy individuals and hospital patients with confirmed acute MI (with documented elevated levels of cardiac troponin T).

Experimental

Chemicals, reagents, enzymes, sorbent and plasma

Hypoxanthine, xanthine, phosphoric acid (HPLC-grade, 85%) and ethyl alcohol (HPLC-grade, denatured) were purchased from Acros Organics (Fair Lawn, NJ, USA). Inosine, dibasic sodium hydrogen phosphate, and uric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Enzymes xanthine oxidase (isolated from bovine milk, Grade III, ammonium sulfate suspension, enzymatic activity ~1.3 Units/mg protein, storage 2–8°C), purine nucleoside phosphorylase (isolated from human blood, lyophilized powder, enzymatic activity ~19 Units/mg protein, storage –20°C) and uricase (isolated from Arthrobacter globiformis, lyophilized powder, ~19.7 Units/mg protein, storage –20°C) were purchased from Sigma-Aldrich. Nucleoside phosphorylase (isolated from bacteria, lyophilized powder, purchased from Sigma-Aldrich) can favorably substitute for PNP (isolated from human blood), and was evaluated due to frequent vendor backorders of PNP isolated from human blood. Pholasin® (luminescent material) and Adjuvant-K™ (signal enhancer) were purchased from Knight Scientific Ltd (Plymouth, UK).

Dibasic sodium hydrogen phosphate was used to prepare an aqueous 20 mM assay buffer solution (final pH 7.4 using

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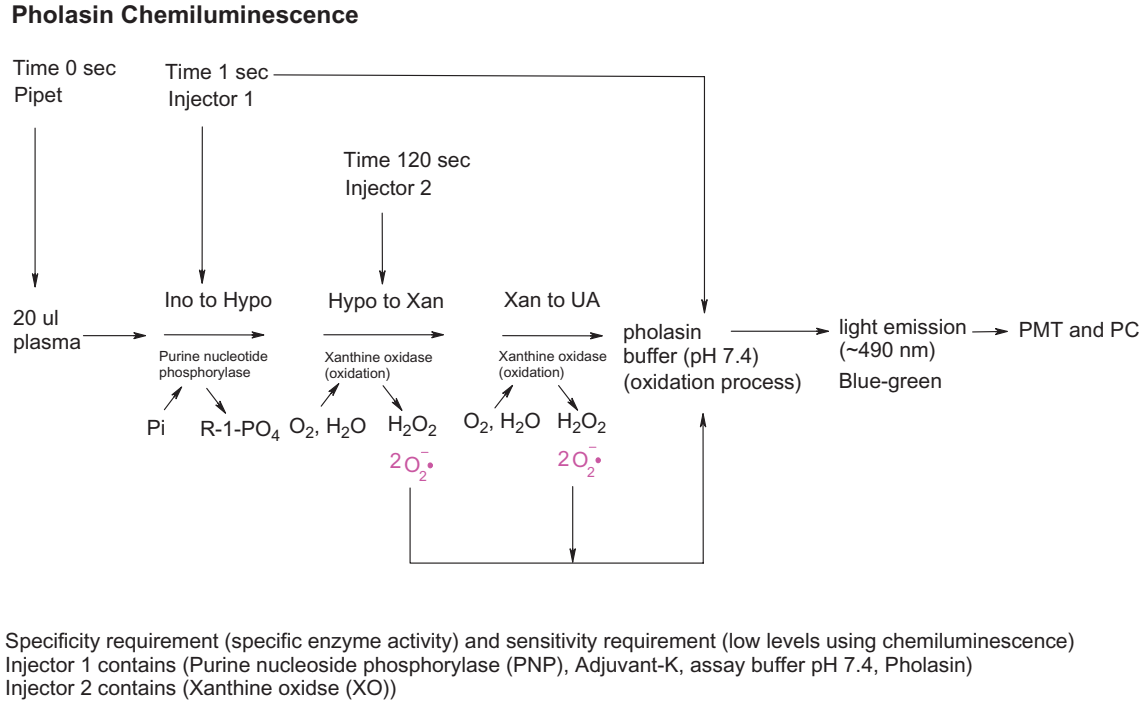

Figure 1. Luminometer injector time sequence for dispensing reagents. Flow diagram representing the addition of reagents, injector time points, and resulting chemiluminescence signal detection, using a photomultiplier tube (PMT) and personal computer (PC) with vendor application software.

concentrated phosphoric acid). Ultrapure deionized water (~18 MΩ cm) for all reagent solutions was prepared in-house and filtered (0.2 µm) prior to use. Micropipette tips (100 µL) containing strong anion exchange (OMIX SAX) resin were purchased from Varian Inc. (Lake Forest, CA, USA). Plasma (heparin anticoagulant) from healthy individuals and cardiac patients with confirmed acute MI (hospital reported elevated cTnT) were purchased from ProMedDx (Norton, MA, USA), which used an IRB approved protocol for sample collection, processing and storage conditions (–70°C).

Apparatus

The luminometer system consisted of a BMG Labtech Inc. Lumistar Optima and Optima software (version 2.1; Durham, NC, USA) and Dell Optiplex 745 PC (Dell, TX, USA). The luminometer was equipped with temperature control (8–45°C), two direct injectors (limit of 3 µL) with variable injection speeds (100–420 µL/s), and microplate shaking capability (e.g. orbital, linear). The luminometer listed specifications for limit of detection (<50 amol/well ATP), spectral range (240–740 nm) and dynamic range (9 decades). All luminescence assays utilized opaque 96-well microplates, incubation temperature of 25°C, lens mode (no emission filter) and the default photomultiplier tube gain setting. The opaque 96-well microplates were purchased from Corning Life Sciences (Lowell, MA, USA), and stored in the dark at ambient temperature. Equipment and data collection set points used for experiments in the flash mode are listed in Table 1. The luminometer rinse solution for the direct injector syringes was prepared using an ethyl alcohol : deionized water mixture (75 : 25%, v/v). Weekly rinses were performed to reduce potential material (e.g. salt and enzyme residue) accumulation in the syringes, reagent tubing and injector needles.

Preparation of standards and reagents

Stock standards of inosine (93.2 µM), hypoxanthine (183.7 µM), xanthine (164.4 µM) and uric acid (148.7 µM) were prepared in deionized water, stored at 4°C with stability of at least 3 months. Working calibration standards for each component were prepared in deionized water immediately prior to use. The working XO solution was prepared by pipetting 80 µL of the aqueous stock XO (from bovine milk suspension) and dilution to 2.0 mL using assay buffer (pH 7.4), resulting in ~1.35 Units XO/mL. The working XO solution was stable at ambient laboratory temperature (22°C) and could be stored at 4°C overnight with minimal loss in enzyme activity; however, the working XO solution should not be stored frozen (e.g. –0°C), as a complete loss of enzyme activity was observed upon freeze–haw and subsequent use.

To prepare PNP and uricase solutions from solid and lyophilized PNP (human blood isolate) and uricase, 1.0 mL of assay buffer (pH 7.4) was added with gentle vortexing into solution. After reconstitution using 1 mL of assay buffer (pH 7.4), the PNP and uricase stock concentrations were ~18.7 and ~110 U/mL, respectively. A working solution of PNP (~701 mU PNP/mL) was prepared by pipetting 75 µL of the aqueous stock PNP material and diluting to 2.0 mL using assay buffer (pH 7.4). A working solution of uricase (~1.1 U uricase/mL) was prepared by pipetting 20 µL of the aqueous stock uricase material and dilution to 2.0 mL using assay buffer (pH 7.4). Both the PNP and uricase working solutions were stable at ambient laboratory temperature and could be stored at 4°C overnight with minimal loss in enzyme activity.

For preparation of the Pholasin® (50 µg) luminescent material, 5.0 mL of assay buffer (pH 7.4) was added with gentle vortexing, resulting in a ~10 µg/mL solution. The prepared Pholasin® reagent was stable at ambient laboratory temperature and

Table 1. Luminometer and data collection set points

Parameters	
Kinetic window	1
No. of intervals	112
Interval time (s)	2.0
Measured start time (s)	0.0
Measured interval time (s)	2.0
Time to normalize results (s)	1.0
Volume group	1
Pump syringe vol (mL)	0.5
Smart dispensing	on
Positional delay (s)	0.2
Volume (µL)	20
Injection start time (s)	120
Shaking after injection (s)	103
Shaking mode	orbital
Shaking width (mm)	1
Additional shaking (s)	2

overnight at 4°C, and was stored protected from light to eliminate potential basal luminescence. The reconstituted Pholasin® solution was transferred and stored in ~1 mL plastic screw top cryogenic vials at −20°C.

Collection and processing blood samples

Following CJW Medical Center (Chippenham Hospital, Richmond, VA, USA) approval and Health Insurance Portability and Accountability Act (HIPAA) guidelines, a limited number of samples for evaluation were obtained from hospital ED patients ($n = 20$). The blood samples were collected using lithium heparin vaccutainer™ tubes, as per hospital emergency room protocols for patients presenting with non-traumatic chest pain, and potential MI or acute myocardial ischemia. Samples were centrifuged at ~1000 ***g*** for 10 min, with plasma drawn off and transferred into tubes for routine hospital clinical testing. One tube was immediately stored frozen at −20°C (long-term storage at −70°C) and used for inosine and hypoxanthine analysis. Prior to sample analysis, the plasma was thawed to ambient temperature, mixed thoroughly by inversion, and centrifuged at 1000 ***g*** for 10 min to eliminate fibrinous material.

Method development and optimization

Development and optimization of the luminescence method included: evaluation of a hypoxanthine calibration curve in plasma; adjustment of XO enzyme concentration to reduce analysis time (≤5 min); enzyme incubation time (e.g. PNP) to maximize repeatability and increase sensitivity; and evaluation of EDTA and heparin anticoagulants on quenching of the luminescent signal. The analysis utilized 20 µL of plasma sample in a final microplate well volume of 200 µL (effectively making a 1 : 10 dilution of the sample). Endogenous plasma uric acid was also evaluated to determine its quenching effect as this substance has antioxidant capacity, and is normally found in human plasma at levels ranging from 226 to 464 µM (15), with patients experiencing hyperuricemia and gout conditions at even higher levels (e.g. ~500 µM uric acid) (27).

Table 2. Typical volumes used for the luminescence experiments. Substances were pipette into a 96-well microplate manually or automatically using direct injectors

Reagents	Vol. (µL)
Hypoxanthine	10.9
Pholasin	50.0
Assay Buffer	59.1
Xanthine Oxidase	40.0
Adjuvant-K	20.0
Plasma	20.0
Total well volume	200.0

The HPLC results from healthy individuals (ProMedDx) and non-traumatic chest pain patients (Chippenham Hospital) from our previous research (14,16) were used to estimate plasma levels of inosine and hypoxanthine for development of the chemiluminescence method. Since the luminometer is a detection device, and does not separate a mixture of components (as does HPLC), it was necessary to utilize enzyme PNP to convert inosine to hypoxanthine, and then measure the resulting total plasma hypoxanthine level (i.e. inosine plus hypoxanthine). Using XO, hypoxanthine is rapidly converted to xanthine, followed by XO conversion of xanthine to uric acid (final product). The luminometer measures light signal generated from the XO metabolic by-product, SAR, reaction with luminescent material Pholasin®.

A standard curve of hypoxanthine in plasma was evaluated with a concentration range of 0.5–30 µM, which incorporated the majority of measured inosine and hypoxanthine levels from healthy individuals, and non-traumatic chest pain patients ($n = 20$ for each group). Xanthine was determined and found at consistent levels (~6 µM using HPLC analysis) in plasma samples from healthy individuals and non-traumatic chest pain patients ($n = 20$ for each group).

Typical volumes of reagents and plasma used for chemiluminescence experiments on inosine and hypoxanthine are listed in Table 2. The plasma (20 µL) was manually pipetted into the opaque microplate well, followed by the addition of incubation reagents (e.g. assay buffer, Pholasin®, PNP, Adjuvant-K™). The incubation reagents were either manually pipetted or dispensed using one injector, with a second injector used exclusively for addition of XO. The XO solution was automatically injected after 120 s of sample incubation time, which allowed sufficient time for PNP to convert inosine to hypoxanthine, and begin the chemiluminescence measurements. Figure 1 is a schematic listing the reagents and timing of injections used for chemiluminescence experiments.

Chemiluminescence computations

All computations were performed using BMG Excel software (vendor-supplied macros) with data acquisition and processing set points listed in Table 1. The background (baseline) chemiluminescence signal can be contributed from reagents (e.g. buffer, Pholasin®, PNP, plasma, Adjuvant-K™) and electronic noise, and was calculated by measurement of the relative luminescence units (RLU) signal for ~20 s, prior to injection of the XO solution. After injection of XO, the peak chemiluminescence RLU signal generated from the reaction of Pholasin® and superoxide anion radicals was calculated from the maximum peak height RLU signal.

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The rationale for using peak height RLU signals for computations, instead of peak area RLU signals, was due to several of the cardiac patient samples having a peak area RLU signal that was slow to return to background (baseline) levels. The cause of the slow RLU signal return to baseline is unknown, but may be due to interferences from medications (e.g. aspirin, beta blockers) that are administered to the patients as part of their medical treatment. The chemiluminescence method was developed to allow for rapid comparison of hypoxanthine peak height RLU signals between healthy individuals (negative control) and non-traumatic chest pain patients, who may be experiencing acute cardiac ischemic conditions.

Estimated biomarker RLU signal cut-off level

The use of plasma substances (e.g. cardiac troponins) as diagnostic biomarkers typically involves the development of a reference 99% cut-off level, which was beyond the scope of this research, as it would best be determined using a large number of samples (e.g. hundreds). In the present study, an estimate of a reference 99% RLU signal cut-off level was determined from a limited number of healthy individuals ($n = 6$), and was calculated using the mean peak height RLU signal and 2.326 standard deviation ($\alpha = 0.01$, one-tail, 99% confidence). To determine potential usefulness of the rapid luminescence method, comparisons using the estimated biomarker reference cut-off level were made between healthy individuals, non-traumatic chest pain patients and patients with confirmed acute MI.

Results and Discussion

To set up the new luminometer system, a commercial luminescence test kit was purchased (ABEL 61M Antioxidant Test Kit, Knight Scientific Ltd) to evaluate antioxidant capability using xanthine/XO and Pholasin®. The kit included Pholasin® (50 µg), xanthine standard material, XO [~10.25 mU/mL] and buffer (proprietary) for plate (glow) mode kinetics. The luminometer system was qualified using the commercial antioxidant test kit with successful replication (Fig. 2) of the xanthine/XO plate mode kinetics profile, kindly provided by Knight Scientific Ltd. The plate mode analysis time was approximately 30 min, as it was developed primarily for antioxidant and other glow mode techniques.

Method development and optimization studies included: determining the optimal level of XO, evaluation of PNP incubation time, the effect of using blood anticoagulants (e.g. heparin, EDTA), the effect of endogenous plasma uric acid, the amount of plasma analyzed, the effect of the signal enhancer Adjuvant-K™, and the linear range for hypoxanthine standards prepared in plasma. The enzymes PNP and XO were used for substrate metabolic conversion steps, with XO by-product SAR, reacting with Pholasin® to emit luminescent light. Optimization of these enzyme levels were performed prior to other method development parameters (e.g. anticoagulant suppression study, plasma volume study), to ensure that comparisons were made using optimal enzymatic conditions, with the exception of temperature. It was determined for this project that all luminometer evaluations would be performed at 25°C. The enzyme kinetics may have been superior at 37°C; however, the choice of 25°C was made with the mindset that use of other luminescence devices (e.g. hand-held luminometer), purchased without temperature control capability, might be utilized for this type of analysis.

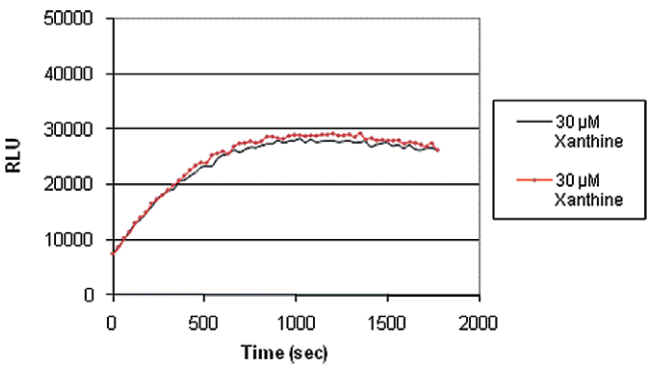

Figure 2. Instrument setup and operation qualification. Overlay plot demonstrating plate mode kinetics using 30 µM xanthine in buffer, and enzyme xanthine oxidase (XO) (duplicate samples, analysis time ~30 min).

Figure 3(A) depicts the optimization of the XO enzyme level to significantly increase the rate of hypoxanthine conversion (flash mode kinetics), which reduced analysis time and enhanced method sensitivity (i.e. increased peak height). The commercial kit from Knight Scientific (glow mode kinetics) was set up for xanthine/XO analysis and studies on material antioxidant capabilities, thus it was necessary to increase the XO level to additionally incorporate plasma hypoxanthine levels, but even more importantly to reduce the analysis time to less than 5 min (i.e. switch from glow mode to flash mode kinetics). The starting level of XO enzyme used for glow mode kinetics evaluation was approximately 10.25 mU/mL (after reconstitution with assay buffer). Xanthine oxidase was adjusted to increase the concentration, resulting in a final working solution of approximately 1.35 U/mL (~100 fold increase), which reduced the analysis time from 30 min to less than 5 min. The RLU plateau observed when using xanthine/XO in glow mode kinetics (Fig. 2), was transformed into a Gaussian peak shape when using flash mode kinetics [Fig. 3(A)]. For this evaluation, hypoxanthine (10 µM) was fortified into plasma (heparin) with samples evaluated in duplicate at 25°C. The amount of XO (1.35 U/mL) injected into the microplate ranged from 5 to 40 µL XO. As seen in Fig. 3(A), increasing the level of XO significantly improved the Guassian peak shape and increased RLU peak height.

Figure 3(B) is an overlay demonstrating results of the inosine and PNP incubation time study. Inosine (10 µM) was fortified into plasma with incubation times of 60 and 120 s, with samples evaluated in duplicate at 25°C. After PNP incubation times of 60 and 120 s, XO was injected with RLU comparison to a reference standard of 10 µM hypoxanthine. As seen in Fig. 3(B), using the incubation time of 120 s resulted in an improved RLU peak shape (Gaussian) and increased peak height. The results indicate that approximately 120 s of PNP incubation time provided sufficient time for conversion of inosine to hypoxanthine at 25°C. For all subsequent experiments, the PNP incubation time was set at 120 s.

For future studies, a possible way to further reduce analysis time would be to fortify PNP into the blood collection tube (e.g. BD heparin vacuutainer), with inosine to hypoxanthine conversion occurring during the blood to plasma centrifugation step. If the centrifugation technique utilizing PNP in the vacuutainer resulted in complete inosine to hypoxanthine conversion, it would eliminate the need for the 120 s PNP incubation time and reduce analysis time to approximately 30 s (assumes injection of XO at 0.1 s and measurement of peak height RLU response).

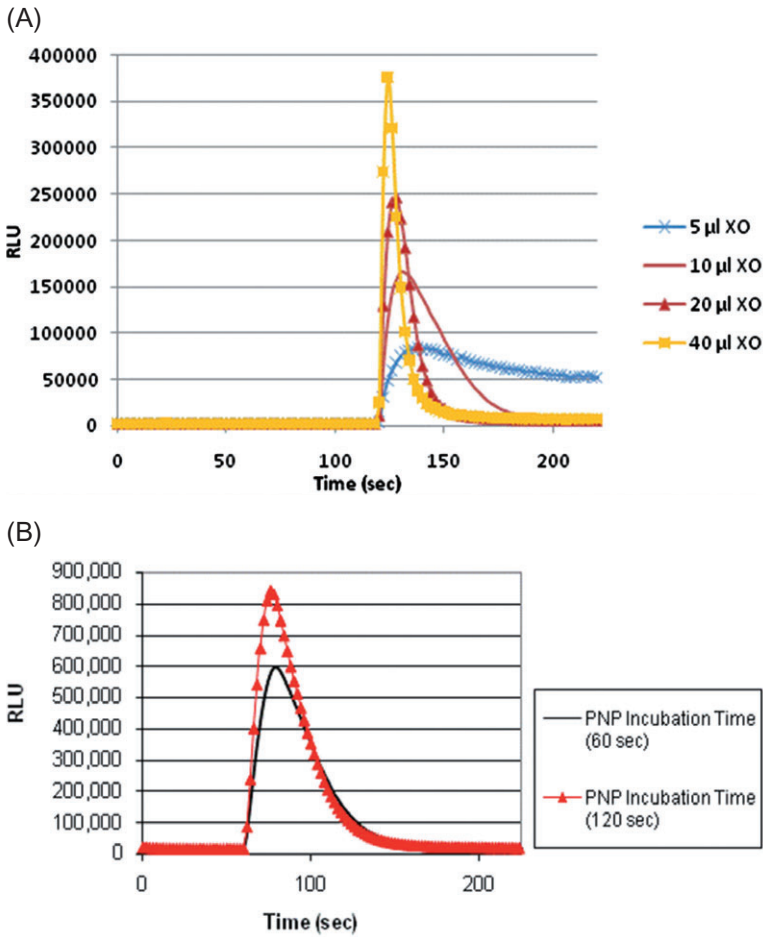

Figure 3. Results of optimization studies on XO enzyme level and PNP incubation time. Overlay plot (A) depicts optimization of the XO enzyme level to increase the conversion rate (kinetics) of hypoxanthine to xanthine, and xanthine to uric acid. Overlay plot (B) depicts inosine and enzyme purine nucleoside phosphorylase (PNP) incubation time study.

An evaluation was performed on several substances that may be found at elevated levels in the study plasma samples, which could potentially cause suppression to chemiluminescence response. Substances such as organic acids [e.g. ethylenediaminetetraacetic acid (EDTA), uric acid] can potentially quench chemiluminescence signal, as they have known antioxidant properties. The antioxidants can chemically react with free radicals (e.g. SAR), thus reducing the amount of free radicals available for the chemiluminescent reaction with Pholasin®. Hypoxanthine (30 µM) was fortified into 5 µL plasma with samples evaluated in duplicate at 25°C, and comparisons were made between blood anticoagulants heparin and EDTA. Figure 4(A) demonstrates the significant negative effects of EDTA on the chemiluminescence response. The EDTA quenched the peak height RLU by approximately 40%, with a resulting non-Gaussian peak shape.

A study of plasma uric acid effect on chemiluminescence response was performed. Uric acid can be found in plasma at relatively high levels (normal range ~350–475 µM) and has antioxidant properties, thus it was important to evaluate the potential effect on chemiluminescence signal. Hypoxanthine (10 µM) was fortified into deionized water with samples evaluated in duplicate at 25°C. Deionized water was used to eliminate the suppression effects of other endogenous plasma components (e.g. albumin, organic acids). As seen in Fig. 4(B), the effect of uric acid at physiological normal levels significantly reduced the chemiluminescence signal (~50% quenching), with an elevated level of uric acid (e.g. ~74 µM) that may be found in gout patients causing even higher signal suppression.

To reduce negative effects from the substances (e.g. uric acid, albumin) that can bind to the superoxide anion radicals and cause signal suppression, sample pretreatments using dilution with deionized water or use of strong anion exchange (SAX) resin was evaluated to reduce the matrix interference. For sample dilution study, hypoxanthine (2 µM) was fortified into 5, 10 or 20 µL plasma (heparin) with samples evaluated in duplicate at 25°C. As seen in Fig. 5(A), a 1:100 dilution of the plasma sample with deionized water, as well as the use of the SAX treatment, demonstrated that removal of the potential interfering substances could improve chemiluminescence response and sensitivity. Since small organic acids (e.g. uric acid) found in plasma should be present in the salt form (e.g. urate at pH 7.4), use of the SAX resin should effectively bind to the urate and remove it from plasma, resulting in an increased chemiluminescence signal.

An evaluation of smaller plasma volumes (e.g. 5 µL) was made by addition of assay blank (e.g. 15 µL) to maintain the 20 µL sample volume. Plasma samples (5, 10 and 20 µL) were fortified with 2 µM hypoxanthine, and analyzed in duplicate at 25°C. The results indicate that smaller volumes of plasma would result in higher peak RLU signal (Fig. 5B). One possible explanation for the increase in chemiluminescence signal when using smaller plasma

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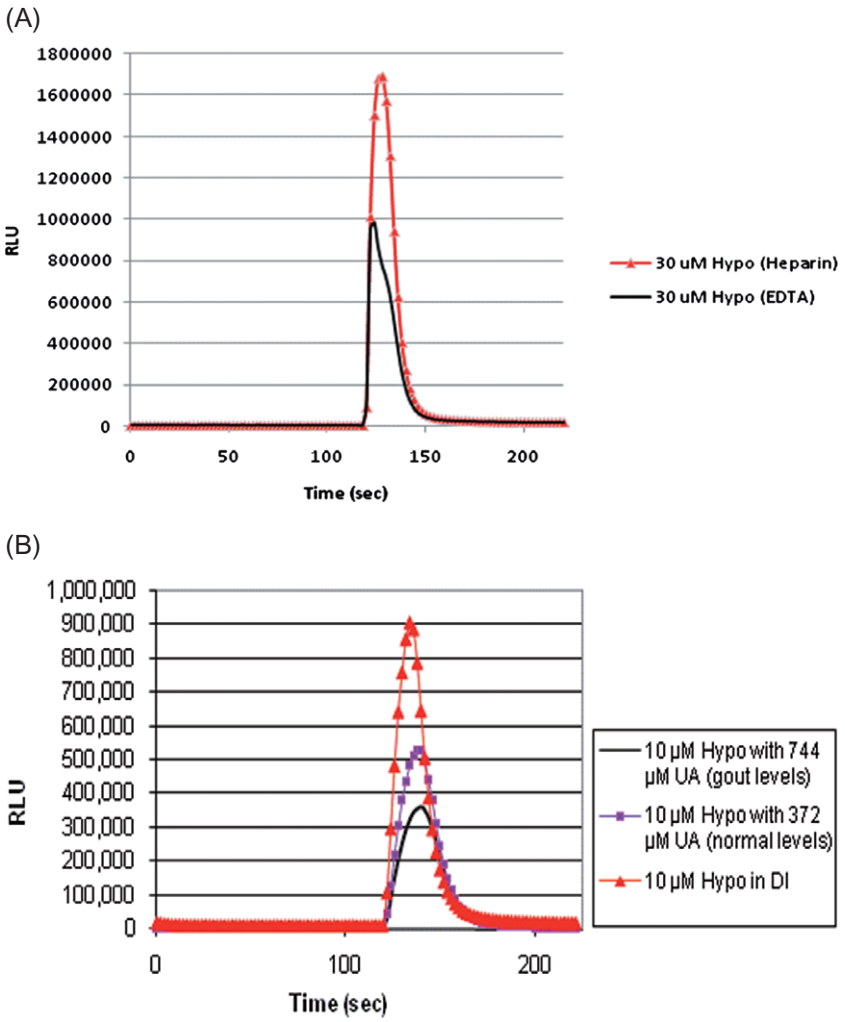

Figure 4. Effects of blood anticoagulant and uric acid on chemiluminescence response. Overlay plot (A) demonstrates the significant suppression effect of EDTA (anticoagulant) and overlay plot (B) demonstrates the significant suppression effect of uric acid (human physiological levels) on chemiluminescence signal.

volumes (e.g. 5 or 10 µL) is that plasma normally contains large amounts of albumin (~40 mg/mL) and organic acid (e.g. uric acid), which cause signal suppression. Both uric acid and albumin can bind to SAR causing a reduction in chemiluminescence signal, hence using a smaller plasma volume would have less interference from these endogenous components.

Another approach to eliminate uric acid was to utilize the enzyme uricase (~1.1 U/mL from bacteria, Sigma, USA). The rationale for an enzymatic approach was to convert uric acid to allantoin during the PNP incubation time (120 s); however, this technique proved unsuccessful with a resulting broad non-Guassian peak shape. A possible explanation for this result is that XO enzyme is deactivated (product inhibition) by the presence of large amounts of uricase metabolic by-product, hydrogen peroxide. Since a by-product of XO activity is also production of hydrogen peroxide, this can lead to significant accumulations of hydrogen peroxide, which can suppress XO enzymatic activity. One possible solution to eliminate the elevated levels of hydrogen peroxide is to use horseradish peroxidase, which catalyzes hydrogen peroxide to water and oxygen, but this additional enzyme would only add to the complexity and cost of the analysis, and was not further studied.

A study was performed using chemiluminescence signal enhancer, Adjuvant-K™ (proprietary substance from Knight Scientific, UK). Plasma from healthy individuals (n = 7) was evaluated, and then fortified using Adjuvant-K™ with samples evaluated in duplicate at 25°C. Figure 6 is an overlay plot demonstrating the positive effect of signal enhancer Adjuvant-K™ on chemiluminescence signal. The use of Adjuvant-K™ increased the chemiluminescence signal in plasma samples by approximately 22 fold, with reproducible sample replicates. The significant sensitivity improvement helped to offset the suppression effects from endogenous plasma components (e.g. albumins, uric acid), and allowed for evaluation of untreated plasma samples using the rapid screening chemiluminescence method.

A standard curve of hypoxanthine in plasma was evaluated with a concentration range of 0.5–30 µM. This range represented inosine and hypoxanthine levels found in healthy normal individuals (n = 20) and non-traumatic chest pain patients (n = 20), as determined from our previous research (14,16). Figure 7(A) (peak height response) and Fig. 7(B) (peak area response) are graphs plotting linear (0.5–10 µM range) and non-linear (10–30 µM range) chemiluminescence signal response for hypoxanthine in human plasma (20 µL volume). The measurement of peak height

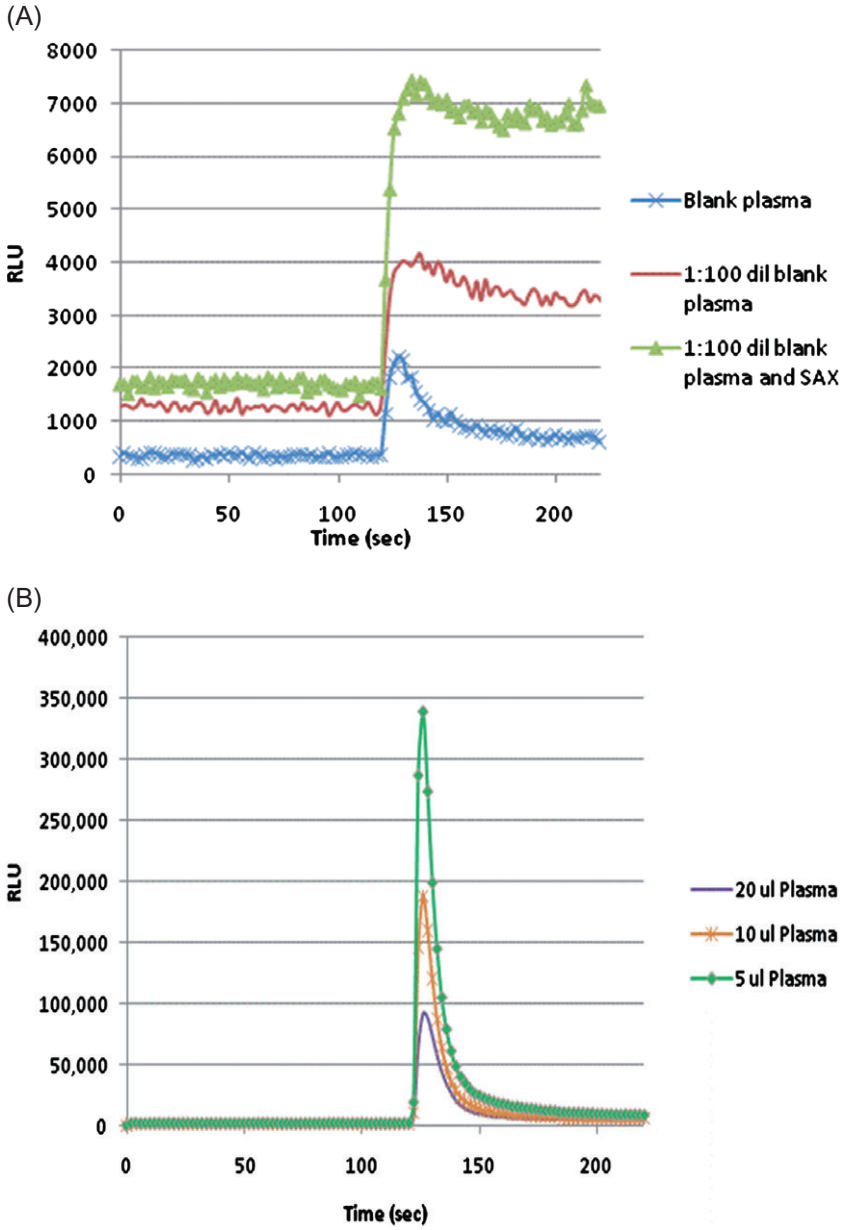

Figure 5. Effects of sample dilution and strong anion exchange on chemiluminescence response. Overlay plot (A) demonstrates the effect of various treatments on plasma (heparin). A dilution of plasma with deionized water (1 : 100), and use of strong anion exchange (SAX) can reduce the antioxidant effect of endogenous organic acids (e.g. uric acid) on the chemiluminescence signal, thus increasing sensitivity. Overlay plot (B) demonstrates the effect of plasma (heparin) sample volume used for analysis. The chemiluminescence signal peak height RLU was inversely related to the volume of plasma used for analysis.

response (Fig. 7A) was performed by measurement of the chemiluminescence signal background (pre-XO injection) and maximum peak height signal (post-XO injection). Measurement of peak area response (Fig. 7B) was performed using trapezoidal rule computation on the chemiluminescence signal for hypoxanthine (i.e. estimates area under the curve). The non-linear portion of the calibration curve may be attributed to accumulation of the XO by-product, hydrogen peroxide (H_2O_2), at higher hypoxanthine levels (e.g. >10 μM). It is known that H_2O_2 inhibits enzymatic activity of XO, which decreases the kinetics and can lower the peak height RLU, which may explain why the peak height response was more negatively impacted than the peak area response.

Using quantitative HPLC-UV and based on a limited number of samples (n = 20) that were available for evaluation, it was estimated that a theoretical cut-off level for healthy individuals was ~5 μM total hypoxanthine. Since a 5 μM hypoxanthine level was on the linear portion of the calibration curve, no additional work (e.g. sample treatment, evaluate other calibration models) was deemed necessary to meet a fit-for-purpose method development criteria. Individuals presenting with non-traumatic chest pain and screened hypoxanthine level above the 5 μM cut-off value would require immediate medical attention to determine the cause and proper course of treatment.

Plasma sample evaluations were made to demonstrate the utility of the rapid screening chemiluminescence methodology. Figure 8A is an overlay consisting of three healthy normal individuals and three patients with confirmed acute MI (hospital documented elevated levels of cTnT). All acute MI patient sample

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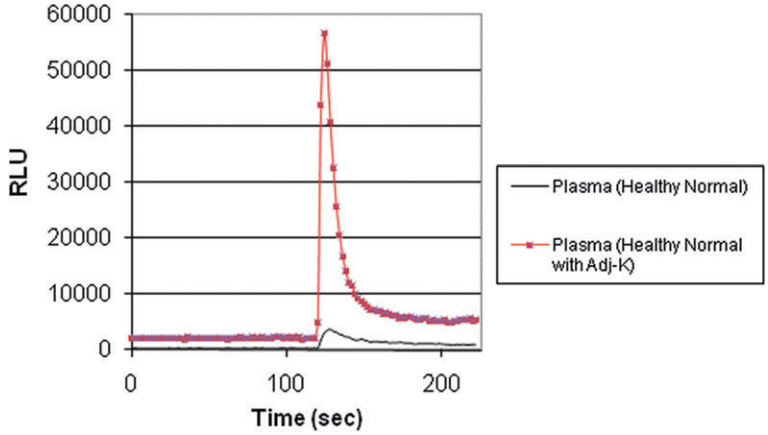

Figure 6. Effect of signal enhancer Adjuvant-K™ on chemiluminescence response. Overlay plot demonstrating the positive effect of chemiluminescence signal enhancer (Adjuvant-K™). The sensitivity improvement using Adjuvant-K™ allowed for untreated plasma samples to be analyzed using the rapid chemiluminescence methodology.

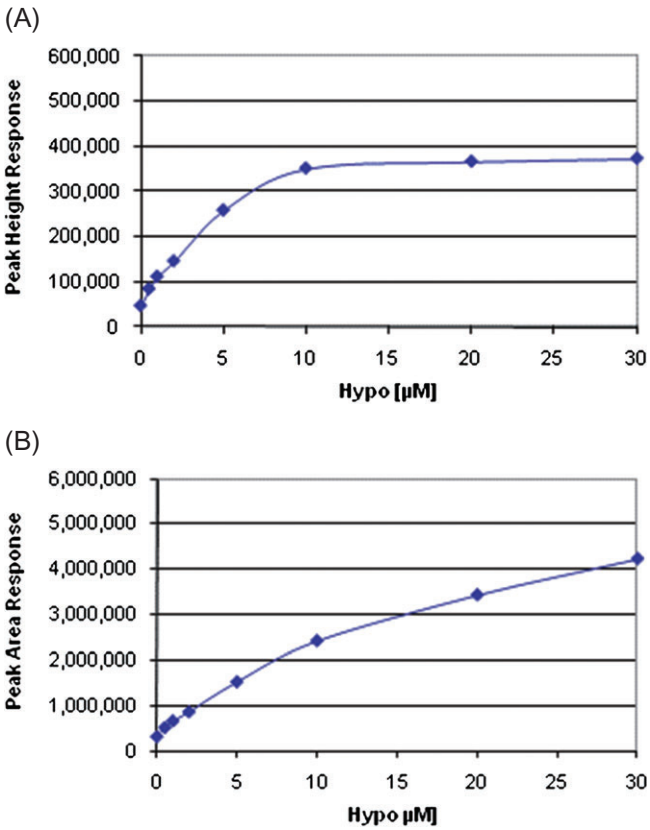

Figure 7. Results for hypoxanthine standards prepared in plasma linearity evaluation. Graphs plotting the linear (0.5–10 µM range) and non-linear (10–30 µM range) chemiluminescence response for hypoxanthine in human plasma. Instrument measurement of peak height response (A) was performed using the baseline (pre XO injection) and maximum (post XO injection) RLU signal. Measurement of peak area response (B) was performed using trapezoidal rule computation on the chemiluminescence (RLU) signal raw data.

RLU signals were above the estimated 99% cut-off reference signal (~7200 RLU), for healthy individuals ($n = 6$ used for reference cut-off statistics). Figure 8(B) is an overlay consisting of three healthy individuals and three ED patients with non-traumatic chest pain, with all samples utilizing Adjuvant-K™. The Adjuvant-K™ was used to demonstrate its significant enhancement to chemiluminescence signal. Each ED patient sample RLU signal was significantly above the estimated 99% cut-off reference signal (~178,000 RLU), for healthy normal individuals ($n = 6$ used for reference cut-off statistics). Quantitative HPLC-UV (14,16) results for total hypoxanthine level (inosine plus hypoxanthine) are listed for each individual (Fig. 8A, B), which confirmed the basal levels of total hypoxanthine detected in healthy individuals and elevated levels in the cardiac patient samples using the rapid chemiluminescence methodology.

Conclusions

A rapid screening chemiluminescence method was developed for the detection of inosine and hypoxanthine in human plasma. Using 20 µL of plasma (heparin) and instrument injectors, the method allowed for rapid (<5 min) detection of hypoxanthine levels, which may be useful as a biomarker for acute cardiac ischemic conditions. The method was utilized for evaluation of plasma samples from healthy individuals, non-traumatic chest pain patients, and cardiac patients with confirmed acute myocardial infarction (hospital-documented elevated plasma cTnT levels). The results clearly demonstrate the potential use of chemiluminescence methodology as a diagnostic tool, used by ED personnel on non-traumatic chest pain patients suspected of undergoing acute cardiac ischemia.

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Conflict of interest

Virginia Commonwealth University (Richmond, VA, USA) filed for a patent related to methods of diagnosing acute cardiac ischemia, on behalf of D. Farthing, H. Thomas Karnes, D. Sica, T. Gehr, M. Unverdorben, L. Xi and L. Gehr.

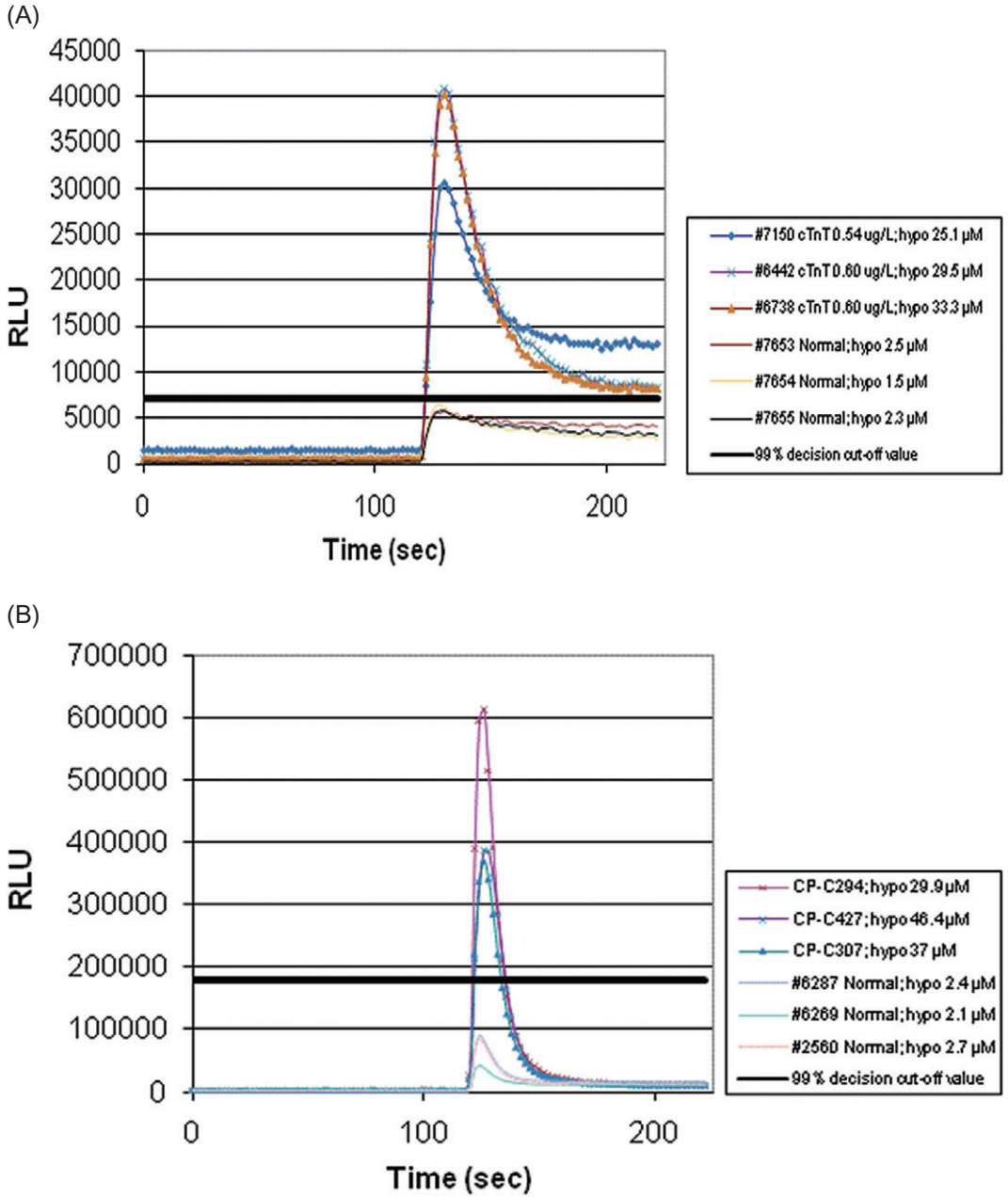

Figure 8. Overlay plots of healthy individuals, chest pain patients and cardiac MI patients, demonstrating the rapid screening chemiluminescence methodology. Overlay plot (A) depicts healthy individuals (normal) and patients with confirmed acute MI (hospital documented elevated levels of cTnT) ($n = 3$ each). Each cTnT patient sample was above the estimated 99% cut-off reference value for healthy individuals. Overlay plot (B) depicts healthy individuals (normal) and emergency department (ED) patients with non-traumatic chest pain ($n = 3$ each). Each ED patient sample RLU signal was above the estimated 99% cut-off reference value for healthy individuals. The use of Adjuvant-K™ signal enhancer significantly increased the chemiluminescence signal (B).

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